GM Fish in the Context of Biosafety

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Abstract

The induction of transgenesis in fish is now an established technology and many different fish species have been modified in this way. These species include goldfish, medaka and zebrafish, which serve as model experimental systems, especially in developmental genetics, and commercial species such as rainbow trout, Atlantic salmon, coho salmon, chinook salmon, arctic charr, carp, African catfish, channel catfish, tilapia, northern pike, and mud loach. The parameters modified or whose modification has been attempted are growth enhancement, improved disease resistance, improved cold tolerance and freeze resistance, altered glucose metabolism, sterility and the exploitation of fish for the production of pharmaceuticals.

Since fish are highly mobile, the release or escape of GM fish could lead to the transfer of transgenes into wild stocks, if conspecifics are present in water adjacent to the cultured GM fish. The various risks associated with escape are considered, as are the potential benefits to aquaculture of GM fish culture.

Containment of GM fish may be achieved by limiting culture to safe and enclosed water systems, or by the imposition of complete sterility on the GM fish. Such sterility could in some cases result from triploid induction: in other cases it could probably be achieved through gene manipulation. Legislation and regulations relevant to the development and exploitation of GM fish are outlined and discussed.

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Riassunto

L'introduzione delle nuove biotecnologie per la modificazione genetica dei pesci è oggi una realtà e molte specie sono già state modificate utilizzando le tecniche dell'ingegneria genetica. Tra le specie interessate ricordiamo il pesce dorato (*Carassius auratus*), il pesce del riso (*Oryzias latipes*) ed il pesce zebra (*Brachydanio rerio*), utilizzati prevalentemente come modelli sperimentali nello studio della genetica dello sviluppo, oppure altre specie di interesse commerciale come la trota arcobaleno, il salmone dell'Atlantico ed altri salmonidi, la carpa, il pesce gatto, la tilapia, il salmerino alpino, ed il luccio. I caratteri modificati o quelli per i quali si è tentata la modificazione comprendono la crescita, la resistenza alle malattie, la resistenza al freddo ed al congelamento, il metabolismo del glucosio, la sterilità e lo sfruttamento per la produzione di farmaci.

Dal momento che i pesci comprendono specie estremamente mobili, il rilascio nell'ambiente o la fuga di un pesce transgenico può portare al trasferimento dei transgeni nella popolazione selvatica, soprattutto se la controparte non transgenica è presente nelle acque adiacenti all'allevamento. Devono quindi essere considerati i rischi associati alla possibilità di fuga, cosi come i benefici potenziali per l'acquacoltura derivanti dall'allevamento di pesci transgenici.

Il contenimento dei pesci transgenici può essere ottenuto limitando l'allevamento a sistemi d'acqua sicuri e chiusi, o mediante il conferimento della sterilità all'organismo transgenico. Tale sterilità può in taluni casi risultare dall'imposizione della triploidia: in altri casi può probabilmente essere ottenuta attraverso la manipolazione genica.

Anche gli aspetti riguardanti legislazione e regolamentazione dello sviluppo e dello sfruttamento dei pesci transgenici meritano grande attenzione e sono sottolineati e discussi in questa pubblicazione.

1. INTRODUCTION

Fish accounts for 16% of consumed animal protein worldwide and approximately one billion people across the world rely on fish as the primary source of animal protein (Knibb, 2002). Total fish catch (inclusive of molluscs, crustaceans and finfish) exceeded 92 million metric tonnes in 1999, while agriculture contributed a further 33 million metric tonnes (FAO, 2001).

The beneficial health bonus of fatty fish consumption, especially involving the omega 3 polyunsaturated fatty acids found in fish such as salmonids, mackerel, eel and tuna, will surely be a further incentive for the upward movement of the index of demand for fish as food.

Following a plateau peak in 1996/7, capture fisheries are now in decline, chiefly as a direct result of over-fishing (Hutchings, 2000); it therefore seems that aquaculture is set to make a much greater contribution to the world economy. However aquaculture itself attracts increasing criticism as being a source of environmental pollution and a common cause of ecological degradation, suggesting that its rapid growth will not come without attendant problems.

Whenever the future of aquaculture is considered, it is commonplace to think of it in the light of technologies which will allow intensification of the culture, partly because there are strict physical limits, at least in the immediate future, to the wide extension of aquaculture to new geographical regions. One possible road ahead would be to use transgenic technology to produce new and better strains of fish for future aquaculture. However, such a development could bring a host of new and difficult problems. In this review, the potential of the GM technology as applied to fish is considered, and the benefits and potential hazards of such development evaluated.

2. PREVIOUS REVIEWS

The topic of transgenic fish has been frequently reviewed. The following are a selection: Iyengar *et al.* (1996), Donaldson (1997), Dunham and Devlin (1999), Hackett and Alvarez (1999), Maclean and Laight (2000), Maclean (2003a), Maclean *et al.* (2002), and Dunham (2004).

3. PRESENT STATE OF PLAY

3.1. History of the Technology and Definitions

When cloned genes became widely available in the early 1970s, a long cherished dream of biologists became feasible, namely to manipulate

genetic material by introducing novel genes to an organism on a single gene basis. An early breakthrough allowed Gordon *et al.* (1980) to transform mouse embryos following injection of purified DNA, and this was soon followed in 1982 by the dramatic experiments of Palmiter *et al.* (1982) in which mice with enhanced growth were produced following egg injection with a growth promoting gene construct.

Almost immediately this raised the prospect of applying gene manipulation to fish. Certain complications were apparent, in that the fertilised fish egg contains tiny pronuclei which defy microinjection. Thus while mammalian eggs allow nuclear injection, extension of the technology to fish required placement of the cloned gene copies in the fish egg cytoplasm in the hope that some of the copies would find their way into the nucleus, thus allowing chromosomal integration and consequent genetic transformation of the organisms. This goal was initially realised concurrently by Zuoyen Zhu in China – Zhu *et al.* (1985), and Norman Maclean in the UK – Maclean and Talwar (1984), resulting in a joint publication in 1987 – Maclean *et al.* (1987).

A somewhat confusing terminology has arisen in line with the transgenic revolution. GM is taken to mean "genetically manipulated", a GMO is a "genetically modified organism" and LMO is a "living modified organism". These acronyms tend to be used interchangeably, all referring to transgenic organisms, and fall short of the ideal in that technically, triploid fish are genetically manipulated, but are commonly excluded from the GM umbrella. Thus GM is perceived to include only organisms which are genetically modified by the "GM technology", namely artificial insertion of so-called novel genes. But since genes from the same organism can scarcely be deemed novel, even this definition is not without its problems.

3.2. Methodology

A range of methods have been used to introduce cloned genes into fish. Microinjection into the fertilised egg is still the most widespread choice, but electroporation, liposome mediated gene transfer, and gene guns have all been tried, and electroporation is still favoured by some (Hostetler *et al.* 2003), especially with species such as medaka (*Oryzias latipes*). Eggs of salmonid and tilapine fish species are rather difficult to microinject because of the tough chorions and the opaque nature of the eggs. On the other hand species such as carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and catfish (*Ictalurus punctatus*) provide relatively transparent eggs with soft chorions, and can be readily injected even with hand-held needles. Microinjection of unfertilized fish oocytes has also been successfully achieved in medaka by Ozato *et al.* (1986).

To take tilapia (*Oreochromis niloticus*) as an example of a fish widely used in our own laboratory, the methodology is roughly as follows (see Rahman and Maclean, 1992; Maclean *et al.*, 2002). Transgene copies, which consist of a promoter sequence of choice, spliced to a coding gene (either cDNA or genomic) and a 3 prime polyadenylation sequence, are produced in bacterial plasmids and recovered by restriction enzyme digestion, so that only linear copies of the transgene construct are injected into the eggs. The DNA is dissolved in NaCl Tris buffer at a concentration such that the injection volume of approximately 250 picolitres per egg will carry 2 X 10^5 copies of the transgene. Eggs are fertilized *in vitro* after stripping of reproductive male and female fish, and injected within one or two hours fertilization. Microneedles used for injection are drawn to an end with an internal diameter of approximately 5 microns. The injection needle is linked to a picoinjector (Medical System Corp., Greenvale, USA) so that injection volume and pressure can be precisely controlled.

In the case of tilapia, a well trained operator will be able to inject about 50 – 70 eggs within the window of the one cell stage, which lasts at most two hours after fertilization. Following injection, eggs are incubated and the hatched fry reared for subsequent assay. Batches of fry may be pooled for polymerase chain reaction (PCR) assay, but in order to determine the incidence of transgene integration into the genome, it is best to wait until young fish are about six weeks old, by which time fin clips can be used to test for integrated copies by PCR. Since the transgene copies often join end to end in solution, it is common to find that the integrated sequence is in fact a concatemer of multiple copies of the original construct.

From 100 fertilised eggs injected, a common outcome is that approximately 80 will survive to the fry stage, of which perhaps 50 will exhibit some transgene expression (if, say, a reporter construct is used, to allow easy detection of early expression). However this early expression is chiefly transient expression of unintegrated copies and is not indicative of true integration. When fry of six weeks are monitored, all unintegrated transgene copies will have disappeared (except in rare cases of nuclear persistence of unintegrated copies, as has been recorded in carp, *Cyprinus carpio*, by Zhang *et al.*, 1990), and expression assays and PCR for DNA transgenes are diagnostic of true integration. The number of positive fish at this time is likely to be between 1 to 10 out of the original 100 eggs injected.

Such positives are invariably mosaic, however, with respect to the cellular distribution and expression of the transgene copies, presumably as a result of the delayed integration of the transgene copies until the embryo consists of 4 cells or more. This mosaicism also raises problems of germ

line transmission of transgenes from these original G0 fish. This is invariably less than the incidence of mosaic positives, and is likely to involve between 0.1 and 1.0% of the original 100 eggs and their hatchlings.

These germ line transmitters, because of mosaicism, may also transmit to quite low numbers of G1 progeny, maybe even less than 1% in a cross with a wild type fish. However, occasionally, if multiple chromosomal integration events have occurred in a particular G0 fish, then more than 50% G1 positives may be recovered. All G1 positives are, of course, hemizygous, in that a transgene is only incorporated into one of a pair of homologous chromosomes. However, by crossing two G1 positive fish in the same line of fish, G2 progeny will consist of 25% homozygous transgenics, 50% hemizygous transgenics, and 25% homozygous wild type (Rahman *et al.*, 2000).

The scenario outlined above is very much a generalization based on micro-injection of tilapia eggs. Higher numbers of G0 transmitting fish can be routinely produced in species such as medaka by particular electroporation procedures (Hostetler *et al.*, 2003) or by the use of methods involving coinjection with nuclear localization signal peptides (Collas and Alestrom, 1998).

Exploitation of the yeast meganuclease enzyme involving its coinjection and insertion of a recognition sequence in the modified transgene sequences has also been used to good effect to greatly increase the efficiency of transgenic induction in medaka (Thermes *et al.*, 2002). However, it is important to stress that these additional methods for improving the efficiency of production of transgenic fish have only been tested with a limited range of fish species and probably do not work well with all.

When transgenic fish were first produced there were considerable anxieties about whether cytoplasmically injected transgenes would ever be chromosomally integrated, whether integrated transgene copies might be subsequently lost, whether integrated transgenes would be routinely silenced as a result of DNA methylation, and whether transgenic fish would frequently suffer from the effects of insertional mutagenesis through random integration. Despite the fact that integration of transgene copies does seem to be random, none of these early anxieties have proved to be substantial problems, and stable lines of transgenic fish of many species have been produced fairly readily and with few attendant problems.

Some further aspects of the technology and variations of the particular approach taken deserve mention. These can be usefully listed as follows:

(i) Reporter genes

It is common to use reporter genes in fish in order to measure the

strength and tissue specificity of particular promoters. The commonly used reporter genes include *CAT* (chloramphenicol acetyl transferase), *LUC* (firefly luciferase), *lacZ* (beta-galactosidase), and *GFP* (green fluorescent protein).

(ii) **Use of transposable elements to enhance integration**
The work of Ivics *et al.* (1997) in reconstructing the 'Sleeping Beauty' transposon for use in GM fish technology is especially noteworthy.

(iii) **Lack of *in vitro* lines of fish embryonic stem cells (ES cells)**
Despite much work in both zebrafish and medaka, there is currently no usable cell line which can be utilised for work with chimeric embryo transfer as a means of achieving gene knock out. This methodology, which has proved so effective and valuable in work with mice (Alberts *et al.*, 2002), is not available in fish systems, and so prevents the development of lines of GM fish in which specific gene function has been ablated.

(iv) **Lack of functional use of RNAi**
The ability to use the so called RNAi (double stranded RNA) techniques in *C. elegans* (Fire *et al.*, 1998) and subsequently in mice (Wianny and Zernicka-Goetz, 2000) to block specific gene expression is unfortunately non-effective in fish (Zhao *et al.*, 2001).

(v) **Somatic transgenesis in fish**
Although transgenesis via the introduction of novel genes into fertilised eggs is the common route for gene transformation and expression in fish, it has proved possible to obtain gene expression by the injection of transgenes into skeletal muscle of adult or sub-adult fish. This not only allows fish to be used as models of gene therapy in humans, but also permits the administration of DNA coding for vaccine protein, thus allowing so-called DNA vaccination. Examples of this work include Anderson *et al.* (1996).

3.3. Species Involved and Lines Produced

Fish species which have been subjected to GM technology fall into two groups. The first group are species which offer no commercial potential but are good model species, allowing experimental work on gene regulation and developmental biology, as well as proving useful for the preliminary testing of gene constructs which will subsequently be used in other fish species of commercial significance. This fish group consists of zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and goldfish (*Carassius auratus*).
The second group are finfish species which may be used as model experimental systems but are also of potential commercial significance. Such species include Atlantic salmon (*Salmo salar*), coho salmon

(*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), Arctic charr (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*), channel catfish (*Ictalurus punctatus*), northern pike (*Esox lucius*), loach (*Misgurnus fossilis*), and mud loach (*Misgurnus mizolepis*). The details of work on these species will be found later in the review.

3.4. Research Objectives – Present and Future

Aside from the use of transgenic fish to explore problems in genetics and developmental cell biology, there are a number of more commercially orientated objectives to consider. These include growth enhancement, improved disease resistance, improved cold tolerance and freeze resistance, more efficient metabolism, sterility and the use of fish as biofactories. Since the application of any of these research goals could impinge on biosafety, they will be briefly considered in turn.

3.4.1. Growth enhancement

The mice that were produced in the Palmiter *et al.* (1982) work, and the further experiments that followed on from this approach, were larger than their non-transgenic littermates. This was because the promoter sequence used to drive the growth hormone (GH) coding gene in the construct resulted in GH expression from the liver rather than, in the non-transgenic, from a small part of the pituitary tissue. This outcome was accomplished by the use of a liver-specific promoter normally associated with a gene for metallothionein. In a similar way, fish of various species have been successfully growth-enhanced by combining a GH coding sequence with a promoter which is widely expressed. Such promoters include those for metallothionein (a metal binding protein), beta actin (a ubiquitously expressed cytoskeletal protein) or antifreeze (a protein made in the livers of a number of species of Arctic fish and subsequently secreted into the blood). Fish growth is very plastic, and in species such as carp, is more or less continuous through life. It is also the case that few fish species have been subjected to long term selection for growth as have cattle, sheep and pigs. For these and other reasons, some fish show dramatic growth responses of more than ten fold when made transgenic for GH constructs. This is especially true of Atlantic salmon (Du *et al.*, 1992) and coho salmon (Devlin *et al.*, 1995). Mud loach also show dramatic growth enhancement (Nam *et al.*, 2001). Since dramatically increased growth may produce skeletal abnormality, it is often desirable to aim more conservatively for a doubling or trebling of growth. It is of course increased growth rate that is

usually commercially attractive, rather than very large final size. Increased growth of tilapia (*Oreochromis niloticus*) of two or three fold at seven months has been achieved (Rahman *et al.*, 1998 and 2001) and this is probably a fairly optimal outcome. There is evidence to suggest that some species such as common carp show less dramatic growth increases, perhaps because they have already been selected for optimal growth over several centuries (Zhang *et al.*, 1990). Since the dramatic increase in size of fish is probably not commercially desirable and may lead to abnormality, it is often beneficial to select GM strains which have only single novel GH genes added to the genome and which only show modest increases in circulating GH levels. Since promoters from many different fish species are now available, there has been a recent trend not only to use constructs of entirely fish origin, but even to use constructs entirely derived from the same species as the resulting GM fish. This so-called 'autotransgenic' approach has been followed successfully by Nam *et al.* (2001) with mud loach, and similar work with tilapia is under way in our own laboratory. Fish of other species in which growth enhancement has been attempted include Arctic charr (Krasnov *et al.*, 1999), northern pike (Gross *et al.*, 1992), rainbow trout (Chourrout *et al.*, 1986), and channel catfish (Dunham *et al.*, 1987).
There is no evidence to date that growth-enhanced GM fish have been used commercially, but some have been exposed to limited field trials in a contained situation (Rahman *et al.*, 2001).

3.4.2. Improved disease resistance

It has not proved easy to design GM fish with improved disease resistance, largely because genes conferring this quality are hard to identify. This is unfortunate in that aquaculture involves the culture of fish at high density and so disease spreads easily and is often hard to combat. Indeed if there is one quality to which GM technology could most usefully contribute in fish, this would surely be it. There are two examples of attempts to develop strains of fish with increased disease resistance. One is the work of Hew *et al.* (1999) in producing GM Atlantic salmon with rainbow trout lysozyme cDNA, driven by an ocean pout antifreeze promoter. This lysozyme has already been demonstrated to have antimicrobial properties against a range of Gram-negative bacteria such as *Vibrio* and *Yersinia* sp. (Grinde, 1989) which are also fish pathogens. Unfortunately no positive outcome has been reported from this work. A more promising outcome has emerged from the work of Dunham *et al.* (2002) in which channel catfish were made transgenic for cecropin genes. Cecropins are natural anti-microbial proteins found in insects. An increased level of disease resistance has been reported for these fish.

3.4.3. Improved cold tolerance or freeze resistance

There are really two separate traits, cold tolerance being the ability to thrive at temperatures above 0°C but below the normal physiological limits for that species, while freeze resistance is the ability to stop ice formation in tissues if the fish is in sea water at less than 0°C. The phenomenon of cold tolerance is of great commercial significance in carp culture in China north of the Yangtse river, where, in abnormally cold winters, severe losses occur with common carp. Similarly, large numbers of tilapia have died in Israel when winter temperatures take water temperature below 10°C.

Freeze resistance is a common and remarkable property of many Arctic and Antarctic fishes, in which either seasonally or continuously, antifreeze proteins are secreted into blood and other tissues and prevent the seeding of ice crystals. Useful reviews of this topic are those of Davies *et al.* (1989a and 1989b). The commercial interest in making GM fish which are resistant to freezing stems from the desire to culture Atlantic salmon in sea cages in northern Newfoundland. Severe losses can occur in some winters when icebergs float southwards.

The evidence that fish can be made more cold tolerant by GM technology rests on the observation that goldfish which are GM with respect to an antifreeze gene construct are protected from the deleterious effects of being cultured at low temperatures (Wu *et al.*, 1998). On the face of it, this is hard to understand, but one explanation offered is that antifreeze synthesis may reduce membrane permeability and so afford some physiological benefit.

Strains of GM Atlantic salmon have been produced which express antifreeze from integrated copies of an antifreeze gene from the winter flounder (*Pseudopleuronectes americanus*) by Fletcher *et al.* (1992) and Hew *et al.* (1999). Although integration, expression and transmission of the antifreeze genes were achieved, the level of antifreeze protein produced remains low and no serious freeze resistance is evident in the GM fish.

3.4.4. Altered metabolism

This parameter is a good example of an area of potential improvement to cultured fish which has been somewhat neglected in terms of attempted GM solution. Salmonid fish, now cultured intensively in China, Japan, Chile, Europe, US and many other countries, are of course essentially carnivorous. They also have rather poor abilities to utilise carbohydrate, especially that of plant origin, despite the presence of the normal insulin-active pathway of carbohydrate metabolism (Wilson, 1994). A research group in Finland is involved in attempting to alter the metabolism of Arctic charr (*Salvelinus alpinus*) with respect to hexokinase and glucose

transporter genes, using GM technology (Krasnov *et al.*, 1999). Since in Europe the intensive culture of Atlantic salmon is almost entirely dependent on their being fed with fish such as sand eels and capelin, the end result of this intensive industry is a profound change to the ecology of the North Sea, especially in terms of sea bird populations. Altering salmonid fish metabolism so dramatically will not be easy, but the results could bring huge benefits.

Dr Heather Hostetler, Dr Bill Muir and others at Purdue University, Indiana, USA (personal communication) have also produced medaka which are GM with respect to a phytase gene from the fungus (*Aspergillus niger*), in order to look for an improvement in their ability to utilise phytate in their diet as a source of phosphorus. Although the GM fish survived better than controls, no change in growth rate was detected.

3.4.5. Sterility

Since, as will be discussed later in this review, sterile fish have a key role to play in the commercial exploitation of GM fish, this is an important parameter to consider. Non-transgenic sterility can of course be accomplished by the induction of triploidy; again this will be discussed later. Sterility via GM will only be a useful genetic development if it can be made reversible, so that brood stock can be retained for production of further sterile progeny. Since there is currently no immediate prospect of gene 'knock-out' in fish via the exploitation of embryonic stem cell lines to accomplish this end, it is necessary to consider other ways of achieving sterility via gene 'knock-down'. This has been reviewed in relation to tilapia in Maclean *et al.* (2002). As discussed in that paper, there are three known approaches to gene knock-down, namely the use of RNAi (double stranded RNA), the use of ribozymes, and the use of antisense RNA.

The present situation is that RNAi is ineffective or problematical with fish (Zhao *et al.*, 2001) and some work in our own laboratory supports this conclusion. The outlook for ribozymes is not greatly different, in that no laboratory has demonstrated its effective use in fish.

Turning to antisense RNA, the protocol here is most obviously to target an essential reproductive hormone such as gonadotropin (GtH) or gonadotropin releasing hormone (GnRH) by antisense knock down of the relevant message, and to treat chosen potential brood stock fish hormonally to restore fertility. A French group have reported some success with this methodology in rainbow trout (Uzbekova *et al.*, 2000), and our group have also had potential success in tilapia (Maclean *et al.*, 2002).

A more considered discussion of the future prospects for GM sterility in fish will be found in section 4.1 of this paper.

3.4.6. Fishpharming – the production of valuable human pharmaceuticals from fish

For many reasons fish make good candidates as model animal systems in which valuable human proteins may be transgenically expressed. Some of these reasons are that fish are generally cheap, easy to culture intensively, often have short generation times and have more or less continuous reproductive activity. In addition, work on fish is often deemed to be more ethically acceptable than using mammalian or avian models, and there is no present evidence for the replication or transfer of prions in and from fish.

There are a number of possible scenarios by which interesting and valuable proteins can be synthesised in and recovered from fish. One is to make such proteins in fish eggs and recover from the eggs, another is to organise synthesis in the embryo and recover from embryos, and the third is to develop lines of GM fish in which a particular organ or tissue is the site of synthesis and thus recovery of the protein from the specific organ is the final step. Although many laboratories have utilised a range of biofactory model species, including bacteria, yeast, plants, insect larvae, chickens and mammals (see Houdebine, 2000 for review), as far as we know only our own group has followed this line of work in fish. Two procedures have been used, both involving the production of human factor VII, a blood clotting factor that is activated by tissue factor following internal tissue injury. In the first, human factor VII was expressed in and recovered from tilapia embryos, following egg injection with transgene copies. Although, only modest amounts of protein were recovered in the pilot experiments, the system seems to us to hold great promise (Hwang *et al.*, 2004).

In the second procedure, in a collaboration with Aquagene Inc., Florida, lines of GM tilapia have been produced in which human factor VII is synthesised in the liver. The transgene used consists of a cDNA for human factor VII driven by a tilapia vitellogenin promoter. Factor VII is secreted into the blood, from which it is readily purified. Although substantial production levels have yet to be achieved and the folding and glycosylation of the resulting protein verified, it has already been shown to efficiently clot human cells, and work is on going.

3.4.7. GM fish for therapeutic use

Some interesting experiments have been carried out by Professor James Wright, Pathology Department of Dalhousie University, Canada (personal communication). The plan is to use tilapia pancreas tissue as a source of human insulin for transplant into diabetic patients. The insulin gene of the tilapia has been 'humanised' by insertional mutagenesis so that the insulin produced in the islet cells of the fish is the same as human insulin.

3.5. Commercial Exploitation

Up to the present time there are no examples known of the commercial exploitation of transgenic fish, except for strains of zebrafish sold through the pet trade which are GM with respect to the reporter gene red fluorescent protein. These fish, marketed as GLOFISH in the US, will be discussed separately at the end of this section. There have been numerous rumours of the commercial production and use of GM common carp in China and GM tilapia in Cuba, but these reports have been officially denied in both cases.

GM Atlantic salmon owned and produced by AquaBounty Farms Ltd, are the nearest to commercial use and the Company has applied to the US FDA for permission to develop and market these fish. The ruling of the FDA on this matter is currently awaited. The application is for the use of all-female triploid fish to be used, thus giving a very high degree of biological containment through sterility.

Semi-field trials of various GM fish have been undertaken, notably with channel catfish in Alabama, USA (Dunham *et al.*, 1992) and tilapia in Szarvas, Hungary (Rahman *et al.*, 2001).

The Glofish Development

Glofish is the trade name given to a line of transgenic zebrafish, GM with respect to a red fluorescent protein, the gene of which has been recovered from jellyfish species. They are marketed by Yorktown Technologies US and the relevant website is [http://www.glofish.com]. Perhaps surprisingly the FDA found no reason to regulate these fish and so they are freely available to the pet trade. A number of experts provide signed statements on the website stating that in their opinion the fish do not pose a potential environmental threat since they appear to be less fit than wild-type zebrafish and so, if they escaped and interbred in the wild, progeny would not survive for long. As stated by Professor William Muir of Purdue University, "GFP has a significant net fitness disadvantage, indicating that one would expect natural selection to eliminate the transgene regardless of where it escaped or was released". Red fluorescent protein (RFP) is closely related to green fluorescent protein (GFP).

3.6. Benefits and Risks associated with GM Fish

3.6.1. Benefits

The main benefit of the GM technology is that it allows genetic traits to be modified, enhanced, or negated on a gene by gene basis. In contrast,

conventional breeding, whereby selection over a number of generations leads to improvement in one genetic parameter, suffers from the fact that other genetic traits may be lost or uncovered accidentally during the selection, so that the new breed has one distinct advantage over the original brood stock but also may have new disadvantages. Examples abound, such as in plants, loss of perfume in garden plants, loss of fungal resistance in crop plants, and in animals the hip problems of golden retriever dogs, the eye problems of Hereford cattle, and the lambing difficulties encountered with Border Leicester sheep.

A particular aspect of the ability to add or delete one gene at a time is also that the novel gene can be teamed with a different promoter, so ensuring an altered pattern of gene expression, and that the gene can be isolated from quite unrelated species, so that the ability to make say, antifreeze protein is bestowed on Atlantic salmon from winter flounder. All of the traits listed and discussed in the earlier part of this section clearly have the potential to be substantial benefits.

3.6.2. Risks

A) There is the possibility that the incorporation of a novel gene on a random basis within chromosomal DNA could lead to unforeseen genetic defects due to insertional mutagenesis. This seems to be infrequent in fish (although, if lethal, it can be easily missed in a low % embryonic survival), presumably due to the large amount of non-functional DNA in the genome.

B) Position effects may result from incorporation, such that genes adjacent to the incorporated novel gene now have changes in their regulation or expression levels. Again this has not been reported in GM fish, but could be overlooked and is certainly a theoretical possibility. An extreme form of this phenomenon is the activation of an oncogene by chromosomal translocation in humans.

C) GM organisms, especially fish, can often incorporate multiple concatenated copies of transgenes, leading to overexpression of the trait of interest. This is especially evident in some growth-enhanced lines overexpressing growth hormone.

D) There is a theoretical possibility that incorporated transgene copies could be subsequently lost, or silenced by DNA methylation. There are certainly examples of non-expressed transgenes in some lines of fish, but not, as far as we know, of gene elimination or silencing of a previously active transgene.

E) GM fish may have the capacity to become a pest species on escape or introduction. This will be discussed later.

3.6.3. Some genetic considerations in evaluating the benefits and risks

A) Transgenes do not automatically acquire new sinister or deleterious properties as a result of being isolated and built into new constructs. So much of the suspicion in the mind of the public about the GM technology is misplaced in this regard.

B) Genes coding for viral proteins or antibiotics are not currently used to produce GM fish, although some viral promoters have been used in the past, as, for example, to produce GM tilapia in Cuba (Martínez *et al.*, 1996).

C) Most gene constructs used in GM fish production, other than in model species such as zebra fish and medaka, are entirely of fish origin and some are autotransgenic constructs in which all sequences come from the same species as that subject to the GM transformation (Nam *et al.*, 2001).

D) As far as is known, transgenes are integrated randomly into the fish genome, although there may be sites that favour integration.

E) Most GM fish do not reveal physiological or anatomical abnormalities, but some have been recorded (Devlin *et al.*, 1995). The truth of this statement depends heavily on the precise definition in this context of the word 'abnormality'.

F) The protein products of transgenes used in fish do not differ from other proteins currently found in fish (except in the case of some reporter gene products) and so are extremely unlikely to raise immune response problems if eaten. However, all GM fish need to be tested on a case by case basis.

G) If GM fish escape or are released, there is the possibility of their breeding with conspecifics and thus the transgene may introgress into wild stocks. There are three scenarios here. One is that the GM fish are sterile or incapable of long term survival, in which case any deleterious effects are transient and due only to competition with wild fish. A second possibility is that the GM fish are fertile but no conspecifics are present in the location into which release or escape has occurred. This could result in novel fish becoming established and also being GM, although their GM status would have little or no relevance. The third is that some gene transfer could occur through GM fish interbreeding with wild type fish, in which case the long term consequences need to be considered very seriously. In general GM fish are considered to be less fit than the wild type (Abrahams and Sutterlin 1999; Jönsson *et al.*, 1996) but some models have suggested that gene introgression could be very deleterious to wild stocks (Muir and Howard 1999; Hedrick 2001).

H) Some sections of the public view GM technology as inherently flawed because it is unnatural. In general this rests on a misunderstanding of other more conventional agriculture as being natural. The level of unnaturalness

involved in GM is arguably less than that involved in current methods of embryo transfer used widely in agricultural stock breeding.
I) Fish do not harbour retroviruses, nor other viruses transmissable to humans. Only inert transposable elements have been identified in fish to date (Ivics *et al.*, 1997) and no active prion proteins are known in fish. However, vigilance is clearly necessary.

4. SAFETY CONSIDERATIONS

4.1. Threats to Aquatic Ecosystems

As outlined briefly in section 3.6.3. of this review, there are a number of alternative scenarios to consider, depending on whether the GM fish are sterile or reproductive, on whether non-transgenic conspecifics are present in surrounding water, and of course depending on the level and type of containment employed.

Effective containment

Enclosed water systems offer one form of effective physical containment, provided that there are rigorous measures in place to control escapes to the wild, or accidental movement of eggs or small fish by bird predation, or theft (a further threat with GM fish is possible interference from Animal Rights Activists, especially in Europe. GM crops have a chequered history in this respect, which should serve as a warning). Countries such as Canada and Finland have numerous landlocked lakes which offer opportunities for contained culture of salmonid fish while countries such as Iceland and Hungary have warm geothermal water sources surrounded, especially in winter, by cold water which would not allow survival of species such as tilapia. This type of containment was exploited by Rahman *et al.* (2001) for the growth testing of GM tilapia.
Where natural conditions fail to offer effective containment, this can be achieved by construction of elaborate barrier systems. This has been implemented for the experimental culture of both carp and channel catfish in the United States, as reviewed by Donaldson (1997).
Sometimes the effective biological sterility of an introduced species allows containment, and much the best example of this is the introduction and release of rainbow trout in European waters, in which breeding records are rare. It appears that the European aquatic environment lacks some cues that are essential for reproduction in this species (Maitland and Campbell, 1992).
Biological containment via induced sterility becomes especially significant in the context of GM fish. Producing sterile fish via triploid induction is long

established and was reviewed by Benfey (1999). Triploidy is normally induced by heat shocking or pressure shocking of the fertilised eggs and these methods have been widely applied to salmonids (Benfey and Sutterlin, 1984). The efficiency of triploid induction varies greatly with species and is often less than 100%. In addition, the triploid fish, although usually showing greatly reduced gonad formation, may be partially fertile, especially in the case of male fish. To optimise sterility, triploid induction can be combined with sex reversal to produce all-female progeny, and this is the scenario used in the production of the AquaBounty Farms lines of GM growth enhanced Atlantic salmon. Whether sterility in even these most optimized conditions can be reliably 100.00% is arguable, however. In fish such as tilapia this procedure is not attractive since the male tilapia is much the more marketable fish.

Transgenesis itself can be implemented to produce sterile fish, as reviewed by Maclean *et al.* (2002) in the case of tilapia. This approach involves blocking expression of a hormone which is essential for gonadal development, such as gonadotropin (GtH) or gonadotropin releasing hormone (GnRH), and rescuing brood stock fish by intra-muscular injection of the relevant reproductive hormone. In the absence of gene 'knock-out' methods in fish, it has been necessary to rely instead on targeting of the specific messenger RNA, so called gene 'knock-down'.

Although double stranded RNA (RNAi) and mRNA targeting via ribozymes have to date proved ineffective in fish, some positive results have been reported for the use of antisense strategy against specific mRNAs. This is particularly true for antisense RNA against GnRH, as reported by Uzbekova (2000) and Maclean *et al.* (2002) in rainbow trout and tilapia, respectively.

For the present the conclusion has to be that sterility via transgenesis is still in the development stage, and sterility by triploidy induction remains, at least in some species and circumstances, problematical.

If sterility is absolutely watertight, then it would allow the use of GM fish even when conspecifics were present, which is always the most risky situation. If conspecifics are not present in the environment, sterility would still be attractive to prevent the GM fish establishing as an exotic introduction.

It can be concluded that the risks associated with these various scenarios are more or less as follows:

Sterility	Risk
Use of completely sterile or completely contained GM fish.	No Risk / Minimal Risk.
Use of fertile GM fish where conspecifics do not occur, thus allowing possible establishment of exotic GM fish.	Risk low but significant, depending on fitness levels and chance of pest status.
Use of fertile GM fish where conspecifics occur in neighbouring water.	Risk high because of transgene introgression, but dependent on fitness levels of GM as compared to wild type fish.

The different situations and risks have been considered by Kapuscinski and Hallerman (1991), Maclean and Laight (2000), Muir and Howard (2001) and Knibb (2002).

4.2. Modelling the Threats

Attempts have been made to use computer modelling to assess the likely level of risk of release or escape of GM fish, and to predict likely outcomes. The papers of Donaldson (1997) and Maclean and Laight (2000) are particularly relevant. In these modelling exercises, much depends on the presumed fitness of the GM fish, whether hemizygous and homozygous for the transgene. Although there is a body of experimentally based literature indicating that GM fish do have reduced fitness when compared to wild type conspecifics, it is not possible to be certain that this outcome will always be the reality. The evidence in this regard is as follows.

Jönsson *et al.* (1996) showed that when rainbow trout were treated by injection with ovine growth hormone, they spent more time than control fish in the upper water columns and showed poorer predator avoidance behaviour than controls. In 1997 Farrell *et al.* demonstrated that growth enhanced GM coho salmon had poorer swimming speed than control fish of the same size, but similar work by Stevens *et al.* (1998) was less conclusive. Work continues on these topics (Johnsson and Björnsson, 2001). Caution is essential in assuming a universal fitness disadvantage in GM fish, especially if dramatic new properties such as freeze resistance were imposed on species such as Atlantic salmon.

The most serious attempts to model and predict outcomes from the

release of GM fish have come from the laboratory of Muir and Howard in Purdue, Indiana. See Muir and Howard (1999 and 2001). These authors have based their assessment partly on computer modelling and partly on lab experiments with medaka. Their Trojan gene hypothesis stems from this work. It envisages growth enhanced GM male fish being preferentially selected for mating by wild type females on the basis of large size, but being of reduced fitness. As the transgene introgresses into the wild stock fish, the fitness is reduced and survival diminishes, leading to possible extinction. This outcome was not precisely followed in the tank experiments with medaka because of technical difficulties. It is crucial to stress that the prediction only holds for growth enhanced transgenic fish in which females select mates on size chiefly or only, and where the transgene brings with it reduced fitness. Unfortunately there has been a tendency to extrapolate from this conclusion to one in which all GM fish are presumed to carry an extinction probability in their genetic enhancement.

4.3. Food Safety Implications

All of the GM modifications to fish involve genes which code for proteins, and clearly if GM fish are eaten, their proteins, including the products of the transgenes, will be digested in the usual way. The proteins produced to date in GM fish include GH, lysozyme, cecropin, antifreeze and reporter gene products such as lacZ and green fluorescent protein. The most recent lines of GM fish that have been developed are transgenic only with respect to sequences from the genome of the same fish, so no novel proteins are involved. Important considerations with respect to the development of GM fish which are destined for the food market are as follows.

(i) If genes coding for antibiotics or antibiotic resistance were included in transgene constructs, then such protein could affect the intestinal flora of the consumer adversely. To our knowledge no such genes have been used in potentially commercial strains of GM fish. The antibiotic resistance gene *neo* (a gene of bacterial origin which confers neomycin resistance and is commonly tested with the neomycin analogue G418), has only been used within the last decade with model species such as medaka and zebrafish.

(ii) Some fish genomes, in common with genomes of species from other animal groups, harbour genes which code for toxic proteins. If any of these proteins are coded in the fish genome (some, such as those of the widely eaten but potentially lethal Japanese puffer fish, Fugu, are acquired by the fish secondarily), then clearly such genes must never be implicated in the GM technology.

(iii) Genes or regulatory sequences of viral origin should be avoided,

although viral promoters have been employed in fish transgenes in the past.

(iv) There is a theoretical possibility that transgene-derived proteins could be immunogenic to some people, in much the same way as some individuals are hyper-sensitive to nut proteins or Crustacean proteins. However, none of the proteins involved in GM fish production are known to present such problems.

(v) Although there are no known reasons why GM fish should prove harmful or have an altered appeal as food, some have been tested in this regard in order to provide public reassurance. This has involved GM tilapia in Cuba (Guillén *et al.*, 1999) and GM trout in Canada (Entis, 1998).

(vi) Reporter genes, although often useful in the early stages of GM fish experimentation, are clearly best avoided in the production of the final fish. Thus, although lacZ protein is no-doubt synthesized by *E. coli* in the normal human gut, it is best to be omitted from construct design.

4.4. Adverse Effects on Fish

A very legitimate aspect of public concern is the possible deleterious effect of intensive agriculture or aquaculture on the animals themselves. There are two aspects to this. One is whether the fish are exposed to adverse conditions during the development of the technology, the other whether the resulting fish are themselves "happy animals". In the UK the Home Office is the Government Arm which monitors all GM fish. In addition, each experiment must be first scrutinised by a local ethical committee prior to the Home Office granting a specific license for the programme of work. Regulations in other countries are highly variable. What is uppermost in the factors considered by the Home Office in granting licenses is the welfare of the fish.

Since the GM technology obviously has the power to alter the physiology and anatomy of the fish, it is clearly important to question the effects of GM induction on the fish. Undesirable complications of growth enhancement have been reported by Ostenfeld *et al.* (1998) in GM Pacific salmon. However, it is important to stress that the GM technology is likely to parallel the outcome of conventional animal breeding by selection. This outcome is that in which the breeder aims for improvement, but not every line produced is improved, and some may show unexpected undesirable properties. But that is the unavoidable outcome of genetic reassortment. But the converse is surely that the transgenic technology will not, and should not be permitted, to produce the somewhat grotesque and anatomically misshapen fish that have been produced in the past by

conventional selection for 'fancy' goldfish. This is especially important in the context of growth enhancement, where the objective should be moderate growth enhancement resulting from small increases in the levels of circulating growth hormone.

5. RELEVANT LEGISLATION AND REGULATION

5.1. The European and UK Perspective

Each European country has its own regulations but most are also bound by EU ones. However, while each member state of the Union is bound to implement the EU regulations, they each interpret them slightly differently. Thus Spain is at an advanced stage of growing GM crops and does not push strongly for labelling. Also it is crucial to remember that not all European states belong to the EU: an important exception is Norway.

In European terms the most important documents are the Cartagena Protocol on Biosafety, signed in January 2000 following the Rio summit and declaration on Environmental Issues, and the directive on deliberate GMO release, signed by the European Parliament on March 2001.

The key UK documents are as follows.

(i) April 1994 issue by the Dept of Environment entitled "Genetic modification of Fish – A UK Perspective". This statement was rushed out in response to the brief activity of Otter Ferry Salmon in Scotland to produce GM salmon.

(ii) Guidance notes published by the Dept of the Environment, Transport and the Regions (DETR/ACRE Guidance Notes 12) in November 1999.

(iii) Report by the Royal Society UK in May 2001 entitled "The Use of Genetically Modified Animals".

This report picks out future developments with GM fish for special mention. "An environmental concern is the escape of GM fish and their breeding with the natural population…..phenotype changes due to the genetic modifications may provide the GM animals with a competitive advantage over their wild relatives for food, shelter, mates, and suitable breeding sites". And again "Despite the potential for sterilising GM fish, the Royal Society of Canada, in its recent report on biotechnology and food, concluded that the consequences of genetic and ecological interaction between GM and wild fish were uncertain as was the utility of attempting to render GM fish sterile. In particular, the Royal Society of Canada recommended a moratorium on rearing GM fish in aquatic net-pens, with approval for commercial production being conditional on rearing of the fish in land-locked facilities. The Royal Society endorses this recommendation".

(iv) Advice of the Advisory Committee on Release to the Environment (ACRE) in July 2001.

5.2. Definition of What is and What is not GM in the EC Documentation

What is included – (1) recombinant DNA technology, (2) technology involving direct introduction into an organism of heritable material prepared outside the organism and (3) cell fusion (including protoplast fusion) or cell hybridisation where live cells with new combinations of genetic material are formed… by means of methods that do not occur naturally.

What is not included – (1) in vitro fertilisation, (2) natural processes such as conjugation, transduction and transformation, (3) polyploidy induction

5.3. EC Rules for Member States on Acceptance

(I) "When product containing a GMO, as or in products, is placed on the market, and where such a product has been properly authorised under this Directive, a Member State may not prohibit, restrict or impede the placing on the market of the GMOs as or in products…."

(II) Labelling – All GMOs and GMO products must be clearly labelled as such. It is worth emphasizing that there is an increasing awareness within UK and EU that with respect to both labelling and testing of GMOs for adverse effects, present regulations for GMOs go far beyond those currently in place for non-GMOs.

5.4. Levels of Risk with GM Fish implicit in DEFRA Regulations in UK

Minimal – tilapia

Low – rainbow trout

Median – carp and salmon

5.5. Particular References and Websites relevant to GM Regulation

1. Directive 2001/18/EC of the European Parliament of 12/3/01 on the deliberate release into the environment of genetically modified organisms. http://europa.eu.int/eurlex/pri/eu/oj/dat/2001/1_106/1_10620010417en00010038.pdf

2. The use of genetically modified animals. The Royal Society 2001. http://www.royalsoc.ac.uk/policy/index.htm

3. Adoption of the Cartagena Protocols on Biosafety 2000 (CPB; http://www.biodiv.org/biosafety)

4. Jank, B., and Gaugitsch, H. (2001). Decision making under the Cartagena Protocol on Biosafety. TRENDS in Biotechnology 19: 194-197.

5. DEFRA Statutory Instrument on Genetically Modified Organisms

(Deliberate Release)
http://www.hmso.gov.uk/si/si2002/20022443.htm
6. ACRE: Advisory Committee on Release to the Environment
http://www.defra.gov.uk/environment/acre/pubs/gmanimals.htm
7. Maclean, N. (2003b). Genetically modified fish and their effects on food quality and human health and nutrition. TRENDS in Food Science and Technology 14: 242-252.

6. CONCLUSION

Nothing is without risk and most scientists and administrators have learnt hard lessons about how to respond to naive questions from the press along the lines of "Is there a risk"? But quantifying risk, especially in the context of GM fish, is extremely difficult. Culture of non-GM fish carries numerous risks, as does the sale and consumption of the resulting food product. It is undeniable that GM fish add to these risks. If and when commercialisation of GM fish occurs, then clearly each GM line will have to be separately authorised. Necessary information prior to authorisation will have to include full details of the transgene sequence used, any known genetic position effects or physiological changes in the fish, and precise details about the novel proteins now present than are not present in equivalent non-GM strains.

As mentioned previously, not the least frustrating aspect of the necessary legislation is that the screening and labelling of GM fish will require information and testing that is not required of non-GM fish. It is therefore impossible to make direct comparisons in most cases. So labelling of GM products, although obviously sensible, does not provide a level playing field.

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