Transgenic Fish - Where We Are and Where Do We Go?

Rex A. Dunham* and Zhanjiang Liu

Department of Fisheries and Allied Aquacultures, Auburn University, Alabama 36849, USA

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Abstract

Transgenic fish have been developed that have improved growth, color, disease resistance, survival in cold, and body composition, and produce pharmaceutical proteins. Transgenes elicit pleiotropic effects, some positive and some negative. In general, transgenic fish appear to have lower fitness than controls and pose little environmental risk, but this research is not fully conclusive. Transgenic zebrafish with altered coloration have been commercialized and growth hormone transgenic salmon, carp, and tilapia are near commercialization. To enhance commercialization and minimize environmental risk, additional technologies such as transgenic sterilization need to be developed. Genomic research has produced an abundance of molecular genetic information including many genes for consideration for gene transfer, highly regulated gene promoters, and knowledge about their expression and function. Functional genomics analysis should be applied in the future to enhance the capacity and versatility of transgenic technology.

Preface

Collaboration with Israel has been historically significant. It is a major component of and has had major impact on genetic enhancement of catfish in the United States. Thus, we would like to begin this manuscript with a heart felt thanks to Israeli scientists for their assistance, collaboration, and friendship. The fruitful cooperation between Auburn University in the USA and Israeli fish genetics teams (Dor, IOLR-Haifa, IOLR-NCM-Eilat, and Tel Aviv University) was initiated 37 years ago, and has been thriving ever since. In 1969, Dr. Rom Moav, followed by Dr. Giora Wohlfarth, visited Auburn University and assisted Dr. R.O. Smitherman in establishing a catfish genetics program and a graduate level course in fish genetics and breeding. Along with Dr. Smitherman, the Israelis fathered the fish genetics program at Auburn. About 1975, Dr. Wohlfarth returned to Auburn as it was in a major growth phase, and once again assisted in the research and planning of this phase of Auburn's catfish genetics program. In the late 1970s and early 1980s, Dr. Smitherman, Dr. William Shelton, and Rex. Dunham of Auburn University collaborated with Dr. Wohlfarth and

* Corresponding author. Tel.: +1-334-844-9121, fax: +1-334-844-9208;
e-mail: dunhara@auburn.edu

Dr. Gideon Hulata on selective breeding of farmed fish. As a result of BARD support, genetically improved fish were produced, leading to the subsequent increase in use of genetically improved fish in the USA and Israeli aquaculture as well as throughout the world. In the USA, several lines of genetically improved catfish and tilapia were released. The first catfish genetics and breeding companies in the USA were spawned from this collaboration. World-wide, monosex tilapia production grew from this collaboration. The USA-Israel collaboration evolved in the late 1980s to include Dr. Rex Dunham, Dr. Tom Chen, and Dr. Dennis Powers of the USA collaborating with Dr. Benzion Cavari, Dr. Wayne Knibb, and Dr. Boaz Moav working on gene transfer in farmed fish. Genetic material has been exchanged, policy on transgenic fish in the USA evolved, and commercialization of transgenic fish in the USA is near. The relationship continued to evolve in the 1990s as Dr. Zhanjiang Liu joined Auburn University and teamed with Dr. Dunham and Dr. Boaz Moav in study of gene transfer in fish. Hopefully, this valuable and productive relationship will continue.

Introduction

The foundation for gene transfer research was laid as early as 1910 when embryologists experimented with injecting cellular material into frog eggs (Gurdon and Melton, 1981). By the early 1970s, it was apparent that gene transfer technology could provide great insight into the function of DNA sequences (Gurdon and Melton, 1981). The first widely publicized work was the transfer of mRNA and DNA into mouse eggs (Palmiter et al., 1982). Gordon et al. (1980) were among the first to microinject a series of recombinant molecules into the pronuclei of mouse embryos at the one-cell stage of development. This pioneering, landmark research in mice provided the impetus for the initiation of genetic engineering research with fish, which followed 4-5 years thereafter.

Zuoyan Zhu in the Institute of Hydrobiology in China was the first to report a transgenic fish (Zhu, 1985). Twenty years later, transgenic fish application is on the verge of making its first and major impact. GloFish, a transgenic zebrafish, *Danio rerio,* containing the fluorescent green, yellow, and red protein genes (GFP, YFP, and RFP, respectively) are now commercialized**.** Commercialization of transgenic edible fish was thought to have taken place in some countries such as Cuba; however, no official documents are available to confirm this. In New Zealand commercial brood stock populations were never used before operations closed, and Chile plans commercialization. In North America, marketing of transgenic salmon may be close, following submission of an application by A/F Protein, Aqua Bounty Farms, Waltham, MA, to the US Food and Drug Administration (FDA) to obtain approval for selling growth hormone (GH) transgenic salmon which contain genetically modified growth hormone genes (Niiler, 1999). FDA approval for consumption of these fish is expected in 2006. Aqua Bounty has potential licensees for their salmon in the USA, Canada, Chile, and Europe. In Europe and Japan, conservative approaches to the development of transgenic fish will likely prevail politically for longer periods of time than in other areas of the world. Because of these concerns, transgenic fish will likely be utilized commercially to a greater extent in developing countries than developed countries in the short term (Bartley and Hallerman, 1995).

Expectations for rapid applications and impact were unrealistic and have been slowed by food safety concerns, environmental concerns, lack of research funding, government regulations, and, just as importantly, the fact that this technology is not completely a short term genetic enhancement program but has many aspects of a long term selection program.

There are many aspects of transgenic fish technology. The areas that will be addressed in this review are performance traits of transgenic fish, pleiotropic effects of transgenes, fish as biological factories, fitness traits, environmental risks, sterilization of transgenic fish, and the future of this technology.

Performance of Transgenic Fish

Growth. Positive biological effects have been obtained by transferring transgenes to fish in some, but not all, cases and the greatest amount of work focused on transfer of GH genes. Due to the lack of available piscine gene sequences, transgenic fish research in the mid 1980s used existing mammalian GH gene constructs. In the early 1990s, most GH research switched to using fish GH constructs.

Four levels of success have been obtained for GH gene transfer. In some cases, no growth enhancement was attained. Mammalian gene constructs (mMT/rGH) failed to affect growth of salmonids (Guyomard et al., 1989ab; Penman et al., 1991), despite the fact that salmonids are very responsive to growth stimulation by exogenously administered mammalian GH protein (McLean and Donaldson, 1993). F_1 Nile tilapia (*Oreochromis niloticus*) transgenic for a construct consisting of a sockeye salmon metallothionein promoter spliced to a sockeye salmon growth hormone gene exhibited no growth enhancement (Rahman et al., 1998), although salmon transgenic for this construct show greatly enhanced growth.

Moderate growth enhancement was reported for some fish species (Zhu et al., 1986; Enikolopov et al., 1989; Gross et al., 1992; Lu et al., 1992; Zhu, 1992; Wu et al., 1994). Gene constructs containing fish GH sequences driven by non-piscine promoters elicited growth enhancement in transgenic carp, catfish, zebrafish, and tilapia (Zhang et al., 1990; Dunham et al., 1992; Chen et al., 1993; Zhao et al., 1993; Martinez et al., 1996). Several species including loach, common carp, crucian carp, Atlantic salmon, channel catfish, tilapia, medaka, and northern pike containing either human, bovine, or salmonid growth hormone genes grew 10-80% faster than non-transgenic sibling fish in aquaculture conditions. Introduction of a CMV-tilapia GH construct into a hybrid *Oreochromis hornorum* resulted in a 60-80% growth acceleration (Martinez et al., 1996; Estrada et al., 1999) depending on culture conditions. This was the first enhancement level obtained.

The next level of enhancement was a 2-6 fold increase in growth from GH transfer. Du et al. (1992) used an all-fish GH gene construct to make transgenic Atlantic salmon, and reported a 2-6-fold increase of the transgenic fish growth rate. Nile tilapia possessing one copy of an eel (ocean) pout promoter-chinook salmon growth hormone fusion grew 2.5-4 fold faster and converted feed 20% better than non-transgenic siblings (Rahman et al., 1998, 2001; Rahman and Maclean, 1999). Preliminary results indicated that homozygous transgenic Nile tilapia produced from the ocean pout antifreeze-chinook salmon GH construct grew similar to hemizygous transgenics. Insertion of other GH constructs into tilapia also yielded positive results, but not as dramatic as with the salmon GH constructs. Two possible explanations for the difference in results are the type of construct and the type of tilapia. Rohu, *Labeo rohita,* containing CMV- or *B* actin-rohu GH gene had a 4-5 fold increase in growth rate (Venugopal et al., 2004). Transgenic Atlantic salmon containing the ocean pout antifreeze promoter-chinook salmon growth hormone (GHcDNA1) gene construct had 3-6 fold accelerated growth compared to non-transgenic salmon (Du et al., 1992; Cook et al., 2000a). Insertion of sockeye MT-B-sockeyeGHcDNA1 (Devlin, 1997) produced a similar result, 5-fold growth enhancement.

The last level of enhancement involved hyper levels of growth enhancement. When introduced into coho salmon, cutthroat trout, *O. clarki,* rainbow trout, and chinook salmon, GH gene constructs using either an ocean pout antifreeze promoter driving a chinook salmon GH cDNA or a sockeye salmon metallothionein promoter driving the full-length sockeye GH1 gene elevated circulating GH levels by as much as 40-fold (Devlin et al., 1994b; Devlin, 1997), resulting in a 5-30-fold increase in weight after one year of growth (Du et al., 1992; Devlin et al., 1994b, 1995ab, 2001) and allowing precocious development of physiological capabilities necessary for marine survival (smoltification). The largest of these P_1 transgenics were mated and produced offspring with extraordinary growth. Similar results were obtained with mud loach

(Nam et al., 2001). Transgenic individuals were 30 times larger than controls. This was an unusual case as not only did these transgenic fish grow faster than normal, they reached a giant maximum size that was 30 times greater than normal.

Universally, F_1 from individual transgenic fish have highly variable performance, requiring family selection for the development of high performance transgenic lines. Varying results among species and families might be related to different gene constructs, coding regions, genomic background, chromosome positions, and copy numbers. Magnification effects can explain some of the growth differences between transgenic and control salmon, however, specific growth rates of the transgenic coho were approximately 2.7-fold higher than older nontransgenic animals of similar size, and 1.7-fold higher than their nontransgenic siblings (Devlin et al., 2000) indicating that the transgenic salmon grew at a faster rate at numerous sizes and life stages. GH levels increased dramatically (19.3-32.1-fold) relative to control salmon, but IGF-I levels were only modestly affected, being slightly enhanced in one experiment and slightly reduced in another.

Domestication is also important in transgenic growth responses. Devlin et al. (2001) first observed that salmonid GH gene constructs had a dramatic effect on growth in wild rainbow trout strains (with naturally low growth rates) but little or no effect in strains where the growth rate was enhanced by selection. In comparison, GH transgenic channel catfish derived from domesticated and selectively-bred strains exhibited only moderate growth enhancement (41%).

However, additional data on transgenic rainbow trout (Devlin et al., 2001) refutes this hypothesis of the effect of wild and domestic genetic backgrounds on response to GH transgene insertion. When OnMTGH1 was transferred to another wild rainbow trout strain, F77, growth was enhanced 7-fold, almost 4-fold greater than observed in a non-transgenic domestic rainbow trout. In this case, the wild transgenic was actually superior to the domestic selected strain indicating that genetic engineering can have a greater, rather than equivalent, effect on domesticated and selected strains. When F77 was crossbred with a domestic strain, growth of the crossbreed was intermediate to the parent strains, a typical result (Dunham and Devlin, 1998). However, the transgenic wild x domestic crossbreed was by far the largest genotype, 18 times larger than the non-transgenic wild parent, 13 times larger than the non-transgenic wild x domestic crossbreed, 9 times larger than the non-transgenic domestic parent, and more than 2.5 times larger than the wild F77 transgenic (Devlin et al., 2001). The combined effects of transgenesis and crossbreeding had a much greater growth enhancement effect than crossbreeding or transgenesis alone. A transgenic with 50% of its heritage from domestic sources was much larger than a wild transgenic, so great response from some domestic genotypes is possible. As seen in transgenic common carp and channel catfish, the effect of GH gene insertion varies among families, and multiple insertion sites and multiple copies of the gene were observed.

Color and reporter genes. The green fluorescent protein gene and other fluorescent pigmentation genes are currently being studied to understand development and gene expression in zebrafish (Amsterdam et al., 1995; Gong et al., 2002). Zebrafish that glow in shades of red, green, yellow, and orange have been developed.

Disease resistance. Momentum is being gained in transgenic enhancement of disease resistance. Expression of viral coat protein genes (Anderson et al., 1996) or antisense of viral early genes may improve virus resistance. Resistance against bacterial diseases may be easier to genetically engineer than for diseases caused by other classifications of pathogens. Bacterial disease resistance may be improved up to 3-4 fold through gene transfer. Insertion of the lytic peptide cecropin B construct enhanced resistance to bacterial diseases 2-4 fold in channel catfish but caused no pleiotropic effects on growth or obvious alterations of other traits (Dunham et al., 2002d). Transgenic and non-transgenic

full-siblings containing the cecropin B construct were challenged in tanks with *Edwardsiella ictaluri*. Both genotypes experienced mortality, but the survival of the transgenic individuals was twice that of the controls. Transgenic channel catfish containing the preprocecropin B construct and their full-sibling controls experienced a natural epizootic of columnaris, *Flavobacterium columnare*. No cecropin-transgenic fish were among the mortalities, and only control fish died.

Similar results were obtained for cecropin transgenic medaka (Sarmasik et al., 2002). F_2 transgenic medaka from different families and controls were challenged with *Pseudomonas fluorescens* and *Vibrio anguillarum*, killing about 40% of the control fish by both pathogens but only 0-10% of the F_2 transgenic fish by *P. fluorescens* and 10-30% *V. anguillarum*. When challenged with *P. fluorescens*, zero mortality was found in one transgenic fish family carrying preprocecropin B and two families with porcine cecropin P1, whereas 0-10% cumulative mortality was observed for five transgenic families with procecropin B and two families with cecropin B. When challenged with *V. anguillarum*, the cumulative mortality was 40% for non-transgenic control medaka, 20% in one transgenic family carrying preprocecropin B, 20-30% in three transgenic families with procecropin B, and 10% in one family with porcine cecropin P1. Cecropin has also shown anti-viral properties *in vitro*. Chiou et al. (2002) examined *in vitro* effectiveness of native cecropin B and a synthetic analog, CF17, for killing several fish viral pathogens, infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus (SHRV), and infectious pancreatic necrosis virus (IPNV). When these peptides and viruses were co-incubated, the viral titers yielded in fish cells were reduced from several to 104-fold. Transgenic rainbow trout containing a synthetic cecropin construct exhibited increased viral resistance (Thomas Chen, pers. comm.).

Grass carp, *Ctenopharyngodon idellus,* were transfected with carp *B* actin-human lactoferrin gene. P_1 individuals were more resistant to *Aeromonas*, exhibited enhanced phagocytosis, and were more viral resistance than controls (Mao et al., 2004).

Shrimp have been genetically engineered with antisense Taura syndrome virus-coat protein gene (Lu and Sun, 2005). When challenged with the Taura virus, transgenic shrimp had 83% survival and controls had 44% survival.

Body composition. It is now possible to directly alter body composition via transgenesis. Zebrafish transfected with *B-actin*-salmon desaturase genes had enhanced levels of omega-3 fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) in their flesh (Alimuddin et al., 2005).

Cold tolerance. Most efforts in transgenic fish have been devoted to growth enhancement although there are also reports of improved cold resistance (Fletcher and Davies, 1991; Shears et al., 1991). Early research involved the transfer of the antifreeze protein gene of the winter flounder (Fletcher et al., 1988). The primary purpose of this research was to produce salmon that could be farmed in arctic conditions, but expression levels obtained were inadequate for increasing the cold tolerance of salmon (Hew et al., 1999). Preliminary results with goldfish show some promise for increasing survival within the normal cold temperature range (Wang et al., 1995).

Transgenic fish serving as bioreactors. Transgenic mammals such as cows, goats, sheep, and rabbits have been used as biological factories to produce pharmaceutical compounds and biomedical proteins such as clotting factors and blood thinners. Such technology is especially important in the modern world since human extracted products have the potential to be contaminated with HIV and hepatitis viruses as well as other human pathogens. These products can also be quite expensive. Transgenically-produced biomedical compounds should be safe from human pathogens, eventually be less expensive, and more widely available.

Fish have potential advantages as bioreactors compared to mammals (Hwang et al., 2004). These advantages include a short gen-

eration interval, low costs for maintaining the animals, easy maintenance, large numbers of individuals, high density culture, and absence of mammalian viruses and prions. Several examples are now available that demonstrate the potential of fish as bioreactors.

MV-human coagulation factor VII was produced in transgenic zebrafish, African walking catfish, and Nile tilapia eggs (Hwang et al., 2004). Clotting activity was detected indicating proper post-translational modifications. Proteins can be collected in eggs or serum, or possibly different proteins in different tissues for other types of genes.

Transgenic Nile tilapia secreted human insulin in Brockmann Bodies (Pohajdak et al., 2004). Islet tissue was used for xenotransplantation and successfully transferred to diabetic nude mice reversing the effects of diabetes.

Single chain goldfish luteinizing hormone (LH) gene was injected into rainbow trout eggs (Morita et al., 2004). At 4-days of age, goldfish LH was isolated from the eggs and the recombinant LH was injected into goldfish. Testosterone levels in male goldfish were elevated after the injections.

Pleiotropic Effects

Improved feed conversion efficiency is one pleiotropic effect of GH gene insertion. Fast growing transgenic common carp and channel catfish containing rainbow trout growth hormone gene had improved feed conversion efficiency than controls (Chatakondi, 1995; Dunham and Liu, 2002). Among common carp, various transgenic families had increased, decreased, or no change in food consumption. Transgenic Nile tilapia had a 20% improvement in feed conversion efficiency, and better used protein and energy than the controls (Rahman et al., 2001). Transgenic tilapia expressing the tilapia GH cDNA under the control of human cytomegalovirus regulatory sequences consumed about 3.6 times less food than non-transgenic controls, and food conversion efficiency was 290% better for the transgenic tilapia (Martinez et al., 2000). Efficiency of growth, synthesis retention, anabolic stimulation, and average protein synthesis were higher in transgenic than control tilapia. Martinez et al. (2000) observed differences in hepatic glucose and levels of enzymatic activities in target organs between transgenic and control tilapia. Feed conversion efficiency of transgenic loach was 50-100% better than controls (Nam et al., 2004). Increased growth from genetic enhancement, whether from traditional selective breeding, biotechnology, or gene transfer, appears to always be a result of both increased feed consumption and improved feed conversion efficiency (Dunham, 2004).

The intestinal surface area of GH transgenic Atlantic and coho salmon was 2.2 times that of control salmon and the growth rate was about twice that of the controls (Stevens and Devlin, 2000b). The relative intestinal length was the same in transgenic and control salmon, but the surface area was greater in transgenics as a result of an increased number of folds. Increased gut tissue is a result of both environmental and genetic effects (Stevens and Devlin, 2005).

The insertion of the rtGH gene altered the survival of common carp (Chatakondi, 1995). The number of F_2 progeny inheriting this transgene was much less than expected. Differential mortality, a true pleiotropic effect, or loss of the recombinant gene during meiosis are likely explanations. Remaining transgenic individuals had higher survival than controls when subjected to a series of stressors and pathogens such as low oxygen, anchor worms, *Lernea, Aeromonas*, and dropsy. Conversely, GH transgenic salmon were more sensitive to *Vibrio* than controls (Jhingan et al., 2003). Survival among GH salmon families is sometimes improved, sometimes decreased, and sometimes unchanged relative to controls (Devlin et al., 2004).

When subjected to low dissolved oxygen (0.4 ppm), mean absolute survival was the same for transgenic and control common carp. However, transgenic individuals had longer mean survival time than non-transgenic full-siblings (Chatakondi, 1995; Dunham et al., 2002b). Ventilation rate could explain the slightly better low oxygen tolerance of the transgenic common carp as the transgenic

channel catfish with the same rtGH construct as the common carp had a lower ventilation rate when subjected to low dissolved oxygen than the controls (Dunham, unpublished).

In the case of salmon, GH transgenics had the same resistance as controls to heat shock (Jhingan et al., 2003). However, oxygen tolerance varied. GH transgenic salmon had an increased need for dissolved oxygen (Stevens et al., 1998; Cook et al., 2000bc) but, after four days of starvation, GH individuals had the same oxygen uptake as controls (Leggatt et al., 2003). After feeding, GH transgenics required 1.4-1.7 fold more O_2, even when the controls consumed an equivalent amount of feed. Adult transgenics had a higher oxygen demand, poorer swimming ability, and longer recovery time compared to ocean ranched salmon (Lee et al., 2003).

Pleiotropy of the GH gene for oxygen tolerance varies from species to species. GH tilapia (McKenzie et al., 2003) have a 58% higher metabolism than controls, compensate for oxygen consumption, and have the same maximum swim speed as non-transgenics. GH tilapia tolerate hypoxia equally as controls despite the higher demand for oxygen.

The GH gene affects body shape. Zhu (1992) reported an increase in muscle thickness and body width in transgenic common carp containing the human growth hormone gene. The effect of rtGH1cDNA (rainbow trout growth hormone cDNA) on body shape, dress-out yield, and body composition were assessed in the F_1 and F_2 generations of transgenic common carp (Chatakondi et al., 1994, 1995; Dunham et al., 2002c). The correlations between head morphometric measurements and length or weight for the F_1 and F_2 generations were negative (Chatakondi, 1995), indicating that the fish head does not grow proportionately to its length or weight. Various head, body, and caudal traits grew disproportionately faster than total body length and this effect was greater in transgenic fish in both generations than in control common carp. Transgenic individuals had relatively larger heads, deeper and wider bodies, and greater caudal areas than controls. Similar changes were seen in GH transgenic Nile tilapia as the head:total length ratio, viscera-somatic index, and hepatosomatic index were greater in transgenic fish than in control fish (Rahman et al., 2001). The change in body shape resulted in a 5% carcass yield for the transgenic common carp (Dunham et al., 2002c).

The condition factor, K, was proportionately higher in most families of transgenic common carp (Chatakondi, 1995). However, families 1 and 7 of the F_1 generation and families 69 and 70 of the F_2 generation had lower condition factors than their controls despite a higher weight increase, similar to results for transgenic salmon, because the length changed more rapidly than the weight in the transgenic salmon (Devlin et al., 2001).

Transgenic wild-strain rainbow trout had the slender body shape similar to that of the wild controls, but their final size at sexual maturity was much larger than in the non-transgenic wild rainbow trout (Devlin et al., 2001), thus no pleiotropic effect on body shape was seen for these fish. However, domestic transgenic rainbow trout derived from a deep-bodied strain, despite minimal growth enhancement, had an even deeper body depth than the controls, caused by either increased muscle or tremendous visceral fat deposits or both. The altered body shape of transgenic common carp resulted in improved dressing percentage in the F_2 generation. A similar result was obtained for transgenic channel catfish containing the same GH construct.

Excessive levels of growth hormone resulted in morphological abnormalities in the head, fin, jaw, and operculum as a result of excessive cartilage and bone growth of the fastest growing transgenic salmon (Devlin et al., 1995a). Insertion of a pOnMTGH1 gene construct into coho salmon altered centroid size (Ostenfeld et al., 1998). The dorsal caudal peduncle and abdominal regions were distinctly enhanced in transgenic fish when compared to controls. Morphological changes of whole body and syncranium were prominent.

GH gene transgenesis also affects gill morphology. The gill morphology of transgenic Atlantic salmon (Stevens and Sutterlin,

1999) and Pacific salmon (Stevens and Devlin, 2000a) differed from that of controls, but the difference was expressed differently in the two species. Pacific transgenic salmon had gill filaments that were similar in length to the controls, but smaller lamellar spacing. Atlantic transgenics had longer gill filaments that were longer than in the controls but similar lamellar spacing. This illustrates that the pleiotropic effects from GH transgenesis can differ, even between closely related species.

The progeny of salmon that grow 30 times larger than normal are subviable and virtually all die. Endocrine stimulation in GH transgenic salmon is elevated to pathological levels, causing excessive, deleterious deposition of cartilage (Devlin et al., 1995ab), analogous to the mammalian acromegaly syndrome. This effect can be sufficiently severe such that impaired feeding and respiration may result in reduced growth and poor viability. Consequently, salmon that ultimately display the greatest growth enhancement as adults are those that have been only moderately (10 times) stimulated (Devlin et al., 1995ab). Progeny from transgenic parents with more moderate accelerated growth do not suffer reduced survival and increased skeletal anomalies.

Despite their minimal growth enhancement, domestic GH transgenic rainbow trout also exhibited cranial deformities (Devlin et al., 2001). The deformities could be a species-specific phenomenon. Despite much more significant growth acceleration compared to slow growing rainbow trout, GH transgenics, P_1, F_1, F_2, F_3, and F_4 GH transgenic common carp and channel catfish do not exhibit deformities. Additionally, no abnormalities were apparent in rapidly growing GH transgenic Nile tilapia, although minor changes to skull shape were observed in some fish (Rahman et al., 1998).

GH transgenic fish exhibit body composition changes, but not as dramatically as mammals. Moisture content in GH transgenic Atlantic salmon was higher, relative to protein and ash, than in normal controls (Cook et al., 2000a). Dunham et al. (2002c) examined body composition changes in GH transgenic common carp for two generations, F_1 and F_2. The carcass composition of transgenic muscle had a lower percentage of lipids and higher protein in both generations (an 7.5% increase in protein and 13% decrease in fat). Moisture was lower in F_1 transgenic muscle but unchanged in F_2 transgenic individuals. Transgenic channel catfish with the same rtGH cDNA also had more protein, less fat, and less moisture in their edible muscle than non-transgenic full-siblings (about a 10% change). Transgenic *O. hornorum urolepis* containing the tilapia growth hormone (tiGH) cDNA had lower levels of cholesterol, free alanine, and aspartic acid in the muscle compared to controls (Martinez et al., 1999). The increased protein level in transgenic common carp and channel catfish muscle resulted in increased amino acid levels. However, the amino acid ratios and fatty acid ratios were virtually identical in control and transgenic common carp and channel catfish, although some amino acids increased in proportion slightly more than others.

GH transgenesis also affects muscle characteristics and activity. GH transgenic catfish had increased numbers of mitochondria in the cell, increased numbers of glycogen globules, increased numbers of muscle fibers, but reduced numbers of fat globules in their cells. Muscle fiber size was unchanged. Perhaps due to the changes in amino acid levels and ratio and the fat and ultrastructure of the muscle, the flavor and texture of transgenic catfish flesh was slightly better than in non-transgenic controls (Dunham and Liu, 2002.). Heterozygous growth hormone transgenic coho salmon had higher numbers of small-diameter fibers in somite muscles (Hill et al., 2000). Both the dorsal and lateral region of the somitic muscle were affected, suggesting that the transgenic salmon grew by greater rates of hyperplasia relative to slower growing nontransgenic fish. Higher levels of activity were found for phosphofructokinase and cytochrome oxidase in white muscle of the transgenic fish, indicating a higher glycolytic and aerobic requirement in the muscle of transgenic fish. The GH gene insertion affected expression of several other genes, and

many of the additional mRNAs in the transgenic fish were specifying myosin light chain 2, consistent with high level of expression in the early stages of muscle fiber construction.

Additional gene expression changes were observed in transgenic GH salmon (Ettensohn et al., 2004). Complement factor Bf-2 was down-regulated. Methionine adenyltransferase was up-regulated. Myostatin 1 was not affected (Roberts et al., 2004). Myostatin 2 was down-regulated in white muscle, but up-regulated in red muscle. Myostatin immunoreactive protein (MIP) decreased indicating decreased processing. These changes in myostatin expression and MIP may be partial explanations for the hyper growth in GH salmon. The level of digestive enzymes was relatively unchanged in GH salmon and does not explain the altered growth.

Color changes in GH transgenic coho salmon (Devlin et al., 1995b; Devlin 1997). Individuals containing opAFP or OnMT salmon GH constructs have lighter skin pigmentation and this is a reliable marker for identifying transgenic salmon prior to first feeding (Devlin et al., 1995b). Control fish possessed the normal brown coloration typical of coho salmon alevins, whereas the GH transgenics had a distinct green coloration.

The most important pleiotropic effect, which is one of the major explanations for the growth differences in transgenic and control salmon, is the accelerated smoltification of the transgenics. The transgenics smolt up to two years early and display enhanced silver coloration and osmoregulatory ability (Devlin, 1997).

In general, the larger the direct effect, the larger and more dramatic the pleiotropic effect for GH transgenic fish. This may or may not be the case for insertion of other genes. GH affects a large number of biochemical pathways and this could be an extreme example of pleiotropy in transgenic fish.

Environmental Risks and Fitness Traits

Commercialization of transgenic aquatic organisms on a large scale may have a variety of ecological implications (Hallerman and Kapuscinski, 1992, 1993). Escape of transgenic aquatic organisms will eventually occur from a commercial facility, and the range of receiving ecosystems is broad.

The risks of transgenic fish should be similar to the risks of domestic fish. Most data indicate that wild fish are more competitive than domestic fish (Dunham, 1996), resulting in elimination of the domestic fish and their potential positive or negative impacts. Utilizing AFLP analysis, Simmons et al. (2006) determined that domestic populations of channel catfish in Alabama, USA, have had no genetic impact on wild populations. However, recent salmonid research indicates that there are situations where domestic fish can have genetic impacts on wild populations. When repeated large-scale escapes of domestic fish occur, genetic impacts can occur from the sheer force of numbers. Transgenic fish would make an impact in this scenario but, again, the consequences should not vary much from that of fish genetically altered by other means.

Reproductive performance, foraging ability, swimming ability, and predator avoidance are key factors that determine the fitness of transgenic fish and should be standard measurements prior to commercial application. Most available data indicate that transgenic fish are less fit than non-transgenic fish and likely to have little if any environmental impact. Extremely fast growing salmon and loach have low fitness and die (Devlin et al., 1994b, 1995ab).

Several models have been developed that estimate and indicate the genetic risk of transgenic fish. Muir and Howard (1999) evaluated a model and created the term ‘Trojan gene effect’, i.e., the extinction of a population due to the mating preference for large transgenic males that have reduced fitness, thus placing a severe genetic load on the population. Their conclusion was that both reduced fitness and increased fitness have potential adverse ecological effects. This modeling was based on experimental results with medaka in aquaria.

Hedrick (2001) developed a deterministic model for the case in which a transgene has a male-mating advantage and a general viability disadvantage, analogous to the Trojan

gene effect of Muir and Howard (1999). Hedrick's results indicate that in 66.7% of the possible mating and viability combinations, the transgene invades the natural population and increases in frequency, while in 50% of the combinations, the transgene goes to fixation. The increase in the frequency of the transgene reduces the viability of the natural population, increasing the probability of extinction of the natural population.

Based on data from a laboratory population of medaka harboring a regulatory sequence from salmon fused to the coding sequence for human growth hormone, Muir and Howard (2001) again concluded that a transgene can spread to a wild population even if the gene markedly reduces a component of fitness. In juvenile transgenics, the growth rate increased while survival dropped, resulting in changes in the development rate and size-dependent fecundity of females. Important factors in the model were the probability of various genotypes mating, the number of eggs produced by each female genotype, the probability that the eggs will be fertilized by the sperm of each male genotype (male fertility), the probability that an embryo will be a specific genotype given its parental genotypes, the probability that the fry will survive, and parent survival. Muir and Howard's (2001) interpretation was that transgenes would increase in populations despite high juvenile viability costs if transgenes had sufficiently high positive effects on other fitness traits. Sensitivity analyses indicated that transgene effects on age at sexual maturity would have the greatest impact on transgene allele frequency. Juvenile viability had the second greatest impact. However, a defect in the simulation was the fact that the effect of predation in the wild could not be included in the model, biasing viability estimates (Muir and Howard, 2001).

Although these modeling experiments based on laboratory data on small model species illustrate potential risk of transgenic fish, some weaknesses exist. The environment was artificial, the mating preference does not exist for many fish including catfish, the models do not account for genotype-environment interactions which are likely, predation is absent as Muir and Howard (2001) indicate and the overall performance of the fish is not accounted for.

Body size does not necessarily result in mating advantages. Rakitin et al. (2001) utilized allozymes and minisatellites to determine that male size, condition factor, and total or relative body-weight loss over the season were not correlated with the estimated proportion of larvae sired by each Atlantic cod male during the spawning season. Similar results were observed in salmon (Doyle, 2003). However, Atlantic cod male reproductive success was affected by female size, with males larger (>25% total length) than females siring a smaller proportion of larvae (Rakitin et al., 2001). In this case, large size was reproductively disadvantageous.

In some cases, reproductive traits have not been greatly affected by GH transgenesis; in others, they have been adversely affected. Fast growing transgenic tilapia have reduced sperm production. Transgenic channel catfish and common carp have similar reproduction and rates of sexual maturity compared to controls (Dunham et al., 1992; Chen et al., 1993; Chatakondi, 1995). Spawning success in transgenic channel catfish and controls appeared similar. When the two genotypes were given a choice in a mixed pond, mating was random and spawning ability was equal (Dunham et al., 1995). Fecundity is not affected by inserting rainbow trout GH cDNA in common carp. Precocious sexual development was not observed in transgenic common carp. However, GH transgenic male tilapia had reduced sperm production. Female GH transgenic Nile tilapia had a lower gonadosomatic index than non-transgenic siblings in both mixed and separate culture conditions (Rahman et al., 2001). The gonadosomatic index in transgenic males was higher in mixed culture and lower in separate culture than in their non-transgenic siblings. Zebrafish containing fluorescent pigment genes had unaltered or lower reproduction (Gong et al., 2003).

Reproduction is a complicated trait in salmon. Mating success is not determined by

size alone but is also affected by color, body shape, courtship, competition, physiology, migration ability, environmental effects, and genotype-environment interactions. GH salmon attain normal adult body size and have advanced hatch time and early growth (Devlin et al., 2004). GH salmon had early sexual maturity (one year) in the laboratory, but the age of maturity in the wild is unknown (Bessey et al., 2004). Transgenic rainbow trout experienced early maturation at 2 years of age, but in the same season as the controls. In the laboratory, there was no enhanced adult size, cultured salmon had higher spermatocrits than transgenics and "wild hatchery fish", transgenics had lower spermatocrits than controls, and milt was equally competitive among transgenic control, cultured, and wild hatchery fish,. However, use of transgenic milt resulted in a lower hatch. GH transgenics had lower spawning and courtship behavior and higher fecundity, but smaller eggs.

Transgenic fish could be more competitive in seeking feed. Devlin et al. (1999) examined the ability of F_1 coho salmon (250 g) containing a sockeye metallothionein-B promoter fused to the type 1 growth gene-coding region to compete for food through higher feeding motivation. Transgenic coho salmon consumed 2.5 times more contested pellets than the controls; the transgenic fish consumed 2.9 times more total pellets than the non-transgenic controls, indicating a high feeding motivation of the transgenic fish. The shortcomings of this trial were that it was conducted in a highly artificial environment with a type of food that will not be encountered in natural conditions. The food-seeking aggressiveness is a likely factor for the increased vulnerability to predation. Similarly, transgenic tilapia outcompeted controls for artificial food (Guillen et al., 1999). Transgenic tilapia had a larger appetite than the controls. Interestingly, wild tilapia out-competed domestic tilapia for food. An important factor in all these experiments was that the fish competed for artificial, not natural, food.

Genotype-environment interactions are important and occur for growth of transgenic channel catfish (Dunham et al., 1995). Transgenic channel catfish containing salmonid growth hormone genes grew 33% faster than normal channel catfish in aquaculture conditions with supplemental feeding. However, there was no significant difference in growth performance between transgenic and non-transgenic channel catfish in ponds without supplemental feeding, indicating equal foraging ability and the inability of transgenic catfish to express their growth potential with limited feed (Chitmanat, 1996). When grown under natural conditions where food is limited, the transgenic channel catfish had a slightly lower survival than the control and grew at the same rate as the non-transgenic controls. The lower survival may have been due to starvation. Transgenic Atlantic salmon had higher metabolic rates and lost protein, dry matter, lipid, and energy more quickly than controls (Cook et al., 2000a).

The foraging ability of transgenic and control catfish is similar under conditions of competition and natural food sources and, as is the case for most genetic improvement programs, genetically engineered fish need adequate food to express their potential.

The faster growing transgenic fish could have impaired swimming, leading to predator vulnerability, problems in capturing prey, reduced mating ability for some species, and reduction in competitiveness for any trait requiring speed. Selection for swimming ability may be one of the primary mechanisms limiting the genetic increase in fish size and preventing fish from evolving to larger and larger sizes.

Silversides, *Menidia menidia*, from Nova Scotia ate more food, had more efficient feed conversion, and grew faster than a population from South Carolina (Billerbeck et al., 2001a). However, the Nova Scotia strain was more vulnerable to predation than the South Carolina strain and predation increased with growth rate and feeding rate both within and between strains (Billerbeck et al., 2001b). Maximizing energy intake and growth rate entails fitness costs in the form of increased vulnerability to predation (Doyle, 2003).

Predator avoidance was slightly better for

non-transgenic than transgenic channel catfish fry and fingerlings exposed to largemouth bass, *Micropterus salmoides,* and green sunfish, *Lepomis cyanellus,* (Dunham, 1995; Dunham et al. 1995, 1999). GH transgenic salmon have reduced swimming ability (Farrell et al., 1997; Stevens et al. 1998) and lack of fear of natural predators (Abrahams and Sutterlin, 1999). GH salmon are willing to take greater risks in the presence of predators and are aggressive feeders. However, predator avoidance data is conflicting (Devlin et al., 2004; Vandersteen Tymchuk et al., 2005). Age and genotype-environment interactions appear to be important in predator avoidance studies of GH transgenic salmon (Devlin et al., 2004). Fastest and slowest growing individuals were eaten in some, but not all, experiments and this might be related to age effects.

The design of an environmental risk/predation study is important. Results could be affected by the habitat or whether artificial or natural food is provided. Selection of same-sized fish to initiate experiments could alter the genetic make-up of the populations and their behavior. The length of the experiment is important. In some cases, salmon GH experiments were conducted for only two days. Dunham et al. (1986) demonstrated significant genotype-environment interaction based on the length of the experiment for hybrid and crossbred catfish in angling vulnerability experiments.

On an absolute speed basis, transgenic coho salmon swam no faster at their critical swimming speed than smaller non-transgenic controls, and much slower than older non-transgenic controls of the same size (Farrell et al., 1997). Ostenfeld et al. (1998) found that coho salmon containing pOnMTGH1 had an altered body contour, centroid size, enhanced caudal peduncle, and enhanced abdominal regions compared to controls. The most prominent alterations were the change in the syncranium and the less elliptical head of the transgenic fish. The overall body shape was less fusiform for transgenic coho salmon. Therefore, the decrease in swimming ability may have been the result of a loss of hydrodynamics and increased drag coefficient caused by the altered body shape. This change in body shape might also have altered the leverage or efficiency of the muscle movements for swimming. The inferior swimming ability of the transgenic salmon should cause them to have inferior abilities to avoid predators, capture food, and migrate to the sea and return to reproduce in natural settings.

All transgenic fish evaluated to date have fitness traits that are either the same or weaker than the controls. The increased predator vulnerability, reduced swimming ability, lack of increased growth when foraging, and unchanged spawning percentage of the transgenic fish indicate that some may not compete well under natural conditions, or cause major ecological or environmental damage. Although transgenic fish may be released into nature unintentionally, ecological effects should be unlikely because of reduced fitness.

The greatest environmental risk that a transgenic fish would have is when the gene insert would allow the transgenic genotype to expand its geographic range, essentially becoming equivalent to an exotic species. About 1% of exotic releases result in adverse environmental consequences (Welcomme, 1988). Altering temperature or salinity tolerance would be analogous to development of an exotic species since this would allow the expansion of a species outside its natural range. This type of transgenic research and application should be avoided. Antifreeze protein genes from winter flounder have been introduced into Atlantic salmon in an attempt to increase their cold tolerance (Shears et al., 1991). If this research were successful, a real possibility of environmental impact would exist. Similarly, if tilapia were made more cold tolerant, a strong possibility of detrimental environmental impact would exist.

Transgenic Sterilization

Data to date indicate that transgenic fish have inferior fitness traits needed for successful establishment if accidentally introduced into the natural environment. Likely, the most desirable transgenic genotypes for aquaculture will be strongly selected in natural settings. The greater the phenotypic change in target traits such as growth rate, the greater

the pleiotropic effects on other traits including fitness traits such as predator avoidance and swimming ability and the lower the probability of genetic impact on wild populations. In reality, transgenic fish may be a more acceptable aquaculture genotype than traditional domestic fish, as high performance transgenics may be eliminated in the natural environment more rapidly than other types of domestic fish, reducing the impact on native populations.

However, it will be difficult to prove this hypothesis without an actual escape. Even with strong data indicating the likelihood of low or negligible environmental risk, many governments will be reluctant to allow commercialization of transgenic fish because of public perception and pressure from the media and environmental groups. Therefore, confinement will be necessary for approval for commercialization. However, most physical, chemical, and biological confinement options are not 100% failsafe. A potential key confinement option is development of genetic sterilization. Successful genetic sterilization eliminates almost all environmental issues concerning application of transgenic fish.

Polyploidy has been proposed as one way to achieve genetic sterilization, however, this approach has drawbacks. Triploid induction is not commercially feasible for all species, it is not always 100% effective, it requires fertile, diploid brood stock, and it has adverse effects on some economic traits, partially negating some of its improved performance. Both transgenic triploid salmon (Jhingan et al., 2003) and transgenic triploid tilapia (Dunham, 2004) have substantially reduced growth compared to diploid transgenics, although growth of triploid transgenics is still much higher than that of controls. Transgenic loach triploids had slightly lower early survival than diploids (Nam et al., 2004).

Redundant mechanisms could be another option for genetic sterilization. Nam et al. (2004) attempted to sterilize transgenic loach by combining triploidy and hybridization. This had adverse effects on growth. Transgenic diploid loach were 30 times bigger than diploids, hybrid diploids, and triploid hybrids. However, transgenic interspecific hybrids and triploid interspecific hybrids were only 14 times larger than the same three non- transgenic controls.

Transgenic sterilization has the potential to render transgenic fish sterile without the drawbacks of polyploidy. Transgenic sterilization would almost completely eliminate environmental risks and may be the most important key to commercialization of transgenic fish. Still, some argue that the potential would exist for escaped transgenic sterile fish to disrupt mating of wild conspecifics, potentially reducing the population. Massive escape could lead to such a scenario. However, unless repeated large-scale escapes occur, this effect would be temporary. Perfect confinement is not possible for all applications of transgenic fish. However, the combination of drastically reduced fitness of domestic transgenic fish, genetic sterilization, transfer of appropriate gene constructs, and appropriate physical confinement wound reduce the risk to such a negligible level that the benefits would be much greater than the risks.

Preliminary research on transgenic sterilization has been promising but this technology is yet to be perfected. Carp β actin-tilapia salmon type GnRH antisense construct was injected into Nile tilapia (Norman Maclean, pers. comm.; Dunham, 2004). Transgenic females were crossed with wild-type males; a reduction in fertility of about half that of non-transgenic control females was observed. Fertility was much more greatly reduced in transgenic males crossed to control females. In some cases, 0% fertility was obtained with an average reduction of about 80% in fertility. Limited data on transgenic females crossed with transgenic males indicated near zero fertility.

Tilapia β actin-tilapia seabream GnRH antisense construct was injected into Nile tilapia without reduction in fertility of heterozygous transgenic males and females. Limited data on transgenic females crossed with transgenic males indicated no reduction in fertility. Reciprocal crosses between seabream and salmon GnRH antisense transgenics gave hatch rates that appeared to be dictated by the salmon GnRH antisense parent.

Transgenic rainbow trout containing salmon type antisense GnRH from Atlantic salmon, *Salmo salar,* driven by either the GnRH or histone 3 promoter had reduced levels of GnRH and appeared to be sterile (Uzbekova et al., 2000ab). Preliminary data indicated that spermiation of transgenic males was only obtained after prolonged treatment with salmon pituitary extract, whereas control males spermiated naturally. Data is still needed for the females.

Another strategy, introduction of "Sterile Feral" constructs, disrupts embryonic development, thus sterilizing brood stock. Preliminary results show promise for this approach (Thresher et al., 2001). Deformities and mortalities were produced with several of the constructs. Gene expression was reversibly repressed with utilization of the doxycycline.

The percentage of deformed zebrafish embryos injected with 3'-*zBMP2* and 5'-*zBMP2* dsRNA was 43.4% and 40.2%, as compared to 9.2% and 2.4% in the corresponding controls. Linearized pzBMP2-As-EGFP anti-sense injected into one-cell zebrafish embryos gave up to 33% deformed embryos. pzBMP2-ds injected into 1-4 cell stage embryos gave up to 45% deformed embryos. pBIT(smad)-BMP2ds resulted in 39% deformed embryos without dox, and 18% deformed individuals with dox. pBIT(smad)-BMP2 sense produced 35% deformed embryos. In these experiments controls yielded 0-10% deformed individuals.

The same approach was utilized in oysters. The promoter was *Drosophila* heat shock protein. The developmental genes for which knockout blockers were developed were the Hox genes controlling a cascade of developmental events. Reporter genes such as green fluorescence protein were used to screen for positive individuals and test for gene expression, repression, and reversibility. Larval oysters injected with HoxCG1 double stranded (DS) RNA had arrested development (79% failed to develop to the D-hinge larval stage). Arrested development occurred in 67% and 33% of oyster embryos transfected with pHSP-oHoxDS/BH plasmid when heat shock or no heat shock was applied, respectively. When the same experiment was conducted with the tetracycline-responsive plasmid phsp-BiT-RFP/dsRNA-HoxCG1, 67% of the oysters had arrested development without dox treatment and 9% had arrested development with dox treatment. GFP and dsRNA-zfBMP transfected oyster embryos expressed green fluorescent protein and had 30% mortality. With the addition of dox, there was no expression of GFP and mortality was dropped to 5%, demonstrating the potential of the Tet-Off™ system. In all these experiments, controls had 0-5% deformities.

One hundred percent deformities and mortalities were not achieved. This was not surprising as not all embryos would have received the genes, and those that did would be mosaics of varying degrees with variable numbers of transgenic cells in the P1 generation. The reporter genes may also have complicated expression.

Templeton (2005) evaluated some of these same zebrafish constructs, SF3(zSMad5 promoter/Bmp2 promoter/dsBmp2 gene and SF4(zSMad5 promoter/Bmp2 promoter/zBmp2 gene), in channel catfish. Similar to the zebrafish results, electroporation of these constructs killed channel catfish embryos, and a substantial percentage of embryos could be rescued by administration of doxycycline.

Where Do We Go?

Transgenic fish with improved color, growth, disease resistance, and body composition as well as transgenic fish capable of producing biodmedical proteins have been produced. Where do we go from here?

Increased research will be needed to model environmental risks, measure fitness and actual environmental risks, and determine food safety for commercialization and application of past and future progress. Success and application of transgenic fish will be dictated by the successful demonstration of food safety, lack or potential lack of environmental risk, appropriate government regulation and labeling, public education, and development of genetic sterilization for transgenic fish. Initial surveys indicate that the

general population in many countries does not understand biology and food production. This could exasperate marketing of transgenic fish products that are proven safe, although many food products partially derived from transgenic plants are currently being marketed in the USA without great public outcry. When appropriate, well-executed public education may be necessary to obtain broad consumer acceptance of transgenic fish from environmental and food safety standpoints and, perhaps, regarding how "organic" a transgenic fish may be. If transgenic sterilization technology is developed that is reversible upon demand, the above application issues will greatly decrease in importance and, in some cases, be fully addressed. Increased emphasis needs to be placed on transgenic sterilization research.

Initial experiments indicate the possibility of controlling reproduction via transgenesis. Future success in this area would potentially have one of the greatest impacts from recombinant DNA technology. This approach could solve not only many transgenic issues but many biodiversity and genetic biodiversity issues as it would allow environmentally safe application of transgenic fish, interspecific hybrids, domestic fish in general, exotic species, and utilization of wild conspecifics outside their native watershed for recreational applications without genetic consequences.

Transgenic fish research and application of transgenic fish has not progressed as rapidly as many envisioned when research on transgenic fish began about 21 years ago. We need to address some of the key reasons for the perceived slowness of this research and its application. As for many other research areas and in particular aquaculture and aquaculture genetics research, lack of funding has been one problem that hindered progress. This is a difficult problem to address, but communication and education such as this BARD workshop are one of the few mechanisms that may open the doors to more funding in the future. Public education and support are key as well.

Lack of control over where transgenes are inserted in the genome and other possible genetic factors such as genetic background and epistasis have lead to various responses in individual transgenic families or lines. This dictates combining transgenesis with traditional techniques such as selection to identify high performance transgenic lines and optimize their gene expression and phenotype. This makes gene transfer a medium to long-term, rather than short-term, breeding program. As for traditional selective breeding programs, most research institutes and scientists do not have the facilities, commitment, or patience for relatively long experiments that are further aggravated by the potentially controversial nature of the research. Long-term commitment is needed to allow transgenic fish technology to reach fruition.

Some transgenic technologies have not been pursued for fish because of a lack of embryonic stem cell lines for fish. This has hindered efforts to conduct gene knockout research and homologous recombination. However, transplantation of primordial germ cells is now possible (Takeuchi, 2003), opening the door to new gene knockout technology. New targeted gene insertion and gene knockout technologies are on the horizon (Cui et al., 2003) to alleviate these problems.

Early work in transgenic fish was also hindered by a lack of fish promoters and much was conducted with viral promoters. If commercialization is the objective, much of that early research needs to be repeated, examining expression with fish promoters that will likely receive much better public perception and marketability. The advancement in genomics will not only provide important genes for gene transfer that likely will have greater public acceptance, but also highly regulated promoters.

Similarly, inducible and tissue specific expression is likely needed for better transgene expression and performance in the future. Progress is being made as several tissue specific promoters have been developed from zebrafish (Gong et al., 2002) including epidermis specific keratin 8, fast muscle specific myosin light polypeptide 2, and pancreatic exocrine cell specific elastase B. For some applications, inducible promoters may be

desirable to allow induction of transgene expression at specific developmental life stages. The inducible HSP70 gene that encodes an enzyme playing an essential role in protein metabolism has been isolated and characterized from *O. mossambicus*; it dramatically increased the rate of mRNA transcription when fish were exposed to transient heat shock (Molina et al., 2000).

Currently, the largest global research activity relevant to current and future transgenic research is genomics and functional genomics. Much smaller research efforts exist for gene transfer and transgenic application in fish, and research funding for this area is much smaller than for genomics. Much is being learned about genetic mechanisms and gene expression regarding the physiology, response to stressors, and response to environmental variables by fish. Great progress has been made regarding gene expression and regulation, gene isolation and sequencing, and gene mapping.

Financial support waned for transgenic research partially because of controversy surrounding the technology and the advent, importance, need for research dollars, and little or no perception of controversy associated with genomics. In some ways, this was actually support for a needed branch of transgenic technology. A huge amount of genomic information has been generated and it is growing rapidly. How are we going to use and apply the knowledge of genomics and functional genomics in the near future? One potential output is that the increased understanding in physiology could lead to new environmental interventions and management in fish culture. This information could allow for the development of and enhancement of marker assisted selection programs. One of the most promising applications of this explosion in genomics is through transgenic technology. In some ways, research support for genomics has been research support for transgenic technology.

Important aquaculture traits such as tolerance of poor water quality, harvestability, carcass yield, increased reproduction, and improved utilization of plant resources have yet to be addressed by transgenic technology. Basic information from genomic research may be the starting point to effectively addressing genetic enhancement of these traits.

One of the greatest future potential benefits of gene transfer in fish will be enhancement of disease resistance. In general, disease is the greatest problem facing aquaculture and damaging its profitability. Additionally, this should be an animal welfare issue. Transgenic fish with enhanced disease resistance would increase profitability, production, efficiency, and the welfare of cultured fish. Preliminary research indicates great promise for enhancing disease resistance. Genetic gains are possible through traditional selective breeding, but it appears that the rate of genetic improvement and the consistency of genetic improvement may be greater with the transgenic approach (Dunham et al., 2002ad). Selective breeding may also have the drawback that diseased organisms may respond to selective forces as well, negating some of the selection response in the fish.

One of first applications of transgenics in fish was the alteration of color in ornamental and aquarium fish with fluorescent pigment genes. If consumers demand color-altered transgenic fish, this could evolve into a major application of transgenesis with a large economic impact. This may also result in additional environmental risk issues and confinement issues. Many, but not all, ornamentals cannot survive in the natural environment. Large aquaculture facilities can be monitored to ensure adequate confinement but it will be impossible to monitor and confine thousands or millions of households. Therefore, new issues and perspectives might need to be addressed.

A topic that is generally avoided is the application of transgenic fish in recreational fisheries. This would involve release of transgenic fish in unconfined areas and confined urban environments. Public opinion will vary in regards to this application. The growth rate and aggressiveness demonstrated for some transgenic fish may be desirable in sport fish applications. Some fishermen are purists and would never want to fish for a genetically modified fish of any type. Other fishermen have

expressed interest in the possibility of genetically modified trophy fish. Some fishermen are unconcerned by the above scenarios/issues; their objective is to have a successful fishing trip, in other words, an acceptable catch per effort rate. Application of transgenic fish in aquaculture and ornamental fish will likely occur much earlier than any recreational fisheries application.

The ultimate aquaculture genotype will likely be developed by combining genetic enhancement programs. In Israel, Hinits and Moav (1999) were able to improve common carp growth by genetic engineering together with crossbreeding more than by crossbreeding alone. Similarly, when salmon metallothionein promoter/salmon GH1 cDNA, OnMTGH1 was transferred to another wild rainbow trout strain, F77, growth was enhanced 7-fold, almost 4-fold more than a domestic rainbow trout (Devlin et al., 2001). The transgenic wild x domestic crossbreed was by far the largest genotype, 18 times larger than the non-transgenic wild parent, 13 times larger than the non-transgenic wild x domestic crossbreed, 9 times larger than the non-transgenic domestic parent, and over 2.5 times larger than the wild F77 transgenic (Devlin et al., 2001). Combined transgenesis and crossbreeding had much greater growth enhancement effects than crossbreeding or transgenesis alone. Channel catfish transgenic for rainbow trout GH had moderate growth enhancement (41%) and were derived from domestic, selectively bred catfish. More research is needed that addresses taking advantage of multiple genetic enhancement programs.

Genetics is no silver bullet. Genetics of aquaculture organisms and production can and will always need improvement. We may never reach the ultimate genotype of cultured fish and shellfish**, but improvements will definitely** be made with genetic research.

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