

5. Genetically modified fish and their effects on food quality and human health and nutrition

Norman Maclean

School of Biological Sciences, University of Southampton, Biomedical Sciences Building, Basset Crescent East, Southampton, SO16 7PX, UK (tel.: +44-23-8059-4403; fax: +44-23-8059-4269; e-mail: nm4@soton.ac.uk)

5.1. Introduction

Fish (finfish) of many species have been subjected to genetic modification over a period of more than 10 years, but only in the last few years have any begun to be considered seriously for the food market. The main species involved are Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), common carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus*), and channel catfish (*Ictalurus punctatus*). Although the main parameter modified to date has been growth rate, other traits that are currently being worked on include cold tolerance, disease resistance, and sterility. This review considers what has been achieved to date, possible commercial uptake, and likely consequences for the environment and human health and nutrition.

In both the developing and the developed world fish constitutes a very important part of diet, in terms of both quantity and contribution to nutritional requirements. The total fish catch in the world (inclusive of fish, crustaceans and molluscs) in 1999 was estimated to be over 92 million metric tonnes (mt), while the yield

from aquaculture (fish farming) was over 33 million mt (FAO, 2000). Most of the capture fishery is from marine areas (84.6 million mt) the remainder being from fresh water. Marine capture fisheries outputs peaked in 1996 and 1997 and, despite increased effort, are now showing signs of decline because of stock depletion from over-fishing (Hutchings, 2000). Locations and amounts of fish capture in 1999 were 40 million mt from Asia, 16 million mt from South America, over 15 million mt from Europe, 7 million mt from North America and 3 million mt from Africa. The north west Pacific region accounted for over 24 million mt. The ten countries with the largest yields (in million mt) from capture fisheries in 1999 were China (17), Peru (8), Chile and Japan (5 each), Indonesia, Russia and USA (4 each), India and Thailand (3 each) and Norway (2). The ten countries with the greatest aquaculture production (in million mt) in 1999 were China (22), India (2), Japan (0.7), Bangladesh, Indonesia, Thailand and Vietnam (0.6 each), Norway and, USA (0.5 each) and Philippines (0.3).

Aquaculture is one of the fastest growing food producing sectors, and is gradually replacing the deficit in world capture fisheries that has been caused by over-fishing. Fish is a major source of animal protein in most developing countries and, in developed countries, the interest in the dietary benefits of fatty fish, such as tuna, eel, mackerel, herring and salmonids, and especially the presence of omega-3 polyunsaturated fatty acids in such fish, ensures a constant, health-driven incentive to consume more fish.

In the context of both the dietary benefits of fish consumption and the increasing contribution made to fish production by aquaculture, it is clear that aquaculture is now undergoing and will continue to undergo a revolution in quality and quantity of production similar to that which has driven the improvement in agricultural stock animals for the past few hundred years. Since fish can be readily improved by application of transgenic technology, it is clearly timely to consider what genetically modified (GM) fish are likely to offer in the future, both in terms of benefits and disadvantages. A very useful recent report by the Royal Society (UK) on the use of GM animals is available.¹

¹ Royal Society (2001) *The Use of Genetically Modified Animals*, London, UK, The Royal Society, available [August 2002] at <http://www.royalsoc.ac.uk/policy/index.html>.

5.2. Contribution of transgenic technology in fish

5.2.1. Details of transgenic technology in fish

Since fish are the most primitive vertebrate class of animals, it is arguable that people have fewer moral reservations about genetic modification being applied to fish than to birds or mammals.

It is as well at the outset of this chapter to set out how transgenic technology is applied to fish and its particular benefits and drawbacks in this class of vertebrates.

By far the most common method of introducing transgenes into fish is by microinjection of one-celled fertilised eggs. Other methods of introduction, such as electroporation, gene (particle) gun, and liposome-mediated gene transfer, have been attempted but have generally proved less reliable than microinjection (Maclean, 1998a). Fish eggs can be hard to inject in species with firm chorions, such as salmonids, but often the egg micropyle provides an easy route into the egg for the microneedle. The fertilisation pronuclei are relatively small, so microinjection has to have a cytoplasmic rather than a nuclear target. Also, since fish eggs are mostly very yolky, deposition of the transgenes in the cytoplasm overlying the yolk requires a high level of manipulative precision. As penetration into the nucleus is relatively rare for the injected DNA copies, high copy numbers are usually injected, from 10^5 to 10^6 per egg. Injection into the egg often requires use of a special micromanipulator to guide the needle into the egg and the use of a 'gene pulsar' to ensure injection of the correct volume of DNA solution under appropriate pressure. The DNA injected is generally linear, without plasmid sequences, and indeed in recent years, most gene constructs injected have been 'all fish', meaning that the regulatory promoter, coding sequence, and 3 prime polyadenylation sequence are all of fish origin. The coding sequence may be genomic or cDNA, but generally the former is preferred. Some GM fish are now being produced that are transgenic only with respect to DNA from their own species; that is they are autotransgenics (Beardmore, 1997; see Sections 5.2.2 and 5.3.2).

Once injected, eggs are allowed to hatch and the embryos and fry are reared. The resulting G_0 fish (generation of fish grown from injected eggs) are invariably mosaic, if transgenic, implying that integration into the chromosomal DNA has occurred subsequent to some rounds of cell division. This also has the knock-on effect that even G_0 fish that are positive for the transgene on Southern blotting or PCR of fin clip DNA may not transmit the transgene to progeny. However, once transgenic G_1 fish (fish produced by crossing G_0 fish with controls or G_0 with G_0) have been produced, mosaicism is no longer a problem. Of course all the transgenics are hemizygous for the transgene, but homozygous transgenics can be produced by crossing males and females within a G_1 line.

The success of producing GM fish is highly variable from species to species and from laboratory to laboratory. As a very broad indication, for every 100 eggs injected, between 5 and 10 G_0 mosaic positives might be obtained but perhaps only 1 or 2 of these fish will transmit the transgene to produce positive G_1 progeny.

As with other GM animal systems, gene expression is determined by the choice of promoter in the transgene, and although occasionally transgenes fail to express following integration, this is not a common problem; subsequent loss of integrated transgene copies also remains a rare phenomenon. However, transgene concatamerisation is frequent prior to integration, so often transgenic fish carry multiple copies of the gene construct, sometimes at different chromosomal sites but most frequently at a single integration site (Rahman, Hwang, Razak, & Maclean, 2000). There is at least a theoretical possibility of using transgenes with inducible promoters in fish, so that the expression of the transgene is only activated when the fish are exposed to the inducer. Promoters from heat-shock genes or metallothionein gene promoters containing metal or hormone response elements offer such a theoretical possibility but none have been used in a commercial or near-market context.

One notable absence from the transgenic technology applied to fish (as compared, for example, to mice) is the facility for gene 'knock out'. Thus there are no established totipotent embryonic stem cell lines from fish, and so selected transgenically altered cells cannot be introduced to early embryos as a means of producing a new desirable transgenic line. Work is in progress with embryonic stem cells of medaka (*Oryzias latipes*; Hong, Chen, & Schartl, 2000) but complete totipotency is yet to be demonstrated. However, gene silencing or 'knock down' by making fish transgenic for an antisense construct directed against a specific message is possible (Maclean, Hwang, & Farahmand, 2002), and this may prove to be of use in the future.

5.2.2. Research with GM fish

Recent reviews of advances in GM fish biology include Alestrom (1996), Donaldson (1997), Dunham (1999), Dunham and Devlin (1999), Hew, Fletcher, and Davis (1995), Maclean (1998a,b), Maclean and Laight (2000).

The range of finfish species used in this work is quite wide; Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), Arctic charr (*Salvelinus alpinus*), tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*), northern pike (*Esox lucius*), channel catfish (*Ictalurus punctatus*) and mud loach (*Misgurnus mizolepis*) feature prominently. In addition to the species listed above, which are of potential aquaculture interest, there has also been considerable work with model species such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and goldfish (*Carassius auratus*).

The objectives of experiments to produce GM fish include the following:

- studies on gene regulation in fish with or without a direct focus on an aquaculture benefit
- attempted growth enhancement
- improved cold tolerance or freeze resistance
- improved disease resistance
- altered metabolism, chiefly to reduce the requirement for fish-based diets by salmonid fish
- sterility
- fishpharming—the use of fish to act as biofactories for production of valuable pharmaceutical products
- DNA vaccination in fish

These objectives and the achievements relating to them are considered in turn, below.

Gene regulation

Gene regulation has no direct bearing on nutritional aspects of GM fish but has several indirect implications. The topic has been reviewed by Hackett and Alvarez (1999) and by Iyengar, Muller, and Maclean (1996). Work on gene regulation frequently has the objective of determining when and where particular genes are expressed during development. For example, the regulatory promoter of the *vasa* gene has been isolated from rainbow trout and, by combining it with a fluorescent reporter gene, it has been used to label and track the migration of primordial germ cells in this species (Yoshizaka, Takeuchi, Sakatani, & Takeuchi, 2000). Other work involves isolation and testing of specific response elements and enhancer sequences to be used subsequently in gene constructs for the production of improved lines of GM fish. The transgenic technology requires the use of either tissue specific promoters, such as those expressing in liver (e.g. antifreeze), or ubiquitous promoters, such as those from beta-actin or histone genes.

In addition, entire gene constructs that are intended to be used to produce future strains of GM fish for aquaculture are normally tested first in fish tissue culture or in model fish species or in both, and the individual promoters and coding sequences are separately assayed to ensure that they will prove satisfactory in later use.

Attempted growth enhancement

Growth enhancement is, without doubt, the trait that is, in finfish, most readily open to influence by gene manipulation, and a number of laboratories have successfully demonstrated impressive results. It should be stressed that overproduction of growth hormone (GH) does not necessarily prove beneficial in livestock and some serious abnormalities have been found in GM pigs (Pursel *et al.*, 1990). Some of the early work with fish involved the use of GH genes from mammals but more

recently GH genes of fish origin have been exclusively used. The promoters chosen to drive the GH gene are sometimes tissue specific, such as anti-freeze from ocean pout or metallothionein from salmon, both of which express strongly in the liver. The growth enhancement of Atlantic salmon and coho salmon has been achieved in this way by Du *et al.* (1992) and Devlin, Yesaki, Donaldson, Du, and Hew (1995), respectively. The enhancement is dramatic, being sometimes more than tenfold, even in mosaic G₀ fish. Tilapia (*Oreochromis niloticus*) also show a good response, with a twofold (Martinez *et al.*, 1996) to more than threefold (at 7 months of age; Rahman, Mak, Ayad, Smith, & Maclean, 1998) weight increase in G₁ fish (see Fig. 5.1). Somewhat less dramatic growth enhancement has been obtained with northern pike (Gross *et al.*, 1992), channel catfish (Dunham *et al.*, 1992), and carp (Chatakondi *et al.*, 1995). Species such as carp have been exposed to artificial selection for growth over centuries and so perhaps have less ‘capacity’ for extra growth enhancement. The work of Nam *et al.* (2001) on growth enhancement of mud loach is also very impressive. The important novel characteristic of growth enhancement must also be accompanied by good food conversion ratios, good palatability and lack of undesirable abnormalities, and these characteristics were all features of the growth enhanced GM tilapia tested by Rahman *et al.* (2001) in Hungary. In recent work by Nam *et al.* (2001), in mud loach, and Maclean and colleagues, in tilapia (*Oreochromis niloticus*), growth enhancement has been achieved using transgenes derived entirely from the relevant species, that is the GM fish are autotransgenics (Beardmore, 1997). The gene construct used in the work on tilapia is illustrated in Fig. 5.2.

Chatakondi *et al.* (1995), Fu, Cui, Hung, and Zhu (1998) and Rahman *et al.* (2001) have studied the



Fig. 5.1. Comparison of growth enhancement and control tilapia from the Hungary trials of Rahman *et al.* (2001). Fish are 30 weeks of age, the three on the left being transgenic and those on the right controls. The average mass of the transgenic and non-transgenic in this group was 653 and 260 g respectively and the fish illustrated were representative of the entire batch.

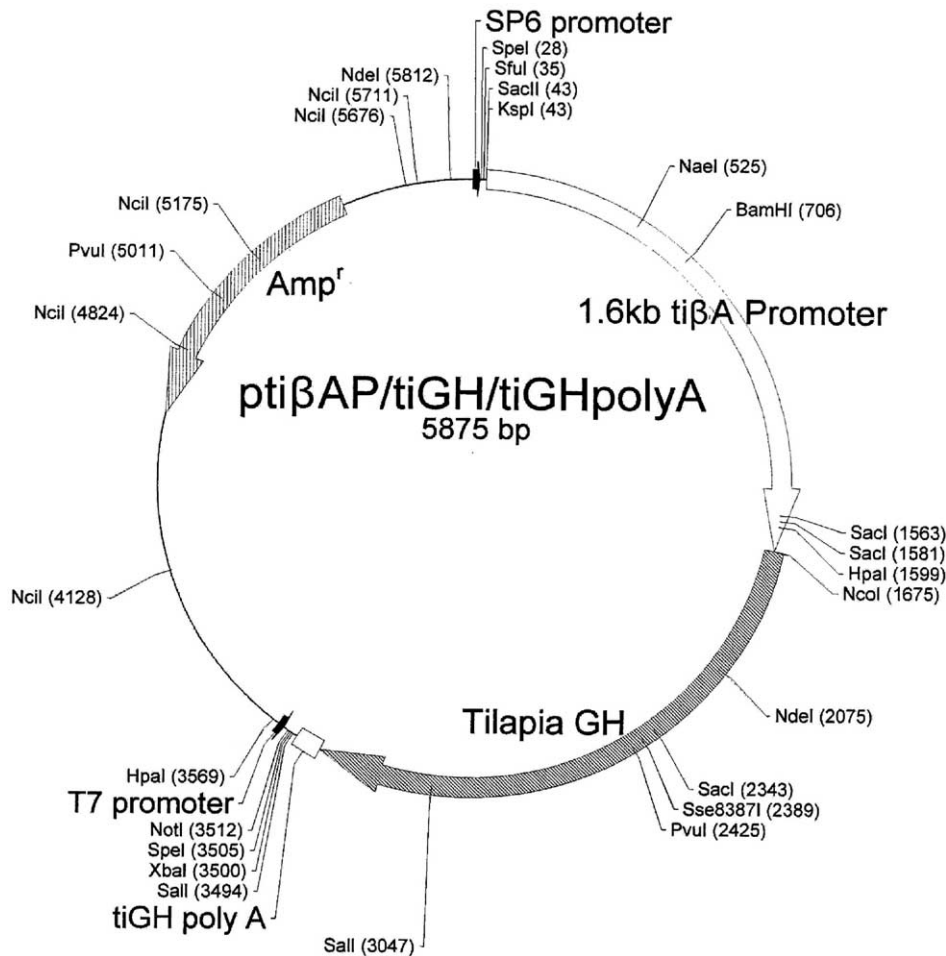


Fig. 5.2. Diagrammatic representation of the 'all tilapia' growth hormone gene construct currently in use in the Southampton Laboratory. The diagram shows the entire plasmid. The linear sequence used to generate the GM fish includes only the tilapia beta-actin promoter (1.6 kb tiβAP) and the tilapia growth hormone coding sequence plus the relevant polyadenylation sequence (tiGH). All these sequences are from a genomic library of *Oreochromis niloticus*.

nutritional requirements of transgenically growth-enhanced strains of fish.

Improved cold tolerance or freeze resistance

Improved cold tolerance and freeze resistance are really two quite separate traits; the first is the more significant in commercial terms. Lack of cold tolerance of species such as common carp leads to severe losses in some winters in China, north of the Yangtze river. Similarly tilapia, which is essentially a warm water species, can be lost in large numbers in cold winters in Israel. Freeze resistance, in contrast, is a characteristic of certain species of Arctic and Antarctic fish species, in which, even when seawater temperatures are below 0°C, the blood does not freeze owing to the release of antifreeze proteins into the blood plasma. Atlantic salmon do not normally produce antifreeze protein, and salmon sea cages in northern Newfoundland suffer severe losses of fish in some winters when the icebergs float southwards.

There has, therefore, been an interest in using transgenic technology to try to raise cold tolerant strains of carp or freeze resistant strains of Atlantic salmon.

Any advance in cold tolerance remains hypothetical. If fish were engineered to possess extra copies of delta-9-desaturase genes, they would be expected to be more tolerant of low temperatures, since the molecular conformation of lipids might be altered, leading to greater membrane fluidity. The enzyme product of this gene has been shown to be cold inducible in carp (Tiku, Gracey, Macartney, Beynon, & Cossins, 1996). There is also some evidence to suggest that antifreeze production may make goldfish more cold tolerant (Wang, Zhang, Gong, & Hew, 1995) and that antifreeze protein can therapeutically protect warm water fish species from the deleterious effects of low temperature, perhaps by reducing membrane permeability (Wu, Hwang, Hew, & Wu, 1998).

Strains of GM Atlantic salmon have been produced that express transgenic antifreeze from integrated copies

of antifreeze genes derived from winter flounder (*Pseudo-pleuronectes americanus*) (Fletcher, Davies & Hew, 1992; Hew *et al.* 1999). Integration, expression and germ line transmission of the transgene were all demonstrated; however, unfortunately, the level of antifreeze protein produced by the salmon was insufficient (by a factor of approx. 100-fold) to give significant lowering of the blood freezing point. This represents one of the best examples of the application of GM technology to a practical problem in fish husbandry, and perhaps at some future time, through the use of stronger promoter sequences or increased copy number, the objective will be realised.

For the time being, neither improved cold tolerance nor freeze resistance in fish have proved to be achievable by application of GM technology.

Improved disease resistance

Aquaculture is certainly plagued by disease problems, since water provides a ready medium for disease transmission and fish are often kept at very high stocking densities in fish farming enterprises. There are two examples of attempts to use transgenic technology to counter fish disease. One is the work of Hew *et al.* (1995) in producing a GM Atlantic salmon with a rainbow trout lysozyme cDNA, driven by an ocean pout antifreeze promoter. Rainbow trout lysozyme has already been demonstrated to have antimicrobial properties against a range of Gram-negative bacteria such as *Vibrio*, *Aeromonas* and *Yersinia* (Grinde, 1989), which are known fish pathogens. A second line of work involves the production of GM lines of channel catfish expressing a cecropin gene from the silk moth (Dunham *et al.*, 2002). Cecropins are antimicrobial proteins expressed by some insects. As with cold tolerance and freeze resistance, work with disease resistance remains at a preliminary stage in GM fish development.

Attempts to make aquacultured fish stocks more resistant to disease also include DNA vaccination (discussed below).

Altered metabolism

One serious problem for the intensive farming of trout and salmon is that, being carnivorous, such salmonid species are substantially fed with diets produced from fish caught at sea. Not only is this patently a rather wasteful methodology in terms of world food resources (see Naylor *et al.*, 2000) but such practices have led to over-fishing of stocks of sand eels and capelin by Norwegian fishermen in the North Sea, with consequent environmental harm to populations of sea birds dependent on these small fish as food (Holmes, 1996). Recognising this problem, some researchers are investigating the possibility of altering the digestive metabolism of salmonid species to allow them to utilise a diet largely based on plant material, especially carbohydrate rich food, which they nor-

mally digest very inefficiently. Although fish synthesise insulin and are well endowed with insulin receptors, their capacity for carbohydrate metabolism is limited, markedly so in salmonids (Wilson, 1994). Attempts are currently underway in Finland to find ways to improve glucose transporter and hexokinase genes (Pitkanen, Krasnov, Reinisalo, & Molsa, 1999). If these experiments prove fruitful, such an approach could, in the medium to long term, have a great impact on salmonid aquaculture and its adverse environmental consequences.

Sterility

The production and use of sterile fish offer a number of substantial benefits to aquaculture. Use of sterile fish may allow the metabolism of the fish to be concentrated on edible muscle rather than gonads; it could drastically reduce the negative environmental impact of GM fish in the event of release or escape; and it also offers a way of preventing fish becoming environmental pest species on escape, whether or not they are transgenic. It is recognised that proposals to produce GM sterile fish sometimes cause concern about terminator genes and the consequent monopoly that their use might offer. However, provided the new sterile strains were made widely available, the legitimate concerns of consumers about potential monopolistic exploitation could be met.

Induced sterility in fish need not involve genetic manipulation. Already there are ways of producing triploid fish by heat or pressure shock treatment of the fertilised eggs. Although triploid fish are substantially sterile there are often two problems. First, some diploid fish can be found amongst the triploids, as the triploid induction of the eggs is less than 100%. Secondly, some of the triploid males can produce some viable sperm, at least in some species (see discussion in Maclean & Laight, 2000); one way around this is to use all female triploids (see Section 5.3.1).

However, transgenic technology also offers ways of producing sterile fish. In the absence of gene 'knock-out' methods, genes responsible for gonad development, such as gonadotropin (GtH) and gonadotropin releasing hormone (GnRH), can be theoretically silenced by making fish transgenic for antisense coding sequences. When these antisense sequences are expressed, an RNA is produced that is precisely complementary to the specific target messenger RNA. RNA hybridisation then occurs, preventing the target message from being translated into protein. Sokol and Murray (1996) and Sokol, Passey, MacKinlay, and Murray (1998) provide useful data about the use of antisense techniques.

Work in progress involves the production of lines of tilapia expressing antisense sequences against two types of GnRH, the so called salmon type and sea bream types, and also expressing antisense sequences against the beta subunit of leutenising hormone (LH), one of

the GtH molecules. To date the results look promising and some sterile fish have been produced (Uzbekova *et al.*, 2001; Maclean *et al.*, 2002). Such sterile fish could be rendered fertile by injection of GnRH hormone to provide fertile broodstock, which would, however, produce all-sterile progeny. Thus the sterility would be reversible. Much of the future exploitation of GM fish in aquaculture may depend on the success of this work.

Fishpharming: the production of valuable human pharmaceuticals in GM fish

Although included here for completeness, ‘fish-pharming’ has no direct bearing on fish as food. Maclean and collaborators are currently conducting experiments to produce human factor VII, a blood clotting factor, in GM tilapia. There is a theoretical possibility that, at some future time, fish could be engineered to express a drug or important food item such as a vitamin, or even to over-express polyunsaturated fatty acid, so that consumption of the fish would bring additional health benefits.

DNA vaccination in fish and alteration of phenotype by gene therapy

It is arguable whether DNA vaccination and gene therapy belong within transgenic technology, since the germ line of the fish receiving the DNA is not transformed and often there is no evidence that the transgene copies have been incorporated into the chromosomal DNA of the fish. The rationale for DNA vaccination is that plasmids that carry a transgene capable of expressing an antigenic protein (to induce active immunity) or an antibody directed against troublesome disease organisms (thus inducing passive immunity) are injected into the body muscle of fish. Anderson *et al.* (1996) and Lorenzen *et al.* (2000) have demonstrated that DNA vaccination of fish can indeed protect against disease, and DNA vaccines are in active development to protect against furunculosis in salmonids.

DNA constructs within plasmids could also be used to express extra exogenous GH following muscle injection in fish. Such fish could well show growth enhancement but might not be perceived as GM fish. Certainly they would not constitute an environmental risk of gene introgression into wild stocks.

5.2.3. The present perspective

At present it is hard to be certain whether GM fish are being commercially exploited anywhere in the world. There have been reports of extensive trials of growth enhanced GM tilapia in Cuba (30 tonnes of transgenic tilapia reaching supermarket shelves; Carr, 1999) but reports are conflicting (Guillen *et al.*, 1999). Similarly, it is possible that growth enhanced GM carp have been farmed in China (Li *et al.*, 1993) but there is no recent information. A trial of GM tilapia was held in Hungary with a water temperature-contained system. The tilapia

were kept in water heated from geothermal hot water via a heat exchanger, but contaminant was ensured because during the Hungarian winter, when the trials were carried out, the water in surrounding rivers and lakes was below the minimum survival temperature for the fish (Rahman *et al.*, 2001).

An early report of a trial of GM channel catfish in Alabama, USA is that of Dunham *et al.* (1992). Also trials have been suggested with GM salmon in North America,² using all female triploids, which should ensure sterility. Discussion of some of these trials can be found in Maclean and Laight (2000).

If proven, watertight, reversible sterility can be induced in fish, there seems no reason to suppose that existing benefits of GM strains will not then be exploited. There are many reasons for using such fish and if, in future, strains can be produced that taste good, grow well, are disease resistant and amenable to aquaculture, without unacceptable adverse consequences, their widespread exploitation can be expected.

5.3. Outcomes and impacts: possible hazards of GM fish

Compared with other GM animals, fish have the serious complication of extreme mobility, so that release or escape could result in adverse environmental impacts. In addition, as with any GM organism, each line of transgenic fish must be evaluated for possible adverse nutritional effects of any novel proteins or other compounds. Furthermore, the consequences, beneficial or otherwise, of the transgenic status on the life of the fish, in terms of stress or altered morphology, should also be considered. In the context of the central topic of this chapter, the nutritional consideration is paramount, but since the future uses of GM fish are dependent on consumer acceptance, all three listed hazards are significant and will be discussed in turn.

5.3.1. Environmental impacts of GM fish

A number of different scenarios need to be considered. One is that the GM fish are fully fertile and, on escape, would readily hybridise with members of their own species, so leading to transgene introgression into the wild population. A second is that the GM fish are fully fertile but are being farmed in a country or continent in which they are not native. However, their escape might lead to the establishment of a wild feral population of the GM fish. Thirdly, the GM fish could be sterile and so the only adverse environmental impact of their escape or release would be a temporary competition with wild fish of the same or different species. A

² Sutterlin, Fletcher, Hew, and Benfly (2002) *Environmental risks in using GH transgenic Atlantic salmon and rainbow trout for commercial marine production in Canada, VA, USA*, International Systems for Biotechnology, Blacksburg, available [September 2002] at <http://www.isb.vt.edu/brarg/brasym96/sutterlin96.htm>.

last possible scenario is culture of GM fish in land-locked lakes or other secure aquaculture facilities. Some consideration of these possible hazards and the risks associated with them is to be found in Hallerman and Kapuscinski (1995), Donaldson (1997), Knibb (1997) and Maclean and Laight (2000).

If GM fish are fertile and can interbreed with their own species on escape, then the question immediately arises about the comparative fitness of the GM fish and the wild non-GM fish. Although in terms of theoretical genetics any altered phenotype is likely to be competitively disadvantageous in terms of selection, this is hard to prove beyond reasonable doubt. Muir and Howard (1999, 2001) and Hedrick (2001) have suggested that if GM fish are larger than wild type, as a result of genetically manipulated growth enhancement, then wild type females might invariably select the males as mates, leading to rapid extinction of the species owing to the reduced fitness. This is a prediction based mainly on computer simulation rather than experimental observation and, as suggested by Maclean and Laight (2000), rests on the, probably false, assumption that GM males will automatically be preferred to wild type because of their larger size. However, be that as it may, there is clearly some risk of deleterious effects of fertile GM fish on wild populations if both coexist, and such a situation should clearly be avoided. Although the risks posed by GM fish in this event would probably be no greater than that already posed by the very frequent escape of farmed salmon, the situation may probably be regarded as unacceptable in the light of current public concern about GM organisms.

If GM fish are fertile but farmed in a country where the species is not native, then at worst their escape will lead to the establishment in the wild of an alien GM species. The likelihood that a GM fish would become a pest is probably best estimated by looking at the probability of an escaped exotic species becoming a pest (Richter, Braun, Mendelson, & Master, 1997). The topic is discussed by Williamson (1996), and the likelihood of any escaped or introduced species becoming a pest can be as high as one in ten if it actually gains a reproductive foothold in an ecological community.

The third scenario is that the GM fish are sterile. Here, of course, much rests on how secure the state of sterility is. As already discussed, fish can be made sterile by a variety of methods, the best known of which is triploidy induction (see Johnstone, 1985; Penman, Skibinski, & Beardmore, 1987; Purdom, 1993). Triploid fish may also perform less well than diploids and some of the benefits of GM growth enhancement may be lost thereby (Razak, Hwang, Rahman, & Maclean, 1999).

As discussed, an alternative method to achieve sterility is to make fish transgenic for an antisense construct directed against one of the gene products in the reproductive hormone cascade, namely GtH or GnRH. Tests on the sterility of such fish and on the reversibility of

sterility, by GnRH injection or exposure, to allow the production of brood stock, are underway, and some promising results have already been obtained (Maclean *et al.*, 2002).

As mentioned above (Section 5.2.2), since the major leakiness of triploid sterility lies with the males, there is some sense in using 'all female' triploid fish as potential GM introductions. Although proposed for the introduction of growth enhanced GM salmon, unfortunately such an approach is less attractive with tilapia, since the males grow larger and faster than the females and are the more desirable aquaculture product.

At least in the short term there is a powerful case to be made that all GM fish used in aquaculture should be sterile fish, thus avoiding problems of gene introgression into wild stocks or establishment of feral pest GM fish. The environmental risks posed by escapee sterile fish are likely to be minimal. It is thus clear that the production of transgenically reversibly sterile strains of fish could greatly reduce the adverse environmental impacts of fish farming, quite apart from opening the way for the use of strains of fish improved by transgenic technology.

A fourth possible way of avoiding the risk of adverse environmental impact from GM fish is to limit their use to situations of effective physical containment, which could be indoor facilities or specialised outdoor facilities such as remote land-locked lakes. This is in line with the recommendation of the Royal Society report on GM animals.³ Certainly, countries such as Canada and Finland are richly endowed with isolated lakes and they could well lend themselves to such future exploitation with GM salmonid fish. An alternative form of physical containment is to grow warm water species such as tilapia in situations, such as are found in Iceland or Hungary, where warm geothermal water is surrounded by cold water rivers and lakes. The trials of GM tilapia in Hungary (Rahman *et al.*, 2001) were based on this rationale and proved entirely satisfactory in terms of containment.

In summary, the exploitation of GM fish in aquaculture will require, at least for the short and medium term, the use of sterile strains or of effective physical containment. Such constraints will certainly limit the economic attractiveness of GM fish but, given the growth rate of the aquaculture industry, will be unlikely to prevent future use entirely. In the context of possible future introductions of GM fish, some work has been done on the comparative fitness of GM fish compared with controls. All such studies to date have suggested that GM fish are less fit in terms of swimming speed and predator avoidance skills (Stevens, Sutterlin, & Cook, 1988; Jönsson, Johnsson, & Björnsson, 1996; Farrell, Bennett, & Devlin, 1997); although

³ Royal Society (2001) *The Use of Genetically Modified Animals*, London, UK, The Royal Society, available [August 2002] at <http://www.royalsoc.ac.uk/policy/index.html>.

Stevens *et al.* found little difference between GM and control fish.

5.3.2. Human nutritional and health problems associated with future use of GM fish

Will GM fish be safe to eat? All of the modifications to date involve transgenes that code for proteins; when the fish is eaten, both the transgenic DNA and any proteins expressed from such genes will be digested in the usual way. The particular proteins so far associated with GM fish production are GH, lysozyme, cecropin, antifreeze, and proteins expressed from some reporter genes such as *lacZ*. In the past, some promoters of viral origin have been used (Martinez *et al.*, 1996), but these should certainly be avoided in future. The most recent GM fish that may have been developed are transgenic only with respect to sequences obtained from fish of the same species (Nam *et al.*, 2001), that is they are autotransgenics; similar work by Maclean and colleagues, in tilapia, is currently at an advanced stage. There seems no reason to doubt that such fish may be safe to eat and could indeed be an improvement on currently available farmed fish, which are regularly exposed to antibiotics, injected vaccines with adjuvants, and food additives, such as carotene in feed pellets. Taste testing has been reported of GM tilapia in Cuba (Guillen *et al.*, 1999) and GM trout in Canada (Entis, 1998).

Some possible health implications resulting from the use of fish gene constructs for production of GM fish are outlined below.

- If genes coding for antibiotic resistance were included in constructs, then it is conceivable that endogenous bacteria in the human gut could acquire such genes by recombination and become resistant. Such gene sequences, although useful for the positive selection of GM fish, have not been used in the production of GM fish in recent years, except in the case of the model fish species, zebrafish and medaka. The antibiotic resistance gene employed is normally *neo* (a gene of bacterial origin), which confers neomycin resistance and is commonly tested with the neomycin analogue G418.
- Some fish species, in common with species from other animal groups, harbour toxins in some of their organs. If any of these are proteins coded in the fish genome, then clearly use of such genes for transgenesis must be avoided.
- There is a theoretical possibility that a protein that is normally harmless could, if it were expressed in a novel tissue, have effects within the tissue that would adversely affect the nutritional quality of the fish. There is also the possibility that a transgene, on its random integration into the fish genome, could lead to the inappropriate activation and expression of fish genes that are in DNA

adjacent to the transgene integration site, either by read-through from the transgene promoter or some other position effect. However, reported examples of such phenomena have not been identified.

What is clear is that both promoters and coding sequences should be selected with care so that any potential risks are minimised. In particular, promoters from the same or a related species of fish should be favoured and, depending on whether the particular promoter is ubiquitous or cell-type specific in its expression, consideration must be given to the possible physiological effects of aberrant expression on fish that is a potential consumer product. A range of reporter genes are used in transgenic fish, including *lacZ* (beta-galactosidase enzyme from the bacterium *E.coli*), *luc* (firefly luciferase gene) and *neo*, and genes for bacterial chloramphenicol acetyl transferase (CAT) and green fluorescent protein (GFP; from jellyfish). Although the risk associated with consuming any of these proteins (other than that from *neo*, as discussed previously) is probably negligible, it still seems sensible to avoid their use in lines of transgenic fish destined for human consumption.

5.3.3. Adverse side effects of transgenic technology on fish

Just as new strains of animals produced by conventional selective breeding can show undesirable traits, such as bone overgrowth, profuse facial hair in dog breeds such as English sheepdogs, and impaired swimming in some varieties of fancy goldfish, so it could be that some lines of GM fish may have attendant behavioural or morphological deficits, such as overproduction of lipid or signs of acromegaly. Some such undesirable complications in growth enhanced GM Pacific salmon have been reported by Ostefeld, Maclean, and Devlin, 1998. In general it cannot be expected that every line of GM fish will show a distinct benefit with no attendant disadvantage. Clearly, as with conventional breeding, different lines will have to be compared through rigorous trials before use and only the best lines adopted for future use. However, there is no reason, based on theory or practice, to suppose that transgenic technology, as such, is any more likely to produce 'problem fish' than conventional breeding by selection.

5.4. Standards and regulations

A number of regulations and statutory controls are operative, covering the safe containment of transgenic fish in certain countries. The former US Agricultural Biotechnology Research Advisory Committee issued '*Performance Standards for Safely Conducting Research with Genetically Modified Fish and Shellfish*'⁴ in July

⁴ Agricultural Biotechnology Research Advisory Committee (1995) *Performance Standards for Safely Conducting Research with Genetically Modified Fish and Shellfish*, VA, USA, International Systems for Biotechnology, Blacksburg, available [September 2002] at <http://www.isb.vt.edu/perfstands/perfstands1.cfm>.

1995); the same body approved a trial, in 1991, of growth enhanced GM common carp (Chen *et al.*, 1996).

In the UK the former Department of the Environment (now Department for Environment, Food and Rural Affairs) issued a research report, '*Genetic Modification of Fish. A UK Perspective*', containing guidelines on GM organisms and their use (DoE, 1994). Regulations drawn up in 1992 and 1995 (Parish, Amijee, Woodward, & Butt, 1997) covering deliberate release of genetically modified organisms, which comply with the EC directive 2001/18/EC⁵ are now in place.

A useful account of the regulatory aspects of release of GM fish is that of Parish, Amijee, Woodward, and Butt (1997). International agreement on harmonisation of the relevant regulations seems long overdue.

The regulations described here apply to the environmental impact of GM fish following their commercial exploitation or intentional release. There are also, of course, necessary relevant regulations that would apply when GM fish enter the food chain (see Chapter 8).

5.5. Methods for evaluating outcomes and impacts of GM fish

5.5.1. Environmental impacts

Risk assessment of the likely or possible environmental impacts of GM fish needs to involve the following considerations.

- Can the GM fish escape from the physical containment under conditions of storm, flood, theft or human interference?
- Can the fish survive in cold or warm water, and in salt, brackish or fresh water?
- Are the transgenic fish sterile or partially sterile?
- Are wild fish of the same species present in the surrounding waters?
- Could the GM fish become established as a novel pest species if they were to escape or be released?

5.5.2. Impacts of GM fish as consumer products

Risk assessment for GM fish as consumer products remain to be devised, and there is a great need for constructive ideas in this area. The following parameters and questions would need to be considered in any such analysis.

- Transgene promoter origin and specificity
- Transgene coding sequence product and its safety for consumption, including secondary effects on nutritional quality.
- What other sequences are included in the transgene construct and will they affect its expression?
- Is (are) the integration site(s) in the genome known?

- Is the copy number of the transgene known and what is the arrangement of the multiple copies?
- Is there any evidence of insertional mutagenesis in any of the fish carrying the transgene?
- How many generations of fish have been produced?
- Is the food intake of the GM fish different from wild type and if so how?
- Is the physiology or anatomy of the fish altered?
- Are the texture and flavour of the fish altered? What trials are appropriate to establish this?
- Are the fish more or less prone to known diseases of such fish species?

Consumer concerns (both those related to safety and, more fundamentally, those of an ethical nature) need to be understood and incorporated into both regulatory processes and commercialisation practices.

5.6. Knowledge gaps

5.6.1. Gene introduction and integration

Most frequently, GM fish are produced by manual injection of fertilised eggs by microneedle or by using a micromanipulator. There is some evidence that in some fish species electroporation can improve both introduction and integration frequency but this needs to be explored further. Similarly, the beneficial effects of using nuclear localisation signal peptides to increase integration frequency require further investigation.

5.6.2. Lack of gene targeting facility in fish

The absence of embryonic stem cell lines in commercially important fish species means that gene targeting by cell selection following transfection is not possible. In the absence of such totipotent cell lines, it would be highly desirable to investigate the possibilities of gene targeting by homologous recombination, and to this end the possible efficacious use of the protein Rad 51 needs to be followed up. Rad 51 is a eukaryotic equivalent of the well known beneficial rec A protein, which facilitates recombination.

5.6.3. Non transferable technology

It needs to be emphasised that advances such as genome sequencing of zebra fish and puffer fish (*Fugu rubripes*), or development of embryonic stem cell lines from medaka, or of DNA microchips for particular fish species, are substantially non-transferable to fish of other species.

5.6.4. Labelling of GM fish

There is a requirement for agreement between countries on labelling appropriate to GM fish and exactly what information the consumer expects.

5.6.5. Retroviruses and other viruses in fish

To date there is little information on whether fish of any species harbour active retroviruses or whether fish

⁵ OJ L117/15, 8.5.90.

act as vectors for viruses that are transmissible to humans. None are presently known. Certainly transposable elements are rare in the fish genome.

5.7. Conclusions

GM fish of a variety of species have been produced. Many involve the use of model fish for fundamental research, although some of the modifications are applicable to species important in aquaculture.

The present and projected increasing demands for fish suggest that GM fish may become important in future in both the developed and developing worlds. However, this will only be possible if consumer acceptance is achieved.

Acknowledgements

Some of the work cited here has been carried out with staff at the author's own laboratory. These include Arati Iyengar, Aziz Rahman, Gyu-Lin Hwang, Alan Smith and others. Funding has been from EC and DFID Fish Genetics programmes. The author is indebted to Professor John Beardmore and Professor Rex Dunham for useful comments on this review.

5.8. References

- Alestrom, P. (1996). *Genetically modified fish in future aquaculture: technical, environmental and management considerations*. Netherlands: International Service for National Agricultural Research.
- Anderson, E. D., Mourich, D. V., Fahrenkrug, S. C., La Patra, S., Shepherd, J., & Leong, J.-A. C. (1996). Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious haematopoietic necrosis virus. *Molecular Marine Biology and Biotechnology*, *5*, 114–122.
- Beardmore, J. (1997). Transgenics: autotransgenics and allotransgenics. *Transgenic Research*, *6*, 107–108.
- Carr, K. (1999). Cuban biotechnology treads a lonely path. *Nature*, *398*(Suppl. 1), A22–A23.
- Chatakondi, N., Lovell, R. T., Duncan, P. L., Aayat, M., Chen, T. T., Powers, D. A., Weete, J. D., Cummins, K., & Dunham, R. A. (1995). Body composition of transgenic common carp, *Cyprinus carpio*, containing rainbow trout growth hormone gene. *Aquaculture*, *138*, 99–109.
- Chen, T. T., Lu, J.-K., Shablott, M. J. O., Cheng, C. M., Lin, C.-M., Burns, J. C., Reimschuessel, R., Chatakondi, N., & Dunham, R. A. (1996). Transgenic fish: ideal models for basic research and biotechnological applications. In J. D. Ferraris, & S. R. Palumbi (Eds.), *Molecular zoology: advances, strategies and protocols* (pp. 401–403). New York: Wiley Liss.
- Devlin, R. H., Yesaki, Y. T., Donaldson, E. M., Du, S. J., & Hew, C.-L. (1995). Production of germline transgenic pacific salmonids with dramatically increased growth performance. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*, 1376–1384.
- DoE (1994). *Genetic modification in Fish: a UK perspective (genetically modified organisms Research Programme Research Report No 2)*. London, UK: Department of the Environment.
- Donaldson, E. M. (1997). The role of biotechnology in sustainable aquaculture. In J. E. Bardach (Ed.), *Sustainable aquaculture*. Chichester: John Wiley and Sons.
- Du, S. J., Gong, Z., Fletcher, G. L., Shears, M. A., King, M. J., Idler, D. R., & Hew, C. L. (1992). Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. *Bio-Technology*, *10*, 179–187.
- Dunham, R. A., Ramboux, A. C., Duncan, P. L., Hayat, M., Chen, T. T., Lin, C.-M., Kight, K., Gonzalez-Villasenor, I., & Powers, D. A. (1992). Transfer, expression, and inheritance of salmonid growth hormone genes in channel catfish, *Ictalurus punctatus*, and effects on performance traits. *Molecular Marine Biology and Biotechnology*, *1*, 380–389.
- Dunham, R. A., & Devlin, R. H. (1999). Comparison of traditional breeding and transgenesis in farmed fish with implications for growth enhancement and fitness. In J. D. Murray, & A. M. Anderson (Eds.), *Transgenic animals in agriculture* (pp. 209–230). Wallingford: CABI Publishing.
- Dunham, R. A. (1999). Utilization of transgenic fish in developing countries: potential benefits and risks. *Journal of the World Aquaculture Society*, *30*, 1–11.
- Dunham, R. A., Warr, G., Nichols, A., Duncan, P. L., Angue, B., Middleton, D., & Liu, Z. (2002). Enhanced bacterial disease resistance of transgenic channel catfish, *Ictalurus punctatus*, possessing cecropin genes. *Marine Biotechnology*, *4*, 338–344.
- Entis, E. (1998). Taste testing at a top Canadian restaurant. *Aqua Bounty Farms*, *1*, 1–4.
- FAO. (2000). *The state of the world fisheries and aquaculture 2000*. Rome: Food and Agriculture Organization of the United Nations Available [Nov 2002] at; <http://www.fao.org/>.
- Farrell, A. P., Bennet, W., & Devlin, R. H. (1997). Growth enhanced transgenic salmon can be inferior swimmers. *Canadian Journal of Zoology*, *75*, 335–337.
- Fletcher, G. L., Davies, P. L., & Hew, C.-L. (1992). Genetic engineering of freeze-resistant Atlantic salmon. In C. L. Hew, & G. L. Fletcher (Eds.), *Transgenic fish* (pp. 190–208). London: World Scientific.
- Fu, C., Cui, Y., Hung, S. S. O., & Zhu, Z. (1998). Growth and feed utilization by F4 human growth hormone transgenic carp fed diets with different protein levels. *Journal of Fish Biology*, *53*, 115–129.
- Grinde, B. (1989). Lysozyme from rainbow trout *Salmo gairdneri* as an anti-bacterial agent against fish pathogens. *Journal of Fish Disease*, *12*, 95–104.
- Gross, M. L., Schneider, J. F., Moav, N., Moav, B., Alvarez, M. C., Myster, S. H., Liu, Z., Hallerlan, E. M., Hackett, P. B., Giuse, K. S., Faras, A. J., & Kapuscinski, A. R. (1992). Molecular analysis and growth evaluation of Northern pike (*Esox luxius*) microinjected with growth hormone genes. *Aquaculture*, *103*, 253–273.
- Guillen, I., Berlanga, J., Valenzuela, C. M., Morales, A., Toledo, J., Estrada, M. P., Puentes, P., Hayes, O., & de la Fuente, J. (1999). Safety evaluation of transgenic tilapia with accelerated growth. *Marine Biotechnology*, *1*, 2–14.
- Hackett, P. B., & Alvarez, M. C. (1999). The molecular genetics of transgenic fish. In E. Fingerma (Ed.), *Recent advances in marine biotechnology* (pp. 77–145). New Hampshire: Science Publishers.
- Hallerman, E. M., & Kapuscinski, A. R. (1995). Incorporating risk assessment and risk management into public policies on genetically modified finfish and shellfish. *Aquaculture*, *137*, 9–17.
- Hedrick, P. W. (2001). Invasion of transgenes from salmon or other genetically modified organisms into natural populations. *Canadian Journal of Fisheries and Aquatic Sciences*, *58*, 841–844.
- Hew, C., Poon, R., Xiong, F., Gauthier, S., Shears, M., King, M., Davies, P., & Fletcher, G. (1999). Liver-specific and seasonal expression of transgenic Atlantic salmon harboring the winter flounder antifreeze protein gene. *Transgenic Research*, *8*, 405–419.
- Hew, C.-L., Fletcher, G. L., & Davies, P. L. (1995). Transgenic salmon; Tailoring the genome for food production. *Journal of Fish Biology*, *47*(Suppl. A), 1–19.
- Holmes, B. (1996). Blue revolutionaries. *New Scientist*, *7th December*, 32–36.

- Hong, Y., Chen, S., & Schartl, M. (2000). Embryonic stem cells in fish: current status and perspectives. *Fish Physiology and Biochemistry*, 22, 165–170.
- Hutchings, J. A. (2000). Collapse and recovery of marine fishes. *Nature*, 406, 882–885.
- Iyengar, A., Muller, F., & Maclean, N. (1996). Regulation and expression of transgenes in fish — a review. *Transgenic Research*, 5, 147–166.
- Johnstone, J. A. (1985). Induction of triploidy in Atlantic salmon by heat shock. *Aquaculture*, 49, 133–139.
- Jönsson, E., Johnsson, J. I., & Björnsson, B. T. (1996). Growth hormone increases predation exposure of rainbow trout. *Proceedings of the Royal Society of London B*, 263, 647–651.
- Knibb, W. (1997). Risk from genetically engineered and modified marine fish. *Transgenic Research*, 6, 59–67.
- Li, G., Xie, X., Xu, K., Zou, J., Liu, D., Wu, C., & Zhu, Z. (1993). Growth of growth-hormone-treated transgenic red carp. *Aquaculture*, 111, 320.
- Lorenzen, N., Cupit, P. M., Elner-Jensen, K., Ahrens, P., Secombes, C. J., & Cunningham, C. (2000). Immunoprophylaxis in fish by injection of mouse antibody genes. *Nature Biotechnology*, 18, 1177–1180.
- Maclean, N. (1998a). Regulation and exploitation of transgenes in fish. *Mutation Research*, 399, 255–266.
- Maclean, N. (1998b). Genetic manipulation of farmed fish. In K. D. Black, & A. D. Pickering (Eds.), *Biology of farmed fish* (pp. 327–354). Sheffield: Sheffield Academic Press.
- Maclean, N., & Laight, R. J. (2000). Transgenic fish: an evaluation of benefits and risks. *Fish and Fisheries*, 1, 146–172.
- Maclean, N., Hwang, G.-L., & Farahmand, H. (2002). Exploiting transgenic tilapia and the tilapia genome. In N. Shimizu, T. Aoki, I. Hirano, & F. Takashima (Eds.), *Aquatic genomics: steps towards a great future* (pp. 365–381). Tokyo, Japan: Springer.
- Martinez, R., Estrada, M. P., Berlanga, J., Guillen, J., Hernandez, O., Cabrera, E., Pimental, R., Morales, R., Herrera, F., Morales, A., Pina, J. C., Abad, Z., Sanchez, V., Melamed, P., Lleonart, R., & de la Fuente, J. (1996). Growth enhancement in transgenic tilapia by ectopic expression of tilapia growth hormone. *Molecular Marine Biology and Biotechnology*, 5, 62–70.
- Muir, W. M., & Howard, R. D. (1999). Possible ecological risks of transgenic organism release when transgenes affect mating success: sexual selection and the Trojan gene hypothesis. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 13853–13856.
- Muir, W. M., & Howard, R. D. (2001). Fitness components and ecological risk of transgenic release: a model using Japanese medaka (*Oryzias latipes*). *American Naturalist*, 158, 1–16.
- Nam, Y. K., Noh, J. K., Cho, Y. S., Cho, H. J., Cho, K.-N., Kim, C. G., & Kim, D. S. (2001). Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. *Transgenic Research*, 10, 353–362.
- Naylor, R. L., Goldberg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C. M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature*, 405, 1017–1024.
- Ostenfeld, T. A., McLean, E., & Devlin, R. H. (1998). Transgenesis changes body and head shape in Pacific salmon. *Journal of Fish Biology*, 52, 850–854.
- Parish, W., Amijee, F., Woodward, R., & Butt, A. (1997). Regulatory effects of proposed releases of genetically modified (GM) fish. In *Symposium Volume of 4th International Marine Biotechnology Conference, Sorrento, Italy* (pp. 22–29).
- Penman, D.J., Skibinski, D.O.F., & Beardmore, J.A. (1987). Survival, growth rate and maturity in Triploid tilapia. In *Proceedings of the World Symposium on Selection Hybridization and Genetic Engineering in Aquaculture, Bordeaux, 27–30 May 1986, Vol. 2*, (pp. 277–288). Berlin: Schriften der Bundesforschungsanstalt für Fischerei.
- Pitkanen, T. I., Krasnov, A., Reinisalo, M., & Molsa, H. (1999). Transfer and expression of glucose transporter and hexokinase genes in salmonid fish. *Aquaculture*, 173, 319–333.
- Purdom, C. E. (1993). *Genetics and fish breeding*. London: Chapman and Hall.
- Pursel, V. G., Boet, D. J., Miller, K. F., Pinkert, C. A., Hammer, R. E., Palmiter, R. D., & Brinster, R. L. (1990). Expression and performance in transgenic pigs. *Journal of Reproduction and Fertility Supplement*, 40, 235–245.
- Rahman, M. A., Mak, R., Ayad, H., Smith, A., & Maclean, N. (1998). Expression of a novel growth hormone gene results in growth enhancement in transgenic tilapia. *Transgenic Research*, 7, 357–369.
- Rahman, M. A., Hwang, G., Razak, S. A., & Maclean, N. (2000). Copy number dependent transgene expression in hemizygous and homozygous transgenic tilapia (*Oreochromis niloticus*). *Transgenic Research*, 9, 417–427.
- Rahman, M. A., Ronyai, A., Engidaw, B. Z., Jauncey, K., Hwang, G.-L., Smith, A., Roderick, E., Penman, D., Varadi, L., & Maclean, N. (2001). Growth and nutritional trials on transgenic Nile tilapia containing an exogenous fish growth hormone gene. *Journal of Fish Biology*, 59, 62–78.
- Razak, S. A., Hwang, G., Rahman, M. A., & Maclean, N. (1999). Growth performance and gonadal development of growth enhanced transgenic tilapia *Oreochromis niloticus* following heat-shock-induced triploidy. *Marine Biotechnology*, 1, 533–544.
- Richter, B. D., Braun, D. P., Mendleson, M. A., & Master, L. L. (1997). Threats to imperiled freshwater fauna. *Conservation Biology*, 11, 1081–1093.
- Sokol, D. L., & Murray, J. D. (1996). Antisense and ribozyme constructs in transgenic animals. *Transgenic Research*, 5, 363–371.
- Sokol, D. L., Passey, R. J., Mackinlay, A. G., & Murray, J. D. (1998). Regulation of CAT protein by ribozyme and antisense mRNA in transgenic mice. *Transgenic Research*, 7, 41–50.
- Stevens, E. D., Sutterlin, A., & Cook, T. (1998). Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. *Canadian Journal of Fisheries and Aquatic Sciences*, 55, 2028–2035.
- Tiku, P. E., Gracey, A. Y., Macartney, A. I., Benyon, R. J., & Cossins, A. R. (1996). Cold induced expression of $\delta 9$ -desaturase in carp by transcriptional and post translational mechanisms. *Science*, 271, 815–818.
- Uzbekova, S., Chyb, J., Ferrier, A., Bailhache, T., Prunet, P., Ales-trom, P., & Breton, B. (2001). Transgenic rainbow trout expressed sGnRH-antisense RNA under the control of sGnRH promoter of atlantic salmon. *Journal of Molecular Endocrinology*, 25, 337–350.
- Wang, R., Zhang, P., Gong, Z., & Hew, C.-L. (1995). Expression of the antifreeze protein gene in transgenic goldfish (*Carassius auratus*) and its implication in cold adaptation. *Molecular Marine Biology and Biotechnology*, 4, 20–26.
- Williamson, M. (1996). *Biological invasions (Population and Community Biology Series Vol. 15)*. London: Chapman and Hall.
- Wilson, R. P. (1994). Growth performance of transgenic tilapia containing an exogenous piscine growth hormone gene. *Aquaculture*, 117, 333–346.
- Wu, S., Hwang, P., Hew, C., & Wu, J. (1998). Effect of antifreeze protein on cold tolerance in juvenile tilapia (*Oreochromis mossambicus*) and milkfish (*Chanos chanos*). *Zoological Studies*, 37, 39–44.
- Yoshizaki, G., Takeuchi, Y., Sakatani, S., & Takeuchi, T. (2000). Germ cell-specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout vasa-like gene promoter. *International Journal of Developmental Biology*, 44, 232–236.