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Abstract

A wide range of genetically modified (GM) animal/trait combinations are being developed with a great variety of purposes: to enhance food production or nutritional quality; to improve human or animal health; as sources of products (pharmaceuticals) or tissues (transplantation) for human therapeutic use; to control vectors of human infectious diseases; and to improve animal farming operations, amongst others. This review will focus on developments in three main animal classes: mammals, fish and insects. Several developments of GM mammals and their derived products shall be reviewed as examples of the potential impacts of this technology on farming practices through to human health. Fish, on the other hand, pose a particular problem because of the possible effects of their entry into natural ecosystems as a result of their unintended escape from fish farming operations. Two main objectives drive the development of GM insects, namely pest management and control of vector-borne infectious diseases. These will require the deliberate release of GM insects directly into the environment, and may require dedicated risk assessment protocols. As products of genetic engineering (GE) technologies, GM animals fall within the regulatory oversight of dedicated biosafety guidelines and legislation. Nonetheless, appropriate approaches regarding their environmental risk assessment are still under discussion and, therefore, relevant regulatory frameworks or guidelines are still in their infancy, with the result that a variety of regulatory burdens appear to block the authorisation process of several applications of GM animals. Research and development in this field is enormously dynamic and with recent findings and innovations beginning to anticipate a significant role for GM animals in addressing some of the fundamental challenges facing humanity.

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Riassunto

Una vasta gamma di combinazioni di animali/caratteristiche geneticamente modificati (GM) è in fase di sviluppo con molteplici finalità, tra cui: migliorare la produzione alimentare o la qualità nutrizionale; migliorare la salute umana o animale; come fonti di prodotti farmaceutici o di tessuti da trapianto per uso terapeutico nell'uomo; controllare vettori di malattie infettive umane; e migliorare le operazioni di allevamento di animali. Questo articolo si focalizza sugli sviluppi che si sono avuti in tre classi principali di animali: mammiferi, pesci e insetti. Viene effettuato un resoconto delle diverse applicazioni di mammiferi GM e loro derivati come esempio del potenziale impatto di questa tecnologia sulle pratiche agricole fino a valutarne gli effetti sulla salute umana. I pesci, costituiscono invece un problema particolare a causa dei possibili effetti del loro ingresso negli ecosistemi naturali come risultato della loro fuga involontaria dagli allevamenti ittici. Lo sviluppo di insetti geneticamente modificati è mosso da due obiettivi principali, la gestione delle malattie e il controllo delle malattie infettive trasmesse dai vettori, i quali richiederebbero l'emissione deliberata di insetti geneticamente modificati direttamente nell'ambiente, e potrebbero richiedere quindi appositi protocolli di valutazione del rischio. Quali prodotti delle tecniche di ingegneria genetica, gli animali GM rientrano nel controllo regolamentare delle linee guida e della legislazione sulla biosicurezza. Tuttavia, gli approcci appropriati sull valutazione del rischio ambientale sono ancora in discussione e, di conseguenza, i relativi quadri normativi o linee guida sono ancora ai loro primordi, con il risultato che una serie di oneri normativi sembrano bloccare il processo di autorizzazione delle diverse applicazioni di animali geneticamente modificati. La ricerca e lo sviluppo in questo campo sono in grande evoluzione e con le recenti scoperte e innovazioni viene evidenziato il ruolo significativo che gli animali geneticamente modificati hanno per affrontare alcune delle sfide fondamentali dell'uomo.

Keywords: Environmental risk assessment, fish, GM animals, insects, mammals, regulations.

1. ENVIRONMENTAL RISK ASSESSMENT OF GENETICALLY MODIFIED ANIMALS.

1.1. Concepts

Environmental risk/biosafety assessment of genetically modified (GM) animals should follow the usual regulatory criteria as for other GM organisms (GMOs): a case-by-case scientific review of the risks (a function of Hazard and Exposure), based on plausible pathways connecting identified hazards (the risk hypotheses) with measurable harm to protected values (the statutory end-point standards). It is broadly accepted that the risk assessment (RA) of GMOs should be science-based and carried out on a case-by-case basis. The Cartagena Protocol on Biosafety (CPB) to the Convention on Biological Diversity (Secretariat of the Convention on Biological Diversity, 2000), often taken as an appropriate reference for the information needed for the RA of *living modified organism*s (as per the Protocol wording), establishes a comprehensive list of "points of consider", which includes:

(a) the biology of the recipient organism or parental organisms,
(b) the biology of the donor organism or organisms,
(c) the vector, the construct and the inserted sequences, including the methods for their detection,
(d) the engineered phenotype,
(e) the intended use of the modified organism, and
(f) the characteristics of the environment(s) into which the modified organisms can enter accidentally or as a consequence of their deliberate release.

When applied to GM animals, the challenge is to translate the above items into a set of risk/safety assessment criteria as well as into appropriate environment endpoints to cover a great variety of species, constructs and traits, since many different genetic modifications and deployment conditions have been already reported for mammals, birds, fish and insects.

On the basis of the intended purpose of the genetic modification, GM animals can be divided into six broad classes (USA FDA, 2009a): (1) to enhance production or food quality traits (e.g. pigs with less environmentally-deleterious waste, faster growing fish); (2) to improve animal health (e.g. disease resistance); (3) to produce products intended for human therapeutic use (e.g. pharmaceutical products or tissues for transplantation (these GM animals are sometimes referred to as "biopharm" animals); (4) to enrich

or enhance animal interactions with humans (e.g. hypo-allergenic pets); (5) to develop animal models for human diseases (e.g. pigs as models for cardiovascular diseases); and, (6) to produce industrial or consumer products (e.g. fibres for multiple uses). It is clear from the above that the environmental and food risk/safety assessments of GM animals will need to consider a great variety of uses.

Based upon the considerable experience with GM plants, there is a basic set of familiar questions a regulatory agency will have to review in order to develop RA methodologies and rules to protect the environment as well as human and animal health. With regard to environmental impacts, there is a great diversity of situations, dictated by the biology of the animal (whether or not GM), the introduced trait, and the way the animal will interact with the receiving environment. With regard to the latter, there are three possible situations: i) animals may be released/used under absolute confined conditions (e.g. a GE animal producing a high value therapeutic protein), or ii) under "standardised confined conditions" (e.g. GM farm animals), where escape, although remote, is still a possibility, or iii) they may be purposely released into the wider environment (e.g. GM mosquitoes for insect-borne disease control). Clearly, the "exposure" factor in the "Risk as a function of Hazard and Exposure" equation will be quite different for each of these three situations.

Under all conditions, the biology of the host species and the transferred GM trait(s) may be a determinant of the eventual outcome, e.g. their potential to: survive and establish in the natural environment; to breed with wild or feral conspecifics; to enhance any invasive behaviour; or to their being exposed to sheer extinction. To these basic questions, additional issues will have to be considered which are specific for GM organisms in general (e.g. mammals, fish, insects) and for some key species and associated traits in particular. Therefore, the assessment of the potential impact will involve consideration of the species intrinsic traits and its ecological features, its life history, its phenotypic plasticity (behavioural, morphological and physiological), its reproductive physiology, and its interactions with biotic and abiotic environmental factors. In some cases, the feasibility of, and the methods for, the detection of the GM animal in the environment may also be a regulatory requirement. Points to consider would be the direct effects (immediate and short term) as well as those derived from the eventual/potential establishment of the GM animal in the accessible environment. There are also environment-specific characteristics which may be interacting with the species, whether or not GM: the presence of conspecific animals,

or competitive or predator species; breeding opportunities with wild or feral populations; effects on biological diversity; susceptibility of the environmental protection goals to the release; reversibility of potential effects; cumulative and/or synergistic effects; routes for gene flow, and; presence of a selective pressure or competitive advantage. Finally, there also release-specific factors, like its scale, risk management practices, likelihood and pathways of dispersal, and mitigation and eradication measures (if needed).

Against this diverse background, only very general RA criteria will be discussed, and the reader is requested to bear mind that for the following sections, the non-GM counterpart is considered the comparator for the assessment of specific GM animal effects on the environment. A few key species and examples will be considered in this review. They have been chosen because of their current or predicted relevance in the areas of human health, food security and/or environmental benefits.

1.2. Regulations

Regulatory oversight of GM animals is in the initial phases of deployment. In the European Union (EU), the European Food Safety Agency (EFSA) was recently commissioned by the European Commission to draft guidance documents regarding the safety assessment of GM animals to be released into the environment. Within its mandate, EFSA contracted three separated "External Scientific Reports" with the aim of identifying information available in the public domain and defining the risk assessment criteria for GM insects, GM fish, and GM birds and mammals, respectively (Benedict *et al.*, 2010; Cowx *et al.*, 2010; Henry *et al.*, 2011). The GMO Panel of EFSA set up three dedicated Working Groups to take these reports into consideration for the development of further respective Guidance Documents on RA criteria. Animal health and welfare aspects were also to be considered. Accordingly, a fourth document on aspects of animal health and welfare has been prepared by EFSA as a "Scientific Opinion", where the analysis of the phenotypic characteristics of the GM animal is compared with the traditionally-bred animal, including health and physiological parameters which are considered important components in the RA (EFSA, 2011). The final guidance document on the environmental risk assessment of GM animals bred for food and feed purposes is expected to be launched for public consultation and it will likely be adopted in 2012.

A guidance document on the Regulation of GM animals has been released by the USA FDA (USA FDA, 2009a). It includes regulation on experimental

use, approval procedures and post-approval responsibility for developers. The agency examines the safety issues (the genetic construct, the safety of food from the animal, any environmental impacts), as well as the extent to which the performance claims made for the animal are met (efficacy).

Regarding regulations in the UK, the Health and Safety Executive, Scientific Advisory Committee on Genetic Modification (SACGM), has published a Compendium of Guidance, Part 5, Genetic modification of animals (HSE, 2007). This document contains regulations concerning the RA (environment and human health issues) and Containment and Control Measures required to work with GM animals. When the modification is for animals intended to be used as food/feed sources, it is recommended that the Secretariat to the Advisory Committee on Novel Foods and Processes be consulted at an early stage of the development.

Several documents from international organisations have addressed scientific aspects relevant to the development of regulations for GM animals, e.g. FAO/WHO Expert consultations (FAO/WHO, 2003, 2007) and a Guidance to RA under the CPB (Secretariat of the Convention on Biological Diversity, 2010). Other documents from national organisations or individual researchers deal with aspects pertinent to regulations: on environmental concerns (USA National Research Council, 2002); on a proposal for a regulatory regime for Canada (Kochhar & Evan, 2007); on how to adapt regulations to be consistent with the CPB in Japan (Yamanouchi, 2005); an overview on RA and the state of research by the Austrian Federal Ministry for Health, Family and Youth (Schmatzberger & Schultz, 2008), intended to provide criteria for the development of national regulations, and; a discussion of environmental RA and management of transgenic fish and shellfish relevant to biosafety regulations (Kapuscinski, 2005).

2. MAMMALS

2.1. Introduction

The first GM mammals resulted from the early finding that transgenic mice could be generated following injection of viral (SV40) DNA into pre-implantation mouse blastocysts (Jaenisch & Mintz, 1974; Jaenisch *et al.*, 1975). Further significant development was achieved with the successful injection of a rat growth hormone gene containing the promoter of the mouse metallothionein-I gene into the pronuclei of fertilised mouse eggs (Palmiter *et al.*, 1982), which resulted in the generation of transgenic mice. Several of the mice had extraordinarily high levels of the fusion mRNA in

their liver and growth hormone in their serum. Since then, several traits have been introduced into mammals, to increase food production and/or quality, develop disease resistance, produce useful pharmaceuticals or adapt animal organs for human xenotransplantation. There are also many examples of genetic modification of small rodents in contained use in biomedical research (Fox *et al*, 2007), which are not directly of concern in this review.

There is a great potential for GM animals to play a significant role in enhancing food production to satisfy the needs of an increasing world population. Moreover, improving living standards globally will increase the demand for meat and other products from livestock origin, thereby generating a powerful drive for livestock production. Genetic engineering of mammals offers opportunities to produce more meat products, with higher quality and improved nutritional properties. Conversely, livestock production interacts forcibly with the environment, via animals management systems and waste disposal practices, and with climate change, through the emissions of greenhouse gases (e.g. methane in cattle farming). All of these interactions are relevant determinants of sustainability and therefore act as potent drivers for innovation in the livestock sector, towards improving the efficiency of food production. Several traits have been introduced in mammals to address these issues. Some interesting examples are described below.

2.2. Food production. Intensive pig farming with less environmental pollution: Enviropig

Hog production under confined conditions requires adequate waste-management measures, as contaminants from animal wastes can enter the environment. A source of environmental pollution is feed management: plant-derived protein sources in feed contain a considerable concentration of phosphorous (P) as phytate (IP6; myo-inositol 1,2,3,5/4,6-hexakis dihydrogen phosphate) complexes (Jongbloed & Kemme, 1990). Most of this P is not available to the animal and ends up in the faeces. In order to meet pig dietary requirements, supplemental P (USA National Research Council, 1998) and/or phytase enzyme which partially hydrolyses phytates to release digestible P, are added to feed (Kerr *et al.*, 2010). The effect of phytase is to reduce the amount of supplemental phosphate needed in the diet (Simons *et al.*, 1990; Ketaren *et al.*, 1993), but still a considerable amount of indigestible P is excreted. Although higher concentrations of phytase added to the feed can further improve the extent of hydrolysis (and the amount of available P from feed), this does not seem to be

cost-effective (Kornegay, 2001). The consequence of these production practices, which call for the appropriate management of pig dietary P requirements (Kerr *et al.*, 2010), is the release of considerable amounts of P into the environment by the pork industry. Phosphorous is an objectionable environmental pollutant, as it leaches into freshwater and marine systems affecting water quality and causing eutrophication leading to algal blooms and the associated death of fish and aquatic animals.

In order to improve the digestion of P from phytates in plant-derived protein sources, pigs have been genetically engineered through the introduction of a construct containing the phytase gene from *Escherichia coli*, and under the control of the promoter of the mouse parotid secretory protein (Golovan *et al.*, 2001). The transgenic pig, dubbed "Enviropig", synthesises phytase in its salivary glands and secretes the enzyme in the saliva. This salivary phytase enters into the digestive tract along with the feed and allows the digestion of phytates, significantly reducing both supplemental P requirements and faecal phosphorous excretion. The faeces of this genetic engineered pig contains up to 75% less P than that of non-GM pigs fed the same diet (Forsberg *et al.*, 2003). In this way, a more efficient management of P nutrition and a lower pollutant output by the pork industry is achieved. The engineered phytase in the pigs does not appear to present any recognisable unintended adverse effects on the animal's health.

The regulatory fate of the Enviropig has not been finished yet. By 2010 this GM pig has advanced one step towards its future use (Canadian Department of the Environment, 2010). Regulations have defined which breed can be used (Yorkshire and Landrace), and stipulated that animals must be raised in a controlled farm facility, segregated from other pigs, with identifications and tags to avoid misplacing animals or them entering the food chain. Food and feed safety dossiers on the Enviropig have been submitted to regulatory agencies, which are currently under review.

2.3. Enhanced offspring growth through improving milk properties

GM technology was used to improve lactational characteristics of pigs, leading to enhanced offspring growth. Endogenous α-lactalbumin synthesis is a limiting factor to milk production early in lactation for first-parity gilts (non-mated female pigs), and consequently is a limiting factor to piglet growth prior to weaning. α-lactalbumin is a key component of the lactose synthase complex in mammary epithelial cells. This enzyme complex is responsible for the production of lactose, the major osmole

in milk and a major determinant of milk volume. Transgenic pigs were produced through microinjection of a bovine α-lactalbumin gene construct into pronuclei of pig embryos (Bleck *et al.*, 1998). The gene construct contained 2.0 kb of the 5′ flanking region, the 2.0 kb coding region (exons and introns), and 329 base pairs of the 3′ flanking region of the bovine gene. The introduction of the bovine protein caused an approximately 50 % increase in the total α-lactalbumin concentration of pig milk throughout lactation. It has been shown that over-expression of bovine α-lactalbumin in lactating gilts enhances lactational performance and litter growth (Noble *et al.*, 2002). Transgenic α-lactalbumin expression in the GM gilt increase milk quantities early in lactation and results in a faster rate of growth for piglets.

2.4. Dairy animals: Improving milk composition

In dairy livestock, the alteration of milk composition can affect suckling growth as milk and colostrum contain a variety of proteins that possess biological activity and perform important functions in the neonate, with regard to regulation of growth, development and maturation of the gut, immune system and endocrine organs (Grosvenor *et al*, 1993). Furthermore, colostrum and milk are natural vehicles for acquiring passive immunity in the offspring and are valuable tools for decreasing neonate mortality from diarrhoea disease in humans. Transgenic alteration of milk composition has the potential to enhance the production of certain proteins and/or growth factors that are deficient in milk (Wall et at., 1991). The over-expression of several of these proteins in milk can improve growth, development, health and survivability of the developing offspring.

The above explains the interest in the addition or supplementation of beneficial hormones, growth factors or bioactive factors to the milk through the use of genetic engineering: dairy animals can be developed as bioreactors for the production of heterologous milk components for the purpose of improving its biological properties for human consumption. Similarly, several relevant active proteins have been expressed in the milk of transgenic animals, such as human lactoferrin in mice (Kim *et al.*, 1999) and cattle (van Berkel *et al.*, 2002; Thomassen *et al.*, 2005; Shu *et al.*, 2007; Yang *et al.*, 2008), human lysozyme in cattle (Yang *et al.*, 2011) and goats (Maga *et al.*, 2003, 2006; Jackson *et al.*, 2010) and porcine lactoferrin in mice (Wu *et al.*, 2007; Chen *et al.*, 2008). One of these products, human lactoferrin expressed in transgenic cows, was granted GRAS ("generally recognised as safe") status by the USA FDA (USA FDA, 2005).

2.5. Pig meat with better n-3/n-6 balance

Quality and nutritional traits have been genetically engineered into mammals (Forsberg *et al.*, 2005). For example, meat products are generally low in omega-3 (n-3) fatty acids, which are beneficial to human health. Moreover, the high omega-6/omega-3 ratios, which results from the extensive use of grains rich in n-6 fatty acids but deficient in n-3 fatty acids as animal feed, may contribute to the prevalence of coronary artery disease and other chronic diseases (Simopoulos, 2008). As livestock are unable to convert n-6 fatty acids into n-3 fatty acids because they lack an n-3 fatty acid desaturase gene, the fat-1 desaturase gene from the roundworm *Caenorhabditis elegans* was introduced into pigs. The GM pigs were produced by the introduction of an *hfat-1* expression vector, which contains a humanised (i.e. modified codon usage) *fat-1* cDNA (Lai *et al.*, 2006). The expression of the desaturase gene was driven by a cytomegalovirus enhancer and the chicken β-actin promoter, and a selection marker cassette was introduced into the expression vector, which was transfected into early-passage male primary porcine fetal fibroblast cells. Transfected cells, expressing lower n-6/n-3 fatty acids ratios were used to clone h*fat-1* transgenic pigs by nuclear transfer (Lai *et al.*, 2002). The transgenic pigs expressing the humanised n-3 fatty acid desaturase gene produce high levels of n-3 fatty acids from n-6 analogues in their tissues, and have a significantly reduced ratio of n-6/n-3 fatty acids. Most piglets appeared normal at birth, and there was no obvious difference in appearance between the transgenic and non-transgenic littermates (Lai *et al.*, 2006).

2.6. Disease resistance: Two examples

2.6.1. Mastitis resistance

The goal to enhance mastitis resistance of dairy cows by enabling the cells of the mammary gland to secrete antibacterial proteins was first demonstrated in a transgenic mouse model. Mice were developed that produce varying levels of lysostaphin in their milk by the introduction of copies of the lysostaphin gene from *Staphylococcus simulans* (a benign Staphylococcus species) into the cells of a developing embryo (Kerr *et al.*, 2001; Kerr & Wellnitz, 2003). Lysostaphin expression was regulated by the 5′ flanking region of ovine β-lactoglobulin as promoter. Lysostaphin is an enzyme that hydrolyses the peptidoglycan component of the cell wall of gram positive bacteria. This protein has potent anti-staphylococcal activity and its secretion into milk confers substantial resistance to infection caused by intra-mammary challenge with *Staphylococcus aureus*, a major mastitis pathogen. Proteins, such as lysostaphin, have desirable properties for use as antibacterial compounds active as secreted in milk, as they are not

typically used as injectable or oral therapeutics to treat human infections. Therefore, there is no risk of inducing resistance to clinically relevant antibacterial compounds used in the treatment of human infections.

This proof of concept was later demonstrated in dairy cattle (Wall *et al.*, 2005). As mastitis causes huge economic losses and considerably impairs livestock welfare, the demonstrated viability of this transgenic approach to combat such a prevalent disease in dairy cattle shows great potential (Donovan *et al.*, 2005). The environmental impact of mastitis-resistant cows does not seem to be significantly different from that of their non-GM counterparts.

2.6.2. Protecting cows from Bovine Spongiform Encephalopathy (BSE)

An interesting achievement was the production of cattle lacking the prion protein (Richt *et al.*, 2007) in an attempt to obtain protection against BSE. Following the finding that disruption of the expression of the PrP^C (the nornal cellular prion protein) in mice, a species that does not naturally contract prion diseases, results in no apparent developmental abnormalities, PrP^C-deficient cattle were produced, to find out the phenotypic effects of ablating PrP^C function in natural host species of prion diseases. At over 20 months of age, the cattle were clinically, physiologically, histopathologically, immunologically and reproductively normal. Moreover, brain tissue homogenates were resistant to *in vitro* prion propagation., PrP^C-deficient cattle may be a useful model for prion research and could provide industrial bovine products free of prion proteins (Richt el al., 2007).

2.7. Mammals as bioreactors: Pharmaceuticals

Transgenic dairy animals can be used as vehicles for the production of high value proteins in milk. As early as 1989, it was demonstrated that the introduction into sheep of a fusion gene construct comprising β-lactoglobulin sequences fused to those encoding anti-hemophilic human factor IX, resulted in the efficient and specific expression in the mammary gland of the corresponding protein, which was secreted into milk (Clark *et al.*, 1989). After this early demonstration, it was soon realised that transgenic dairy animals offer a cost-effective system for the production of complex proteins expressed in milk (Echelard & Meade, 2003).

Basically, these GM animals are obtained using an expression vector comprising a gene coding for the target protein under the control of mammary gland-specific regulatory sequences, which is inserted into the germline of the selected production species. Pro-nuclear microinjection of

one-cell embryos or transfection into a primary cell population suitable for somatic cell nuclear transfer have both been used to generate transgenic founders. When integrated as a dominant genetic trait, the milk-specific expression is inherited by the progeny of the founder animal. By this general strategy, large quantities of target proteins can be produced which are secreted by the mammary glands of the dairy animal.

Milk is a convenient matrix for subsequent downstream processing to extract the purified protein. Yields are high (1 g/L or higher) and downstream processing has several advantages compared with other recombinant proteins production technologies, such as recombinant bacteria, where the target protein may aggregate within the bacterial cells to form inclusion bodies from which the active protein must be recovered in the correct native folded state through appropriate protocols (Sahdev *et al.*, 2008). Furthermore, recombinant bacterial systems are not suitable if complex post-translational modifications are needed to achieve the active protein, and yeasts often perform post-translational modification patterns which are different from the human counterpart. Moreover, although transgenic expression systems based on mammalian cells are able to perform complex post-translational modifications, there are species- and tissue-specific characteristics for these modifications that may affect the properties of the expressed proteins.

Recombinant human antithrombin (rhAT, commercial name ATryn) was the first transgenic milk-derived compound to be granted market approval: by the EU (EMA, 2006) and by the USA (USA FDA, 2009b). It is used for the prevention of venous thromboembolism during surgery of patients with congenital antithrombin deficiency. This was the first positive review by a regulatory agency for a transgenic biopharmaceutical, from either plant or animal sources (Echelard *et al.*, 2006). Expression in the milk of transgenic dairy goats was employed. The promoter region of the goat β-casein gene was linked to hAT cDNA. This transgene was introduced into the chromosomes of goat embryos, which were then transferred to surrogate mothers. The resulting transgenic goats produce the gene product, rhAT, in their milk. Transgenic offspring from the line selected for commercial development consistently express rhAT in their milk at approximately 2 g/L (Echelard *et al.*, 2006). Other therapeutic proteins have been expressed in milk of farm animals (Salamone *et al.*, 2006) and it is likely that this field will continue to be very active in the future. The use of GM animals as sources of valuable pharmaceuticals is being considered by several regulatory documents (Schmitt, 2004; USA FDA, 2009b).

2.8. Mammals as source of organs: Xenotransplantation

Xenotransplantation is a potential solution to the worldwide shortage of organs, and pig is considered to be the most likely species for clinical transplantation into humans. The Galα (1,3)Gal carbohydrate linkage, synthesised by α1,3-galactosyltransferase (GT) was found to be the major xenoepitope causing hyperacute rejection (HAR) of a donor organ (Sandrin & McKenzie, 1994).

In an effort to avoid HAR, deletion of the 1,3GT gene to remove the Galα (1,3)Gal epitope was considered in order to produce GM donor animals for xenotransplantation. This was initially achieved in mice using homologous recombination (Tearle *et al.*, 1996), and later in pigs using nuclear transfer technology (Lai *et al.*, 2002; Phelps *et al.*, 2003).

Consequently, the generation of α1,3-galactosyltransferase knock-out pigs was thought to avoid HAR and encouraging results were found supporting this assumption (Tseng *et al.*, 2005). Surprisingly, Galα (1,3) Gal is still present in α1,3-galactosyltransferas knockout pigs (Milland *et al.*, 2005). It was then realised that to fully exploit the use of carbohydrate modification in xenotransplantation, more information was required about glycosyltransferase biology, as other enzymes with these activities are involved in the expression or masking of the Galα (1,3)Gal epitope and therefore are relevant to xenotransplantation. These glycosyltransferases are structurally similar to each other and catalyse the transfer of either a galactose residue or an N-acetylgalactosamine residue in an 1,3 linkage to their respective acceptor molecules.

At present, GM pigs lacking 1,3-Gal epitopes are still considered to be the basis for further genetic modifications that can address other rejection mechanisms and incompatibilities between the porcine and primate blood coagulation systems. However, it is also recognised that various strategies for the genetic modification of pigs will be needed to facilitate tailoring them to be donors for organ transplantation (Klymiuk *et al.*, 2010).

Although the potential benefits are considerable, the use of xenotransplantation raises concerns regarding the potential infection of recipients with both recognised and unrecognised infectious agents and the possible subsequent transmission to those in which they come into close contact, and into the general human population. Of public health concern is the potential for cross-species infection by retroviruses, which may be latent and lead to disease years after infection. Moreover, new

infectious agents may not be readily identifiable with current techniques (USA FDA, 2010). Therefore, in addition, transgenic strategies will have to be developed to reduce the potential risk of infections by endogenous porcine retroviruses (Miyagawa *et al.*, 2005; Wilson, 2008; Chapman, 2009). A future challenge will be to combine the most important and efficient genetic modifications in multi-transgenic pigs for clinical xenotransplantation.

3. FISH

3.1. GM fish: A special case of biosafety

The main environmental concern with GM fish considers the hazard of fish entering natural ecosystems. Potential routes by which GM fish can enter the natural waters include: escape during transportation; loss from research or experimental facilities; indiscriminate introductions to improve fishery performance, and; escape from commercial aquaculture facilities. The following discussion pertains to the latter situation, that is, GM fish escaping from fish farming systems.

In the assessment of the environmental risk derived from the release of GM fish into the wild, the main potential hazards are ecological disruptions due to GM fish outperforming non-GM fish with regard to competitive abilities in resource acquisition, increased predation, enhanced survival, increased somatic growth and reproductive performance under the same conditions, and facilitation of GM fish invasion into habitats that limit the non-GM conspecifics (Cowx *et al.*, 2010). All of these ecological effects are likely to be potentially more severe where wild conspecifics are present in the receiving environment, due to the potential effects of the transgene(s) to be passed into the wild gene pool, should GM and non-GM fish interbreed (Cowx *et al.*, 2010).

Against the background of the above concerns, it has been argued that GM fish pose serious threats to wildlife. Focusing on fish farming, these threats may be realised if GM fish escape from fish farms and upset the oceans' delicate ecology, causing ecological disruption or species extinction. In the case of such an escape, several phenotypes of the GM fish would led to undesirable scenarios: i) GM fish with increase cold-, salt- or heat-tolerance could expand into new territories; ii) higher disease resistance and better use of nutrients could allow GM fish to out-compete wild relatives and change predator-prey relationships, therefore invading new ecological niches where wild species would usually not survive; iii) by mating with wild fish, escaped GM fish could spread the transgene amongst the

wild population, which could cause conflicting effects on fitness factors required for the species to survive. As an example of the latter effect, it has been observed that non-GM, domesticated fish that have been bred and selected for growth in captivity where predators are absent and food is abundant, have lost their ability to find food and to avoid predators in the wild. If these fish breed with wild fish, their genes may introgress in the wild gene pool and cause a general decrease in fitness of the entire population (Lynch & O'Hely, 2001).

For the estimation of the exposure to the hazard of GM fish entering into natural ecosystems, a "net fitness methodology" has been proposed (Kapuscinski, 2005), by which the six fitness components (fecundity, fertility, juvenile viability, age at sexual maturity, mating success, and longevity) are measured over the critical points in the entire life-cycle of the organism. As a second step, the joint effect of all six fitness traits are quantified to predict transgene fate in the ecosystem.

This approach has been tested in studies with a growth-enhanced transgenic line of a model fish species, the Japanese medaka (*Oryzias latipes*) and model simulations in which GM fish escape into a population of wild relatives (Muir & Howard, 2001). The results have shown that different combinations of values for six fitness traits may lead to three different predictions of transgene fate:

- Purge scenario. The transgene is lost at some time after the initial escape of GM fish,
- Spread scenario. The transgene is spread through a wild population of relatives with no impact on the size of the introgressed population,
- Trojan gene scenario (Muir & Howard, 1999). Initial transgene spread that then triggers a decline in the size of the introgressed population; such a scenario occurs when the transgene has an antagonistic effect on different fitness traits.

Environmental harm assessments should also consider potential genotype-environment interactions, for example, food availability (Devlin et al., 2004), shifting the population towards individuals with the greatest food-gathering abilities.

Focusing on the fitness components model, it is clear that the purging and disappearance scenarios are the environmentally safest situations, although at least transiently they may not always be impact free (natural

selection must operate over a number of generations). Of particular concern is the potential harm from the Trojan gene effect, because its predicted population decline constitutes an environmental harm. Loss of a wild fish population would clearly lead to loss of unique genes. If transgenes conferring the Trojan gene effect spread through a threatened or endangered population, this would increase the chance of extinction. The loss of an entire population, in turn, might reduce the resilience of the aquatic biological community, for instance through simplification of the food web, unless the community contains other species that serve the same ecological function (Olden *et al*., 2004). As explained below, the risk implied in the Trojan gene effect has considerable weight in the regulatory assessment of GM fish.

3.2. Increased food production: Growth-enhanced fish

Several relevant desired phenotypes have been genetically engineered into GM fish, including, *inter alia*, enhanced growth rate (Rahman *et al*., 1998; Zhu & Sun, 2000; Nam *et al*., 2001), resistance to bacterial diseases (Dunham *et al*., 2002, 2004a, 2004b; Sarmasik *et al*., 2002; Mao *et al*., 2004; Yazawa *et al*., 2006), tolerance to cold temperatures (Wang *et al*., 1995), improved nutrient use and biocontrol of invasive species (Angulo & Gilna, 2008; Kapuscinski & Patronski, 2005). For a comprehensive list of transformed species and associated traits, see EFSA (2010).

The only transgenic fish that is commercially available today is a zebrafish that glows when illuminated, due to skeletal muscle expression of a fluorescent protein genetic construct (Gong *et al*., 2003) and sells under the brand name GloFish™. It is designed for aquarium owners and was originally developed at the National University of Singapore as a living indicator for environmental pollution. As it is not meant for human consumption, the USA Food and Drug Administration saw no need to regulate it under its mandate.

The growth-enhanced trait has attracted great interest as it could be an answer for increasing the yield of fish farms in addressing the need for greater availability of high quality protein, whilst at the same time overcoming environmental concerns (Muir, 2004). Accordingly, it is interesting to see fish farming of growth-enhanced fish within the broader context of food security. The increase of the availability of high quality protein has been a goal over the last several decades, and has been addressed with different technological approaches (Brown, 1968; Stillings & Knobl, 1971). In spite of these efforts, one of the most important protein sources, fish, is still

primarily gathered from the wild, with adverse consequences (Muir, 2004). Marine fisheries increase yields by over-fishing, which is threatening the sustainability of (or perhaps already irreversibly damaging) the source in many fishing grounds. In addition, fishing fleets and technology are highly capital-intensive, which put the ocean's fisheries under increasing stress (Muir, 2004). Fish farming, a feasible alternative, currently provides only about 30% of global fish production and is expected to reach 50% in 2030 (FAO, 2000). However, a desired expansion of aquaculture, even if it overcomes some resistance due to environmental concerns, would still not be enough to meet the increasing demand of this protein source (Tidwell & Allan, 2001).

Although hunger and malnutrition are multi-factorial problems, which recognise a complex set of causes, such as dietary deficits in energy, proteins, essential micronutrients, etc, the development of abundant and high quality protein sources can make a significant contribution to the solution of these problems, now suffered by a large proportion of world population (FAO, 2010). Against the background of the predicted growth of the human population (United Nations, 2010), it has been proposed that meeting the increasing demand for fish while protecting marine fish supplies can only be achieved by applying the experience from the agricultural revolution to increase the contribution of aquaculture (Tidwell & Allan, 2001). The underlying assumption is that the lessons of the technology-driven success of GM crops (James, 2010) can be translated to GM animals, as in the former "green revolutions". This is therefore the rationale behind the efforts to develop fish with increased yield and/or quality traits.

One GM fish with enhanced growth rate that is striving to reach commercial status is an Atlantic salmon, genetically modified with a construct containing a growth hormone gene from Chinook salmon (*Oncorhynchus tshawytscha*) under the control of regulatory sequences of the antifreezeprotein gene promoter from ocean pout (*Macrozoarces americanus*). This GM fish, dubbed *AquAdvantage* (AA) Atlantic salmon, is produced by the USA company Aqua Bounty Technologies (Aqua Bounty Technologies, Inc., 2010) but still has to obtain regulatory clearance.

From the previous discussion on the biosafety and environmental risk assessment of GM fish, it is clear that the enhanced growth rate trait depicts a particular example of regulatory hurdle, and in part explains why the growth-enhanced AA is still waiting for approval some fifteen years after the initial submission to the USA FDA (Van Eenennaam *et al.*, 2011).

Although the food quality objections (endogenous allergens, increased levels of insulin-like growth factor 1, unfavourable polyunsaturated fatty acid content with low omega-3 to omega-6 ratio) have been shown to be unfounded, proving that the fast growth phenotype is not associated with any food safety concerns (Van Eenennaam & Muir, 2011), the environmental concerns are still unresolved, in particular the possibility that the growth-enhanced trait may generate a Trojan gene effect into wild fish populations.

In recent work (Moreau *et al.*, 2011), the reproductive performance of the AA salmon has been compared with wild fish in pair-wise competitive trials within a naturalised stream mesocosm. The results of this study show that, despite displaying less aggressive behaviour, captively-reared non-GM fish were superior competitors to their transgenic counterparts in critical aspects of reproductive performance, with respect to a first-generation invasion scenario. These results seem to indicate a low risk from the Trojan gene effect, but the further reproductive performance of GM salmon still remains difficult to predict from this study. In order to assess that risk, the effects of the GM fish with a growth-enhanced trait in ecologically-relevant scenarios could be also assessed by testing the relative performance in the most favourable environment for the GM fish. This would give a worst-case scenario, so if the RA for the GM fish does not reveal an unacceptable risk under these conditions, then in all other less favourable conditions the GM fish will be an even lesser risk (Van Eenennaam, personal communication).

Proof of this concept has been reported (Devlin *et al.*, 2004, 2006) comparing the fitness of GM salmon in hatchery and stream conditions. The GM fish presented the greatest risk in a hatchery condition and almost no risk under stream condition, as the hatchery conditions were the more favourable for the GM fish. Therefore, it would appear that, at least for the well characterised growth-enhanced trait, it is reasonable to conclude that the Trojan gene effect does not constitute a significant risk.

4. INSECTS

4.1. Environmental Risk Assessment

There are two main objectives for the development of GM insects, namely: as biological control agents (as a pest management strategy), and; for the control of vector-borne infectious diseases. In both cases, their use would imply the release of GM individuals into the environment.

The environmental RA of GM insects presents considerable challenges. In

accordance with Annex III of the CPB, the CBD has produced a Guidance Document which includes an assessment of GM mosquitoes (Secretariat of the Convention on Biological Diversity, 2010) due to their potential to reduce transmission of vector-borne human pathogens, particularly those that cause malaria, dengue and chikungunya (Sperança & Capurro, 2007; WHO, 2010). Control, including the eradication of such diseases, is a recognised public health goal, and it is expected that these strategies will imply massive releases of GM individuals into wide areas.

In addition to the usual issues considered for the environmental RA of GMOs, several specific questions will also have to be assessed for the release of GM insects. For example, for certain regions and environments where GM mosquitoes are planned to be released, there is a need to gather more information regarding gene flow among vectors and into the wild target population (Marshall, 2008), their mating behaviour, the interactions between vectors sharing one habitat, trophic consequences on ecological communities, how pathogens respond to the introduction of new vectors, and a comprehensive knowledge of the population dynamics of the species to be affected by the release. Such information will be needed to establish a baseline in order to successfully assess the risks of GM mosquitoes, as well as the possible management strategies. Summaries of the environmental RA of four case study species, namely the mosquitoes *Aedes aegypti* and *Aedes albopictus*, and the fruit flies *Ceratitis capitata* and *Bactrocera oleae*, have recently been published (Benedict *et al*., 2010).

4.2. Pest Management

In the pest management context, the population of undesirable insects can be reduced by the massive releases of sexually-compatible male insects which can introduce sterility into a target pest population (the sterile insect technique; SIT). Usually these male insects are obtained by treating mass-reared individuals with irradiation (e.g. gamma rays, X-rays) to introduce chromosome damages in their germ-line cells, which in turn prevent the generation of viable offspring upon mating with wild females of the target population. Repeated releases of irradiated, single gender insects are required to either suppress the target population or to reduce damage below economic thresholds (Benedict et al., 2010). The SIT has been successfully used in field programmes as an effective and very powerful method of insect pest management (Gonzalez & Troncoso, 2007; Robinson *et al*., 2009).

Field measurements in SIT programs need to use reliable visual marking methods in order to assess the attributes critical to SIT in the field: ability to find a mate and to initiate copulation; as well as dispersal and persistence

in the release area. Recent improvements addressing this issue have resulted in the first open-field experiments (June-August of 2007) with a genetically engineered insect (Simmons *et al.*, 2011). The purpose of the genetic modification was to develop a genetically engineered strain of pink bollworm, *Pectinophora gossypiella*, with a heritable fluorescent protein visual marker to improve discrimination of sterile from wild moths. Pink bollworm was transformed with a construct comprising a fluorescent protein marker cassette (the red fluorescent protein, DsRed2) and its performance in the SIT strategy was compared with the standard, non-GM equally-irradiated strain. The findings have shown that the genetically engineered strain performed well relative to a standard strain: key parameters such as survival, dispersal and mating competitiveness were comparable between the genetically engineered strain and a standard strain used in SIT programmes.

The level of sterility induced in a pest population to be released (which is related to the efficacy of the control method by SIT) corresponds to the irradiation dose and therefore some programmes use very high levels of sterility. However, at the high levels of irradiation required for this purpose, the mating competitiveness of released insects is reduced. Although this can be partially overcome by adjusting the radiation dose rate to an adequate level of males competitiveness (Helinski *et al.*, 2009), there is an interest in inducing sterility by more specific, genetic engineering methods (WHO, 2010). Recent advances in insect transgenesis has resulted in their improved efficacy for population suppression and replacement strategies (Scolari *et al.*, 2011). Using these new techniques, the fitness of the transformant individuals is not impaired, so that once released in the field, they can efficiently compete with or even out-compete their wild-type counterparts for mating in order to reduce the population size, or to spread desirable genes into the target population (discussed below).

A derivative of the SIT strategy is the release of insects carrying a dominant lethal trait, RIDL (Alphey *et al.*, 2002). Using RIDL techniques, organisms can be manipulated to be conditionally sterile or lethal and released into the environment to disrupt mating or to reduce the fecundity of the F1 generation of the wild population. During production, a dominant lethal or fecundity disrupting gene that the strains carry is repressed by rearing the insects in the presence of a suitable co-repressor which specifically inhibits transcription through the promoter needed to express the gene. The released insects mate with specimens of the target population and thereby transmit the lethal gene. In the absence of the inhibitor, transcription is de-

repressed, and the transgene is expressed and causes lethality or sterility at some stage during the development of the offspring.

4.3. Control of vector-borne infectious diseases

An example of the RIDL approach is a new "sterile-male-release" control strategy based on the release of mosquitoes carrying a conditional dominant lethal gene. Transgenic strains of *Aedes aegypti* were engineered to have a repressible female-specific flightless phenotype using either two separate transgenes or a single transgene, based on the use of a female-specific indirect flight muscle promoter from the *Aedes aegypti Actin-4* gene (Fu *et al*., 2010). These strains eliminate the need for sterilisation by irradiation, permit male-only release ("genetic sexing", needed for the SIT, is not required in this case), and enable the release of eggs instead of adults. These strains are expected to facilitate area-wide control or elimination of dengue if adopted as part of an integrated pest management strategy.

The first field demonstration of the use of RIDL was the release of strain OX513A in an open field trial to control the dengue-carrying *Aedes aegypti* mosquito (Oxitec, 2010). The field trial took place in Grand Cayman Island with sterile male releases from May to October in 2009 with additional pre- and post-trial monitoring. After the demonstration that released RIDL males mated successfully with their local counterparts in the open environment, the sterile male mosquitoes (which do not bite or spread the disease) were hatched and released by the Mosquito Research and Control Unit in the Cayman Islands. After initial releases, male sterile mosquitoes reached the required release level in July of said year. A significant reduction in the local mosquito population was observed from August. As per the developer's press release, all of the trial objectives were successfully met, including the main goal of suppressing the local *Aedes aegypti* population (Oxitec, 2010; Subbaraman, 2011). The same *Aedes aegypti* strain is currently being tested in Brazil (Lima Oliveira *et al*., 2011).

Strategies for the control of insect-borne diseases are priorities in worldwide public health programs (WHO, 2010, 2011). For the control of infectious diseases, two approaches have been explored: i) biological control methods, such as RIDL described above, or ii) the replacement of the population of infective individuals by insects that have lost the ability to transmit the disease (Deredec *et al*., 2008). Population displacement strategies are particularly being pursued, as exemplified here by malaria. The approach was to generate transgenic mosquitoes that express anti-parasitic genes in their midgut epithelium, thereby protecting them against

the parasite. Alternatively, genes can be introduced into the germ-line of insects, whose expression will somehow disrupt the cycle of the parasite in the insect host, thus rendering them inefficient vectors of the disease (Ito *et al.*, 2002). These GM insects become "refractory" vectors, unable to transmit the disease (Marshall & Taylor, 2009).

Engineering mosquitoes with genes conferring refractoriness to the malaria parasite can also be approached by the identification of genes needed to transmit the disease, and then replacing or altering their function. For example, in order to perform the infectious cycle, the parasite needs to bind to specific receptors in the gut wall of the insect. Accordingly, GM mosquitoes can be engineered to express proteins which will occupy these receptors, blocking transmission of the parasites, i.e. making them refractory to them (Ito *et al.*, 2002). In another example of this approach, the site-specific integration of an anti-malarial gene into *Anopheles gambiae* was done, which resulted in the significant protection *against Plasmodium yoelii nigeriensis*, highly reducing average parasite intensity (Meredith et al., 2011). Similar protection was observed against *Plasmodium falciparum* in some experiments, although protection was inconsistent. The antimalarial gene that has been introduced in this case, named *Vida3*, is a novel synthetic form an antimicrobial peptide sequence produced by insects, which was designed to increase its original antimicrobial activity (Arrighi *et al.*, 2002).

Research is quickly progressing in the discovery of genes conferring refractoriness and their development into efficient GM insects addressed at reducing the impact of insect-borne diseases (Riehle *et al.*, 2007). There is also ongoing research using RNAi silencing methods (Brown & Catteruccia, 2006; Franz *et al.*, 2006). However attractive these approaches appear, to generate refractory insects that do not transmit the disease is not in itself of practical value because these strains would need to be released on a scale that would be infeasible given the wide areas that are inhabited by vectors of human tropical diseases (Sinkins & Gould, 2006). Therefore, for a strategy of large-scale population replacement with refractory mosquitoes, a gene drive system will be required, which will efficiently spread the desired gene(s) into the target population.

Gene drive systems are naturally occurring "selfish" genetic elements that are known to spread within populations in a non-Mendelian fashion, even when they provide no fitness benefit to the host organism. Transposable elements (TEs) were amongst the first candidate gene drive systems considered in this context. These elements are able to spread quickly

through a population due to their ability to replicate within a host genome, increasing their copy number and hence to be inherited more frequently in the offspring's genome. This increase in inheritance enables TEs to spread even in the presence of a fitness cost to the host. Autonomous TEs encode transposase enzymes which enables them to effect their own transposition, whereas non-autonomous elements need cross-mobilisation. For population replacement strategies, a TE-based gene drive system could then be designed with the use of an autonomous element with a tightly-linked refractoriness-producing effector gene. Criteria to be met by efficient drive mechanisms have been described (James *et al.*, 2004). Basically, they should be able to spread the effector gene to effective levels of fixation and be resistant to the loss of the linkage to the refractoriness gene. Also, the spreading should be sufficiently fast so as to prevent the loss of efficacy by mutational inactivation of the carried gene, or by the development of resistance or evasion of the pathogen.

For a gene drive system to be efficient enough, it should carry the gene causing refractoriness and thereby spread this trait into the vector population at a time scale appropriate for controlling the transmission of the disease (Sinkins & Gould, 2006). Unfortunately, gene drive systems based on TEs constructs have been shown not to perform adequately for population replacement strategies. TEs tend to repress their activity over time, as they accumulate mutations leading to inactivation of mobility, their activity is strongly decreased by increasing the size of the refractory gene(s) they carry, and are vulnerable to losing internal sequences during replication (Marshall & Taylor, 2009). Therefore, attention has shifted to different gene drive systems. Amongst them, some promising drive mechanisms currently being investigated include Medea elements, homing endonuclease genes (HEGs) (Deredec *et al.*, 2008), engineered under-dominance constructs, meiotic drive and the intracellular bacterium *Wolbachia*.

Great attention has been given to Medea (Chen *et al.*, 2007; Ward *et al.*, 2011), a selfish genetic element that is able to spread through a population through its ability to cause the death of all offspring of heterozygous females that do not inherit the allele. Medea is a maternal-effect selfish genetic element based on a naturally-occurring element, which drives population replacement and is resistant to recombination-mediated loss of drive or disease refractoriness functions genes. Medea uses microRNA-mediated silencing of a maternally-expressed gene essential for embryogenesis, which is coupled with the early zygotic expression of a rescuing transgene (Chen *et al.*, 2007). The silencing of the essential embryogenesis gene

causes the death of all progeny lacking the Medea allele, and the rescuing transgene rescues Medea-bearing progeny from an otherwise imminent death. In this way, the proportion of Medea-bearing individuals is increased with each generation. By this mechanism, all offspring of heterozygous females that do not inherit the Medea allele are driven out from the population, and hence its name, an acronym for maternal-effect dominant embryonic arrest, with reference to the mythological Greek figure who murdered her own children. It is hoped that Medea could efficiently spread attached refractory gene(s) conferring resistance to malaria (Marshall & Taylor, 2009).

In considering population displacement strategies, there are also environmental requirements for the gene drive systems: it should not cause unexpected effects on the vector or on non-target species and it should be possible to remove the construct from the population in the event of unanticipated negative effects. In addition, ethical and ecological factors must also be considered. Most of these have been discussed in the context of the environmental RA under the CPB. A Guidance for Contained Field Trials of Vector Mosquitoes Engineered to Contain a Gene Drive Systems has been published (Benedict *et al*., 2008).

5. CONCLUSIONS

Mammals, fish and insects have been genetically modified for the introduction of several desired traits. The main drivers have been food production (output and quality), the reduction of undesirable impacts on the environment, animal disease resistance, the production of pharmaceuticals or organs for human xenotransplantation, pest management and the control of vector-borne human and animal diseases. The challenges for defining environmental risk assessment criteria and methodologies for GM animals are great: to the wide variety of animal/traits combinations, special situations need also to be considered. The appropriate approaches for some of these special cases are still under discussion or in the process of being developed. Moreover, regulatory frameworks or even guidelines are in some cases still in their infancy. In spite of this, research and development shows enormous dynamism and the reports of new findings and innovations represent a fascinating course, anticipating a relevant role of GM animals amongst the technological tools available to humanity.

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