

**ABSTRACT:** Although capture fisheries have experienced slow to stagnant growth in recent years, the world population has been increasing, with subsequent rises in demands for marine-based foods. One possibility for alleviating potential food shortages and price increases is through aquaculture, which has experienced rapid worldwide expansion. A major focus of research in the aquaculture industry is on the use of biotechnology to increase food availability and reduce production costs, specifically through the manipulation of the genes and chromosomes of cultivated species. Examples include transgenic fish with properties such as increased growth rates, feed conversion efficiency, disease resistance, cold tolerance, and improved metabolism of land-based plants. However, use of transgenic organisms in aquaculture is a very controversial topic due to a number of environmental and human health concerns such as escapement and introduction of genetically modified organisms into the food chain. In response, some transgenic research has also been focused on inducing sterility to reduce the risk of transgenic organisms breeding with wild species. A method of chromosome manipulation, referred to as polyploidy, provides the option of creating sterile organisms, some of which also exhibit increased growth rates. This review paper will discuss recent advances in biotechnology research, specifically in regards to the manipulation of genes and chromosomes, for enhanced cultivation of fish and invertebrates. Major environmental and human health concerns will also be addressed.

## Introduction

Advances in biotechnology over the past several decades have provided the tools necessary for artificial manipulation of genes and chromosomes in living organisms. The creation of transgenic fish and shellfish is a topic of great interest in aquaculture research due to the potential improvements in production that this technology can offer (Zbikowska 2003; Dunham 2004). Major areas of transgenic research in fish include use of growth hormones (GHs) to increase growth and feed conversion efficiency; use of antifreeze proteins (AFPs) for enhanced cold tolerance and freeze resistance; use of antimicrobial peptides for increased disease resistance; use of metabolic genes to promote low-cost, land-based diets; and genetic methods for inducing sterility. Although transgenic work in fish is well established, research with marine invertebrates is only in the initial stages due to complications with introduction and expression of foreign genes. Early research with marine invertebrates has led to the development of successful gene transfer methods, with studies focused on improving disease resistance. In addition to transgenic research, advances in chromosome manipulation (polyploidy) also show potential for improving production in the aquaculture industry, particularly in the case of shellfish. Use of polyploidy in aquaculture can result

in sterility, along with enhanced growth and survival rates and increased quality of final products.

The aquaculture industry is the fastest growing of the animal food-producing sectors, increasing at an average rate of 8.9% per year since 1970 (FAO 2004). Although landings from capture marine fisheries increased about 5-fold in the period from 1950 to 1990, annual growth has become slow to stagnant over the past 15 years (FAO 2000, 2006). As a result of factors such as population growth, urbanization, and rising per capita incomes, world fish consumption more than tripled over the period of 1961 to 2001, increasing from 28 to 96.3 million metric tons (FAO 2004). The global demand for fish and fishery products is predicted to continue to increase in the years to come—from 133 million tons in 1999 to 2001 to 183 million metric tons by the year 2015. Taking into account indications that capture fisheries are close to or have already reached their potential, the world is looking toward aquaculture and its technologies to fulfill the expanding food demands: by 2020, aquaculture is expected to supply 41% of the global food fish production (compared to 3.9% in 1970 and 29.9% in 2002) (Delgado and others 2002; FAO 2004).

Despite predictions of a growing aquaculture industry, stagnant world capture fisheries and increased populations are projected to lead to a global shortage of fish and fish products in the years to come (Delgado and others 2002). As a result, prices are expected to rise: it has been predicted that by 2015, fish prices will increase by about 3.2%<sup>1</sup> (FAO 2004), and by 2020, prices for mollusks, finfish, and crustaceans are expected to increase by 4% to 16%<sup>2</sup>

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<sup>1</sup> Compared to prices in 1999/2001.

<sup>2</sup> Compared to prices in 1996/1998.

(Delgado and others 2002). Use of biotechnology in aquaculture has the potential to alleviate these predicted fish shortages and price increases by enhancing production efficiency, minimizing costs, and reducing disease. However, the incorporation of transgenic organisms into the food chain has been met with massive criticism from both the environmental and human health sectors. This review paper will cover current advances in the manipulation of genes and chromosomes for use in aquaculture along with a discussion of the controversy surrounding transgenic technology in aquaculture.

### Transgenics

#### Transgenic fish

By definition, transgenic or genetically modified organisms (GMOs) are those that have had foreign DNA (deoxyribonucleic acid) artificially inserted into their own genomes (Chen and others 1996; FAO 2000). The first successful case of transgenic fish was reported in 1985, when Zhu and colleagues microinjected the human GH gene into the fertilized eggs of goldfish (*Carassius auratus* L. 1758) (Zhu and others 1985). This was followed by successful introduction of human GH gene into the genome of the loach (*Misgurnus anguillicaudatus*) with resulting transgenic fish that grew 3 to 4.6 times faster than the control within the first 135 d (Zhu and others 1986). Since 1985, the field of transgenics has experienced a number of technological advances. Many genetically modified fish species have been established along with various methods for foreign gene insertion (such as microinjection, electroporation, infection with pantropic defective retroviral vectors, particle gun bombardment, and sperm- and testis-mediated gene transfer methods) and detection (such as polymerase chain reaction [PCR]-based assay and Southern blot analysis) (Chen and others 1996; Lu and others 2002; Sarmasik 2003; Collares and others 2005; Pandian and Venugopal 2005; Smith and Spadafora 2005). Transgenic fish are appealing to some because attainment of desired traits is generally more effective, direct, and selective than traditional breeding, and could prove to be an economic benefit for improvement of production efficiency in aquaculture worldwide (Nam and others 2002; Ramirez and Morrissey 2003). In addition, fish species tend to be relatively tolerant to artificial manipulation of their genes during early development, making them ideal subjects for genetic modification (Foresti 2000). However, there are numerous environmental and human health concerns, which will be addressed later in this section, that are associated with the use of transgenic technology in aquaculture (FAO 2000; Fleming and others 2000; Aerni 2004; Naylor and others 2005).

To create a transgenic fish, a DNA construct containing genes for the desired trait(s) along with a promoter sequence is generally introduced into the pronuclei of fertilized eggs. This is followed by *in vitro* or *in vivo* (implanted into the uterus of a pseudopregnant female) incubation of the injected embryos and subsequent maturation into a fully developed transgenic organism (Chen and others 1996). Once transgenes have become integrated into a host organism's DNA, they can be passed on to future generations (Chen and others 1996), with the possibility of 100% transmission using stable isogenic transgenic lines (Nam and others 2002). Zebrafish are often used as a model species in transgenic studies owing to a number of biological and experimental advantages: they are easy to maintain and breed, their embryos are sturdy against experimental manipulations, they develop rapidly, they have a large number of offspring, and they are transparent at some stages of development, facilitating the use of fluorescent transgenes (Dahm and Geisler 2006). Thus far, fish including Atlantic, coho, and chinook salmon, rainbow and cutthroat trout, tilapia, striped bass, mud loach, channel catfish, common carp, Indian

major carp, goldfish, Japanese medaka, northern pike, red and silver sea bream, walleye, and zebrafish have been genetically modified to produce select traits (see Table 1) such as increased growth, increased feed conversion efficiency, cold tolerance, and disease resistance, all of which will be thoroughly discussed in the following subsections.

**Growth hormone.** GH is a polypeptide that is excreted from the pituitary gland, binds specific cell receptors, and induces synthesis and secretion of insulin-like growth factors (IGF-1 and IGF-II), resulting in promotion of somatic growth through improved appetite, feeding efficiency, and growth rate (Hsieh and others 1997; de la Fuente and others 1998). In fish, the central nervous system (CNS) normally controls GH excretion levels, which are highly variable, occurring seasonally and in bursts. However, the AFP gene of ocean pout (*Macrozoarces americanus*) is expressed year-round in the liver. As a way of bypassing CNS control on GH expression, transgenic research typically involves linking the GH gene to the AFP gene promoter (Fletcher and others 2004). Enhanced secretion of GH and subsequent fish size augmentation could greatly reduce production costs associated with aquaculture by reducing the time to market size and lowering exposure to risks such as disease and predators (Cook and others 2000). Although attempts to introduce GH exogenously have also been met with some success, commercial application of strains of fish containing transgenically introduced GH, once developed, could prove to be more cost-effective (Chen and others 1996). Increased growth has been the most thoroughly researched of the possible transgenically induced fish traits, and it is predicted that this technology will soon be applied to commercial aquaculture production (Wu and others 2003). However, a major remaining challenge is to determine the optimum balance of GH required for obtaining maximum growth without causing harmful effects to the organism (de la Fuente and others 1998), as excess GH can cause problems such as acromegaly (associated in humans with excess bony growth in the jaw) and head enlargement (Rahman and others 2001).

Since the initial transgenic introduction of human GH gene into goldfish and mud loach (Zhu and others 1985, 1986), extensive research has been performed on the use of GH in a wide variety of aquatic species. New, improved techniques have been developed for introduction of transgenes into host genomes, and there has been a focus on the use of GH genes originating from fish rather than humans, with the hope of increasing consumer acceptability (Levy and others 2000). Negative perceptions associated with the use of viral promoters to express transgenes have also driven many researchers to replace them with fish-based promoters for the creation of "all-fish" transgene constructs (Galli 2002). These all-fish GH-transgenic strains have been developed in a number of species, including common carp (*Cyprinus carpio*), silver sea bream (*Sparus sarba*), red sea bream (*Pagrosomus major*), tilapia (*Oreochromis niloticus*), and Atlantic salmon (*Salmo salar*) (Table 1).

As discussed by Wu and others (2003), an all-fish GH construct has been successfully introduced into the common carp, resulting in increased growth rate and more efficient feed conversion as compared to farmed fish controls. Middle-scale trials of these all-fish GH-transgenic common carp have shown high potential for successful commercial application in aquaculture. In 2003, carps and other cyprinids were the top species group produced worldwide in aquaculture, contributing 17.2 million tons (live weight) to the global food supply (Johnson 2005). Use of transgenic technology in carp aquaculture could prove to be highly beneficial to the industry and further increase the availability of these fish.

Transgenic lines of silver sea bream, an economically important cultivated species in Asia, were developed using a construct

**Table 1 – Genetically modified fish being tested for use in aquaculture<sup>a</sup>**

Species	Foreign gene	Desired effect/results	Status	Country	Reference
Bass, striped ( <i>Morone saxatilis</i> )	Insect genes	Disease resistance	Early stages of research	U.S.	(FAO 2000)
Carp, common ( <i>Cyprinus carpio</i> )	Salmon and human GH; rainbow trout GH	20%–150% growth improvement; improved disease resistance; tolerance of low oxygen level	Research and growth trials	China, U.S.	(Zhang and others 1990; Chen and others 1992; Fu and others 1998; FAO 2000; Maclean and Laight 2000; Dunham and others 2002a) (Zhang and others 2000; Wang and others 2001)
	Grass carp GH and common carp $\beta$ -actin promoter	Increased growth in only 8.7% of transgenic fish; no harmful effects to mice fed transgenic fish	Research	China	
	Antisense-GnRH mRNA	Sterility — 30% of injected fish did not develop gonads	Early research phase, pilot studies	China	(Hu and others 2006)
Carp, grass ( <i>Ctenopharyngodon idellus</i> )	hLF	Increased disease resistance to bacterial pathogen	Research phase	China	(Mao and others 2004)
	hLF + common carp $\beta$ -actin promoter	Increased disease resistance to grass carp hemorrhage virus			(Zhong and others 2002)
Carp, Indian major	Human GH	Increased growth	Research phase	India	(FAO 2000)
Carp, Indian major ( <i>Labeo rohita</i> )	Carp GH + CMV promoter + internal ribosomal entry sites element	4- to 5-fold growth increases compared to controls, but 99% mortality by wk 10	Research phase	India, U.S.	(Pandian and Venugopal 2005)
Catfish, channel ( <i>Ictalurus punctatus</i> )	GH	33% growth improvement in culture conditions	Research phase	U.S.	(FAO 2000)
	GH + Rous sarcoma virus promoter	Some increased growth rates (23–26%), some normal growth rates	Research and growth trials		(Dunham and others 1992; Maclean and Laight 2000)
	Silk moth ( <i>Hyalophora cecropia</i> ) cecropin genes	Bactericidal activity, increased survival when exposed to pathogens	Research phase		(Dunham and others 2002b; Dunham 2005)
Charr, Arctic ( <i>Salvelinus alpinus</i> L.)	Sockeye salmon GH + human CMV (also tried sockeye piscine metallothionein B and histone3 promoters)	14-fold weight increase after 10 mo; CMV promoter resulted in greatest weight increase, with no difference in muscle composition compared to controls	Research phase	Finland	(Krasnov and others 1999; Pitkanen and others 1999b)
Goldfish ( <i>Carassius auratus</i> )	GH + Arctic flatfish AFP	Increased growth	Research phase	China	(FAO 2000)
	Ocean pout type III AFP	Increased cold tolerance			(Wang and others 1995)
	Human GH	Increased growth			(Zhu and others 1985)

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Table 1 – Continued.

Species	Foreign gene	Desired effect/results	Status	Country	Reference
Medaka, Japanese ( <i>Oryzias latipes</i> )	Insect cecropin or pig cecropin-like peptide genes + CMV	Enhanced bactericidal activity against common fish pathogens; germline transmission	Research phase	U.S.	(Sarmasik and others 2002)
Mud loach ( <i>Misgurnus mizolepis</i> )	<i>Aspergillus niger</i> phytase gene + human CMV or sockeye salmon histone type III promoter Mud loach GH + mud loach and mouse promoter genes	Ability to digest phytate, the major form of phosphorus in plants; increased survival on high phytate diet Increased growth and feed conversion efficiency; 2- to 30-fold growth enhancement; 100% germline transmission	Research phase	China, South Korea	(Hostetter and others 2003) (FAO 2000; Nam and others 2002; Kapuscinski 2005)
Loach ( <i>Misgurnus anguillicaudatus</i> )	Human GH	3- to 4.6-fold increase in growth over 135 d	Research	China	(Zhu and others 1986)
Pike, northern ( <i>Esox lucius</i> )	Bovine GH or chinook salmon GH	Growth enhancement	Research phase	U.S.	(Gross and others 1992)
Sea bream, red ( <i>Pagrosomus major</i> )	Chinook salmon GH + ocean pout AFP gene promoter	9.3% increase in length and 21% increase in weight after 7 mo	Research phase	China	(Zhang and others 1998)
Sea bream, silver ( <i>Sparus sarba</i> )	Rainbow trout GH + common carp $\beta$ -actin promoter	Increased growth; successful use of sperm- and testis-mediated gene transfer techniques	Research phase	China	(Lu and others 2002)
Salmon, Atlantic ( <i>Salmo salar</i> )	Arctic flattish AFP Chinook salmon GH	Cold tolerance Greater feed efficiency; 2- to 13-fold growth increase; inheritance through 6 generations	Research phase, trying to achieve freeze resistance Patented and licensed to Aqua Bounty Technologies, currently under regulatory review for commercial use	U.S., Canada, Newfoundland U.S., Canada	(Fletcher and others 1992, Hew and others 1995; FAO 2000) (Du and others 1992; Fletcher and others 1992, 2004; Hew and others 1995; Cook and others 2000; FAO 2000; Kapuscinski 2005) (Hew and others 1995)
Salmon, chinook ( <i>Oncorhynchus tshawytscha</i> )	Rainbow trout lysozyme gene + ocean pout AFP promoter	Bacterial resistance is desired, but no results have been reported	Early research stages	Canada	
Salmon, coho ( <i>Oncorhynchus kisutch</i> )	Mx genes Arctic flattish AFP + salmon GH	Potential resistance to pathogens following treatment with poly I:C Enhanced growth and feed efficiency	Early research stages Research phase	Norway New Zealand	(Jensen and others 2002) (FAO 2000)
Tilapia ( <i>Oreochromis niloticus</i> )	Arctic flattish AFP + chinook salmon GH Chinook salmon GH with ocean-pout type III AFP promoter	10- to 30-fold growth increase after 1 y Increased growth (2.5–4x) and 20% greater feed conversion efficiency; germline transmission; no organ growth abnormalities	Research phase Research and growth trials have been carried out, now seeking regulatory approval	Canada Canada, United Kingdom	(Devlin and others 1995; FAO 2000) (Rahman and Maclean 1998; Maclean and Laight 2000; Rahman and others 2001; Caelers and others 2005)

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Table 1 – Continued.

Species	Foreign gene	Desired effect/results	Status	Country	Reference
Tilapia ( <i>Oreochromis</i> sp.)	Tilapia GH + human CMV	Increased growth and feed conversion efficiency (290%); stable inheritance		Cuba	(Martinez and others 1996, 2000; FAO 2000; Kapuscinski 2005)
Tilapia, Nile ( <i>O. niloticus</i> ) and Tilapia, redbelly ( <i>Tilapia zillii</i> )	Shark ( <i>Squalus acanthias</i> L.) IgM genes	Enhanced immune response and growth; abnormal gonad development at high doses	Research phase	Egypt	(El-Zaeem and Assem 2004; Assem and El-Zaeem 2005)
Trout, cutthroat ( <i>Oncorhynchus clarki</i> )	Arctic flatfish AFP + Chinook salmon GH	Increased growth	Research phase	Canada	(FAO 2000)
Trout, rainbow ( <i>Oncorhynchus mykiss</i> )	Arctic flatfish AFP + salmon GH Human glucose transporter + rat hexinose type II with viral or sockeye salmon promoters	Increased growth and feed efficiency Improved carbohydrate metabolism	Research phase	U.S., Canada Finland	(FAO 2000) (Pitkanen and others 1999a; Kapuscinski 2005)
Zebrafish ( <i>Danio rerio</i> )	Masu salmon n-6-desaturase-like gene + medaka $\beta$ -actin promoter Antisense salmon GnRH + common carp $\beta$ -actin promoter Cre recombinase driven by T7 promoter + fluorescent protein flanked by two loxP sites crossed with T7 RNA Polymerase (gonad specific)	Increased ability to convert ALA to DHA and EPA Sterility in 30% of injected eggs Gonad-specific excision of foreign (fluorescent protein) gene	Research phase Research phase Research phase	Japan China China	(Alimuiddin and others 2005) (Hu and others 2006) (Hu and others 2006)
	4 Japanese flounder ( <i>Paralichthys olivaceus</i> ) promoters (complement component C3, gelatinase B, keratin and tumor necrosis factor) linked to GFP	Tissue-specific GFP gene expression: complement component C3 in liver, gelatinase B in pectoral fin and gills, keratin in skin and liver, and tumor necrosis factor in pharynx and heart	Ready for use in transgenic research	Japan	(Yazawa and others 2005a)

<sup>a</sup>Adapted from The State of the World Fisheries and Aquaculture (SOFIA) (FAO 2000) and Ramirez and Morrissey (2003).

Note: AFP = anti-freeze protein gene, can also refer to the AFP promoter when used in combination with the growth hormone gene; GH = growth hormone gene; CMV = cytomegalovirus promoter; hLF = human lactoferrin gene; IgM = immunoglobulin; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; ALA =  $\alpha$ -linolenic acid; GnRH = gonadotropin-releasing hormone; GFP = green fluorescence protein.

containing rainbow trout (*Oncorhynchus mykiss*) GH complementary DNA (cDNA) with a common carp promoter (Lu and others 2002). Using 2 novel methods of introduction, sperm- and testis-mediated gene transfer, between 56% and 76% of the animals carried the transgene, and several showed faster growth than controls (Lu and others 2002).

Red sea bream has high economic value in China, but production is limited by a relatively slow growth rate (Zhang and others 1998). With hopes of developing this industry, Zhang and others (1998) introduced an all-fish gene construct containing chinook salmon (*Oncorhynchus tshawytscha*) GH with an ocean pout AFP gene promoter into fertilized eggs using electroporation, a technique that allows for treatment of over 10000 eggs in 10 min. After 30 d, 29% of the fish carried the foreign DNA sequence and after 2 mo, 38% were found to carry the sequence. Compared to controls at 7 mo, the average body weight and length of the transgenic individuals increased by 21% and 9.3%, respectively.

Tilapia is an economically important aquaculture species that is farmed in over 60 countries worldwide (Rahman and Maclean 1998). All-fish transgenic tilapia were successfully created by microinjection of chinook salmon GH linked to an ocean pout AFP promoter. The transgenic tilapia were found to grow almost 4 times faster than nontransgenic siblings, and the transgenic sequence was successfully transmitted through the germline (Rahman and Maclean 1998). A more recent, long-term trial found a 2.5-fold increase in growth and 20% greater food conversion efficiency, with more efficient utilization of protein, dry matter, and energy, as compared to nontransgenic siblings (Rahman and others 2001). In an investigation into the expression sites of the AFP type III promoter/chinook salmon GH construct in tilapia, messenger ribonucleic acid (RNA) containing the transgenic GH was expected to be mainly found in the liver (Caelters and others 2005). However, the resulting expression pattern was more reflective of natural rather than transgenic GH, with transgenic messenger RNA being detected in a number of other organs, including the gills, heart, brain, kidney, spleen, intestine, skeletal muscles, and testes. Since no growth abnormalities were observed, the authors suggested that this gene transfer method mimics the natural expression of GH in organs during development, resulting in growth-enhanced fish with normal proportions.

Transgenic tilapia containing the GH gene driven by the human cytomegalovirus (CMV) were compared to nontransgenic siblings on a number of metabolic and physiological parameters (Martinez and others 2000). The results showed several significant differences, with transgenic tilapia consuming 3.6-fold less food and having 290% greater feed conversion efficiency. In addition, growth efficiency, average protein synthesis, anabolic stimulation, and synthesis retention were higher for the transgenic tilapia. The authors also reported distinct metabolic differences for transgenic juveniles, concluding that GH-enhanced transgenic juvenile tilapia are biologically more efficient than nontransgenic juveniles.

Studies into the use of CMV as a promoter of the GH gene have also taken place with fish such as Arctic charr (*Salvelinus alpinus* L.) and the Indian major carp *Labeo rohita* (Pitkanen and others 1999b; Pandian and Venugopal 2005). Pitkanen and others (1999b) reported that CMV showed a greater ability to promote transgenic growth as compared with 2 piscine promoters, metallothionein B and histone 3. A further investigation into the use of CMV as a GH gene promoter showed no difference in the muscle composition of transgenic fish as compared to nontransgenic siblings (Krasnov and others 1999). However, the transgenic fish did show some metabolic features that are often observed in farmed salmonids, such as an increased metabolic rate and faster utilization of dietary lipids, particularly in the case of triglycerides. In transgenic studies involving the Indian major carp *L. rohita*,

an element known as internal ribosomal entry sites (IRES) was included in an expression vector containing the CMV promoter and Indian major carp GH (Pandian and Venugopal 2005). This IRES element enables expression of 2 genes—the gene coding for the protein of interest (GH) along with a reporter gene (enhanced green fluorescent protein [EGFP]). Although the resulting transgenic carp exhibited 4- to 5-fold increases in growth compared to nontransgenic controls, 99% mortality was observed within 10 wk of hatching.

Transgenic Atlantic salmon containing chinook salmon GH have also been developed (Du and others 1992; Fletcher and others 1992; Cook and others 2000). In 1 study, all-fish growth-enhanced transgenic Atlantic salmon produced by microinjection were found to have a 13-fold increase in body weight compared to nontransgenic controls (Du and others 1992). In a later study, all-fish growth-enhanced transgenic Atlantic salmon were found to grow at rates 2.62 to 2.85 times greater than nontransgenic controls, with a 10% greater feed conversion efficiency (Cook and others 2000). Transgenic salmon also ingested more, exhibiting 2.14- to 2.62-fold greater daily feed consumption, with lower body protein, dry matter, ash, lipid, and energy and higher moisture than nontransgenic controls (Cook and others 2000). The results of a recent study into the genetic expression and interactions of GH, IGF-I, and their receptors indicate involvement of the hormones in the areas of vertebral growth and bone density (Wargelius and others 2005). It was reported that plasma GH most likely activates growth in bony tissues, while IGF-I appears to be important in bone matrix production.

Fletcher and others (2004), whose laboratory began studying GH gene transfer in 1989, reported successful expression and inheritance of the GH gene through 6 generations of Atlantic salmon. The transgenic salmon reach market size approximately 1 y earlier than nontransgenic farmed salmon. This technology was patented and licensed to Aqua Bounty Technologies™ of Waltham, Mass., U.S.A., which has recently announced the development of transgenic broodstocks of salmon, trout, and tilapia under the trade name of *AquAdvantage*™ (<http://www.aquabounty.com/products.html>). These fish, which contain the GH gene from chinook salmon, express GH protein year-round instead of seasonally and are reported by the company to reach market size at least twice as fast as their nontransgenic counterparts (Dove 2005). The transgenic fish are also neutered to prevent interbreeding with wild stocks. *AquAdvantage* salmon, which grow at a rate 4 to 6 times greater than nontransgenic Atlantic salmon, are currently under regulatory review in both the United States and Canada (Castle and others 2005).

**Antifreeze protein.** The idea of an “antifreeze” system was first described in marine fish inhabiting the coast of Northern Labrador whose body fluids had the same freezing point of seawater (−1.7 °C to −2 °C) rather than freshwater (0 °C) (Scholander and others 1957; Gordon and others 1962). This phenomenon was eventually attributed to a set of peptides and glycopeptides termed AFPs and antifreeze glycoproteins (AFGPs), respectively. These proteins are synthesized primarily in the liver and secreted into the blood and extracellular space, where they bind and modify the structure of microice crystals, thereby inhibiting ice crystal growth and lowering the freezing point of body fluids (Davies and Hew 1990; Raymond 1991). AFPs have diverse structures and are divided into 3 categories (types I, II, and III) depending on their protein sequences. Also, the number of copies and type of AFP genes varies with the fish species: for example, winter flounder (*Pleuronectes americanus*) have 30 to 40 copies of type I; sea raven (*Hemitripterus americanus*) have 12 to 15 copies of type II; and Newfoundland ocean pout have 150 copies of type III (Hew and others 1995).

The ocean pout type III AFP transgene has been successfully transferred and expressed in goldfish (Wang and others 1995). The gene was microinjected into the goldfish oocytes and was inherited through 2 generations. The transgenic goldfish showed significantly higher cold tolerance as compared to controls, suggesting possible use of the transgene for promoting cold resistance in fish.

AFP transgenic technology could be highly beneficial to the aquaculture industry in countries with freezing and subzero coastline conditions. For example, winter water temperatures along the Atlantic coast of Canada can range from 0 °C to -1.8 °C; these conditions restrict the cultivation of salmonids and other commercially important fish to a few select areas at the southern edge of eastern Canada (Hew and others 1995; Fletcher and others 2004). Therefore, research is currently under way to develop strains of Atlantic salmon that could be cultivated over a wider geographic range. This could be accomplished by (1) introduction of a set of AFP transgenes that allow the fish to survive lower water temperatures, or (2) introduction of GH transgenes to produce a rapidly growing strain that does not require overwintering (Hew and others 1995). Although AFP transgenes have been successfully introduced into, expressed in, and inherited through germlines of Atlantic salmon, the cold-tolerant transgenic salmon do not produce AFP in sufficient quantities to achieve freeze resistance (Hew and Fletcher 1992; Hew and others 1995; Fletcher and others 2004). This may be due to the need for higher expression levels and/or the fact that the gene actually codes for an AFP precursor that must first be changed into its fully functional form (Maclean and Laight 2000). The current challenge, according to Fletcher and others (2004), is to create an AFP transgene construct that will be expressed at heightened levels in the epithelial tissues and the liver.

**Disease resistance.** A major limitation facing the aquaculture industry is outbreak of disease, as farmed fish are generally cultured at high densities and under stress, putting them at elevated risk for bacterial infection (Hew and others 1995). One example can be found in the catfish industry, which suffers an average annual loss of over US\$100 million because of disease (Dunham and others 2002b). Channel catfish (*Ictalurus punctatus*) are an economically important species in the United States, accounting for more than 60% of U.S. aquaculture production (269000 metric tons per year) (USDA 2001). However, these fish are susceptible to numerous harmful bacterial infections, the most detrimental being enteric septicemia, caused by *Edwardsiella ictaluri* (Dunham and others 2002b). Antibiotics can help provide disease resistance, but only a limited number have been approved for use in aquaculture (Sarmasik and others 2002). Although there are effective vaccines available for some diseases, many common catfish diseases, including enteric septicemia, do not have truly effective treatment methods (Dunham and others 2002b). Also, use of DNA vaccines is often labor-intensive and can cause high stress to the fish owing to excessive handling (Sarmasik and others 2002). A promising alternative involves use of transgenic technology to produce strains of fish with increased disease resistance. A number of antimicrobial peptides with the potential to improve disease resistance in aquaculture have been isolated from fish. The gene coding for these peptides has not been well characterized; however, a few possibilities are discussed below.

Cecropins are a group of small, antibacterial peptides first identified in the silk moth *Hyalophora cecropia* (Dunham and others 2002b). Cecropins have antimicrobial activity against a wide spectrum of bacteria and have already been incorporated into transgenic plants such as potato and tobacco to provide increased disease resistance (Sarmasik and others 2002). Channel catfish with transgenically introduced cecropin genes demonstrated increased disease resistance and survival when exposed to *E. ic-*

*taluri* and *Flavobacterium columnare*, as compared to nontransgenic controls (Dunham and others 2002b). Transgenic individuals exposed to *F. columnare* in an earthen pond showed 100% survival, which was significantly greater than the nontransgenic controls (27.3% survival). When challenged with *E. ictaluri* in tanks, transgenic individuals also showed significantly greater survival (40.7%) as compared to nontransgenic controls (14.8%) (Dunham 2005). Transgenic Japanese medaka (*Oryzias latipes*) containing insect cecropin or pig cecropin-like peptide transgenes driven by a CMV promoter have also shown enhanced bactericidal activity against 2 known fish pathogens, *Pseudomonas fluorescens* and *Vibrio anguillarum* (Sarmasik and others 2002). Cecropin transgene constructs were electroporated into medaka eggs, and survivors containing the transgene were used as founder stocks to establish successive generations of transgenic medaka. When the F2 transgenics were exposed to *P. fluorescens* and *V. anguillarum* at 60% lethal dose, only 10% and 10% to 30% were killed, respectively, while 40% of the controls were killed by both pathogens (Sarmasik and others 2002).

Lysozyme is a nonspecific antibacterial enzyme present in the blood, mucus, kidney, and lymphomyeloid tissues in fish (Hew and others 1995). Rainbow trout contain elevated levels of lysozyme (10- to 20-fold higher than in Atlantic salmon) and a rainbow trout lysozyme cDNA construct with an ocean pout AFP promoter has been created (Hew and others 1995). Interestingly, rainbow trout were recently reported to have 2 distinct types of lysozymes, with only type II having significant bactericidal activity (Mitra and others 2003). The gene for type II lysozyme was amplified and sequenced for future use in transgenic immune system enhancement of farmed fish. Lysozyme transgenes are also being tested in agricultural products such as rice and have been found to increase disease resistance (Huang and others 2002). Although the lysozyme gene may prove to heighten disease resistance in transgenic fish, research is still in the initial phases with no published results to date (Fletcher and others 2004).

Human lactoferrin (hLF) is a nonspecific antimicrobial and immunomodulatory iron-binding protein that has been used widely in agriculture for production of disease-resistant transgenic crops, including potatoes and tobacco (Mao and others 2004). One use of hLF in fish is to increase resistance against the grass carp hemorrhage virus (Zhong and others 2002). This virus induces deadly hemorrhaging in grass carp and is a major setback to the successful farming of these fish in China. To induce resistance against the virus, a DNA construct containing the hLF gene linked to a common carp  $\beta$ -actin promoter was electroporated into the sperm of grass carp (*Ctenopharyngodon idellus*) (Zhong and others 2002). Gene transfer efficiency was reported to be near 50%, and after 5 mo about 36% of the surviving grass carp contained the transgene. After being challenged with the virus, transgenic fry showed a significant delay in the onset of symptoms of hemorrhage, indicating a possible use for hLF gene expression in grass carp. In a more recent study, electroporation of hLF cDNA into grass carp sperm resulted in as high as 55% production of transgenic fish (Mao and others 2004). These fish had increased immunity against infection with the bacterial pathogen *Aeromonas hydrophila* as compared to nontransgenic controls; transgenic grass carp had enhanced phagocytic activities and were able to clear *A. hydrophila* from their systems more quickly (Mao and others 2004). The authors hypothesized that hLF increases disease resistance by stimulating phagocytic activity in transgenic fish.

A series of recent studies have focused on the use of shark DNA to boost immune responses in fish (El-Zaem and Assem 2004; Assem and El-Zaem 2005). Sharks contain high levels of immunoglobulin (IgM) proteins, which act as antibodies and help initiate immune responses to bacterial invasions. Although IgM

can be found at high levels in shark (up to 50% of the serum proteins), it has been reported to be present at much lower levels in fish such as Atlantic salmon, halibut (*Hippoglossus hippoglossus* L.), haddock (*Melanogrammus aeglefinus* L.), and cod (*Gadus morhua* L.) (2%, 8%, 13%, and 20% of the serum proteins, respectively) (Marchalonis and others 1993; Magnadottir 1998). When shark (*Squalus acanthias* L.) DNA was injected into the skeletal muscles of Nile tilapia (*O. niloticus*) and redbelly tilapia (*Tilapia zillii*) fingerlings, fish showed significantly higher levels of total antibody activity, serum total protein, and globulin (El-Zaeem and Assem 2004; Assem and El-Zaeem 2005). In addition, injected tilapia had significant growth enhancement and changes in proximate composition, with decreases in moisture and increases in both protein and lipid content. Injected fish showed high genetic polymorphism, indicating random integration of the shark genes into tilapia muscle DNA. The highest injection dose resulted in deformities in the ovaries and testes of tilapia, suggesting a negative effect on spawning and reproductive abilities. Consequently, the authors pointed to a need for further studies into the effects of injected DNA on following generations.

An additional biotechnological application in the aquaculture industry is the treatment of fish with poly I:C, a potent inducer of type I interferons (IFNs) (Jensen and others 2002). Type I IFNs are known to stimulate expression of myxovirus resistance (Mx) proteins, which are GTPases that inhibit the replication of single-stranded RNA viruses such as infectious salmon anemia virus (ISAV), one of the most economically harmful pathogens in the Atlantic salmon industry. When challenged with ISAV, Atlantic salmon treated with poly I:C experienced increased levels of Mx proteins and reduced mortality as compared to untreated controls (Jensen and others 2002). Since Mx proteins have been successfully cloned from Atlantic salmon, Japanese flounder, rainbow trout, and Atlantic halibut, they could potentially be introduced into transgenic fish. These fish could then be treated with poly I:C in order to induce type I IFNs and Mx protein expression, thereby promoting resistance against pathogens such as ISAV.

**Metabolism of land-based plants.** The use of terrestrial plant-based diets in aquaculture has a number of advantages over more traditional marine-derived diets (Naylor and others 2000). Plant products such as soybean meal and vegetable oils can supply high levels of protein and energy at a lower cost than marine products such as fish meal and fish oil. Also, some argue that use of plants helps to conserve marine ecosystems by reducing the need for the wild-caught small pelagic fish often used to produce fish feed (Naylor and others 2000). However, since plant-based diets differ in composition from the traditional marine diets, it is important to ensure that farmed organisms are able to maintain appropriate levels of nutrients and other beneficial ingredients. For example, terrestrial plants lack some essential amino acids and fatty acids that are found in fish meal and fish oil. To compensate for this, 2 transgenic technologies have recently been applied to fish in hopes of promoting the use of plant-based diets.

The omega-3 polyunsaturated fatty acids (*n*-3 PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to provide a number of benefits to human health, including promotion of visual and neurodevelopment, alleviation of diseases such as arthritis and hypertension, and reduction of cardiovascular problems (Bao and others 1998; Horrocks and Yeo 1999; Grimm and others 2002; Hu and others 2002; Leaf and others 2003). Fish obtain high levels of these fatty acids through the aquatic food chain and are thus a major dietary source of EPA and DHA for humans. However, farmed fish are often fed diets rich in plant oils, which contain the fatty acid  $\alpha$ -linolenic acid (ALA), a precursor to EPA and DHA. The rate-limiting step in the conversion of ALA to EPA and DHA involves the enzyme

*n*-6-desaturase. Recently, a gene construct containing the *n*-6-desaturase-like gene from masu salmon (*Oncorhynchus masuo*) linked to a  $\beta$ -actin gene promoter from Japanese medaka was microinjected into the 1-cell embryos of zebrafish (Alimuddin and others 2005). The expression of the *n*-6-desaturase-like gene in the transgenic zebrafish resulted in a 1.4-fold increase in EPA content, a 2.1-fold increase in DHA content, and a corresponding decrease in ALA content, as compared to the nontransgenic controls. Despite these changes in fatty acid composition in the transgenic fish, total lipid content remained constant. This technology has potential in the aquaculture industry as a means to increase levels of *n*-3 fatty acids in farmed fish, thereby providing consumers with a healthier product. It could also reduce the reliance of the aquaculture industry on ocean-caught feed sources and allow for widespread use of cheaper, plant-based diets rich in ALA. Alternatively, advances in plant transgenics have recently allowed for the production of EPA (up to 15% of total fatty acids) and DHA (up to 3.3% of total fatty acids) in oilseed crops (Robert 2006). Although these transgenic plants produce variable and limited levels of *n*-3 PUFAs, they do show potential for use in aquaculture feed.

In another effort to facilitate the use of plant-based diets in aquaculture, the gene coding for the enzyme phytase was recently expressed in Japanese medaka (Hostetler and others 2003). Phytase breaks down phytate, the major form of phosphorus in plants. Nonruminant animals such as fish are incapable of digesting phytate and therefore excrete it as waste. The excreted phytate is then degraded by microorganisms, creating large amounts of phosphorus, an environmental pollutant. Since fish cannot access the bioavailable form of phosphorus, plant-based diets used in aquaculture must include supplementation with inorganic phosphorus. Attempts to add the phytase enzyme to the feed for reduction of phytate waste have shown limited success due to degradation of the enzyme at the high temperatures required for feed processing. Hostetler and others (2003) reported successful expression of the *Aspergillus niger* phytase gene, driven by either the human CMV promoter or the sockeye salmon histone type III promoter, in Japanese medaka. The transgenic fish were compared to their nontransgenic siblings on 3 different diets all rich in phytate (1—high phytate, low phosphorus; 2—same as 1 but with phytase supplementation; 3—same as 1 but with phosphorus supplementation). Although no significant differences were observed between the weights of transgenic and nontransgenic medaka, there were some differences in survival rates. As predicted, the transgenic fish had equal survival on all 3 diets due to their ability to break down phytate. The control fish, on the other hand, showed the highest survival on the diet supplemented with the enzyme phytase and lower survival on diets 1 and 3, indicating positive effects of the enzyme phytase, either as a feed supplement or as a transgene. Although these results are promising, further research needs to be carried out to examine factors such as phytate excretion levels and additional fitness parameters that are affected by expression of the phytase transgene.

**Sterility and gonad-specific gene excision.** Although use of transgenic fish in aquaculture has the potential to increase food availability and reduce production costs, there is much concern over the possibility of escapement of transgenic fish and contamination of wild populations through interbreeding. Therefore, it is of great interest to develop techniques for preventing introduction of transgenes into wild stocks. Two concepts currently being researched include induction of sterility and gonad-specific transgene excision (Hu and others 2006). Although a method has been developed for production of sterile fish through chromosome manipulation (to be discussed in a later section), sterility is not achieved 100% of the time and the fish often have stunted growth (Wang and others 2003; Dunham 2004). Recently,

sterility was reported using a transgenic method to inhibit expression of the gene coding for the gonadotropin-releasing hormone (GnRH), an important component in gonad development and reproductive function (Hu and others 2006). In a series of pilot studies, reversible fertility was achieved in common carp by inserting a gene coding for an antisense RNA sequence that inhibits expression of the GnRH gene. When the antisense-GnRH messenger RNA (mRNA) was transferred into common carp eggs, around 30% of the offspring did not develop gonads; however, the fish could be made fertile by exogenous administration of hormones.

To achieve gonad-specific transgene excision, 2 types of transgenic zebrafish were created (Hu and others 2006). One line of zebrafish contained the gene coding for Cre recombinase driven by the T7 promoter along with the desired transgene (in this case fluorescent protein) flanked by 2 *loxP* sites. When expressed, the Cre recombinase excises the transgene in between the 2 *loxP* sites. The other line of zebrafish contained the T7 RNA polymerase driven by a protamine promoter specifically expressed in the gonad. When these 2 lines of zebrafish are crossed, the offspring specifically express the T7 RNA polymerase in the gonads, resulting in the expression of the Cre recombinase and excision of the foreign gene. This concept would allow for the creation of transgenic individuals lacking the ability to pass on a foreign gene.

**Transgenic marine invertebrates**

Worldwide a large number of marine invertebrates, including bivalves (clams, oysters, mussels, and cockles), crustaceans (prawns, crabs, and lobster), and gastropods (abalone and Trochus), are cultivated for use in food and food products. In

2002 alone, 13.9 million metric tons of mollusks and crustaceans were produced from aquaculture, accounting for over US\$21 billion (FAO 2004); however, gene-transfer biotechnology in invertebrates remains significantly behind the level it is now in fish (FAO 2000). Although use of selective breeding and chromosome manipulation has advanced the field of marine invertebrate aquaculture, transgenic technology is hindered by several biological factors such as growth rates and breeding properties (FAO 2000; Langdon and others 2003). In this section, current advances and challenges in transgenics will be discussed from the perspective of improving production of specific marine invertebrates in aquaculture (Table 2).

**Oysters.** A high worldwide demand for oysters has given way to major developments and growth in oyster farming technologies over the centuries. The Pacific oyster (*Crassostrea gigas*) alone represented the leading global aquaculture production for a single species in 2002, at 4.2 million metric tons (FAO 2004). Originally from Japan, the Pacific oyster has been successfully introduced as a commercial species along shorelines of Australia, New Zealand, Europe, and the West Coast of North America (since about the 1920s) (Shatkin and others 1997).

A major setback to oyster farmers has been disease caused by protozoan pathogens and the risk of transfer of pathogens such as *Vibrio vulnificus* and *V. parahaemolyticus* from oysters to humans (Buchanan and others 2001). Two possible ways to combat the spread of disease among marine invertebrates are to (1) selectively raise organisms that are more resistant to disease or (2) introduce disease-resistant transgenes into organisms (Lu and others 1996). Transfection of oysters with foreign DNA has proven to be difficult for a number of reasons, including the small size of bivalve gametes and embryos and problems with efficient selection of

**Table 2 – Genetically modified marine invertebrates being tested for use in aquaculture**

Species	Foreign gene	Desired effect/results	Status	Country	Reference
Abalone (species name not given)	Coho salmon GH + various promoters	Increased growth	Research phase	U.S.	(FAO 2000) <sup>a</sup>
Clams, dwarf surf ( <i>Mulina lateralis</i> )	Retroviral insertion	Successful gene transfer method for use as a model (“guinea pig”) species	Retroviral insertion method is patented	U.S.	(Lu and others 1996; Burns and Chen 1999; Kapuscinski 2005)
Crayfish ( <i>Procambarus clarkii</i> )	Replication-defective pantropic retroviral vector	Successful transformation of immature gonads; germline transmission	Research phase	U.S.	(Sarmasik and others 2001)
Oysters (species name not given)	Coho salmon GH + various promoters	Increased growth	Research phase	U.S.	(FAO 2000)
Oyster, eastern ( <i>Crassostrea virginica</i> )	Aminoglycoside phosphotransferase II ( <i>neo</i> <sup>I</sup> )	Increased survival rates when exposed to antibiotics	Research phase	U.S.	(Buchanan and others 2001)
Shrimp	IHHNV promoters	Functional for use in gene transfer; potential use in expression vectors	Research phase	U.S.	(Dhar and others 2005; 2006)
Shrimp ( <i>Litopenaeus vannamei</i> )	Antisense TSV-CP + shrimp ( <i>Penaeus vannamei</i> ) $\beta$ -actin promoter	Stable expression; no biological abnormalities; increased survival (83% vs. 44% in controls) when challenged with TSV	Research phase	U.S.	(Lu and Sun 2005)
Shrimp, black tiger ( <i>Penaeus monodon</i> )	Kuruma prawn EF-1 $\alpha$ promoter + GFP gene or CAT gene	Ubiquitous expression of GFP/CAT	Research phase	Japan, Thailand	(Yazawa and others 2005b)

<sup>a</sup>Adapted from SOFIA (FAO 2000) and Ramirez and Morrissey (2003).

Note: GH = growth hormone gene; IHHNV = infectious hypodermal and haematopoietic virus; TSV-CP = Taura syndrome virus-coat protein; GFP = green fluorescence protein; CAT = chloramphenicol acetyl transferase.

transformed larvae (Buchanan and others 2001). However, techniques have been developed for gene transfer by microinjection of fertilized Pacific oyster eggs and particle bombardment of Pacific oyster eggs, zygotes, and trochophores (Cadoret and others 1997a, 1997b). The first successful introduction and expression of foreign DNA in eastern oyster (*Crassostrea virginica*) larvae was recently reported, with the goal of developing gene transfer techniques to counter disease-related problems in the oyster farming industry (Buchanan and others 2001). The authors used both electroporation and chemically mediated transfection to introduce the gene coding for aminoglycoside phosphotransferase II (*neo<sup>r</sup>*), which promotes resistance to neomycin and related antibiotics, into eastern oyster larvae. Transfected larvae exposed to the antibiotic G418 demonstrated significantly increased survival rates as compared to nontransfected larvae; however, the authors mentioned the need for further modification of techniques for selection of transfected larvae (Buchanan and others 2001).

**Abalone.** While commercial landings of wild-caught abalone in the United States are in decline, abalone aquaculture in California and around the world has been expanding (Powers and others 1995). In 2003, worldwide production of farmed abalones, winkles, and conches was 29000 metric tons (FAO 2006). Despite a growing interest in cultivation of abalone, their slow, highly variable growth rate and relatively long generation time (taking about 4 y to reach market size and sexual maturity) have frustrated industry advancements and have been problematic for genetic studies (Powers and others 1995; Counihan and others 1998). However, over the past decade, there have been some promising transgenic advancements, in particular with abalone gene identification and the development of gene transfer techniques specific for abalone (Elliot 2000). For example, in the first transfer of exogenous DNA into shellfish embryos, transgenes were successfully introduced by electroporation into the embryos of red abalone (*Haliotis rufescens*) (Powers and others 1995). Successful gene transfer has also been achieved by electroporation of the sperm of Japanese abalone (*Haliotis divorsicolor supertexta*) (Tsai and others 1997). These initial advancements may help give way to the development of transgenic strains of abalone with increased growth rates, shorter generation times, and/or improved disease resistance, resulting in a more profitable worldwide abalone aquaculture industry.

**Dwarf surf clams (*Mulina lateralis*).** Dwarf surf clams have served as an important “guinea pig” species in the development of techniques for introduction and expression of various transgenes in marine invertebrates. Dwarf surf clams are advantageous for use in gene transfer research because they have a quick generation time (2 to 3 mo), a lifespan of about 2 y, and high fecundity, with about 0.5 to 2 million eggs per spawning cycle (Lu and others 1996). Research with dwarf surf clams led to the successful development and patenting of a method for gene transfer in mollusks by electroporation of fertilized eggs followed by pantropic retroviral integration (Lu and others 1996; Burns and Chen 1999). The authors reported successful protein expression of the integrated transgene in dwarf surf clams and suggested that the techniques developed in this study could be applied to genetic experimentation with a number of marine invertebrates such as oysters, abalone, mussels, and shrimp. Indeed, use of the pantropic retroviral method led to the first successful reports of foreign gene expression in the primary cultured cells of a penaeid shrimp (Shike and others 2000) and in the cultured dissociated embryonic cells of the Pacific oyster (Boulo and others 2000). Also, Sarmasik and others (2001) reported successful expression of reporter genes using this gene transfer method in the immature ovary or testis of both live-bearing fish (*Poeciliopsis lucida*) and crayfish (*Procambarus clarkii*).

**Shrimp/prawns.** Global production of shrimp and prawns has shown steady growth over the past several years, with a total of 1.8 million metric tons in 2003 (FAO 2006). However, cultivation of shrimp is limited by the reliance of the industry on wild broodstock, and advances in domestication and selective breeding have been slow, in part due to incomplete genetic and biochemical information on shrimp reproductive systems (Preston and others 2000). Although there has been interest in the development of transgenic penaeid prawns with increased growth rates and disease resistance, no system of genetic transformation has been established. As an initial step in this development, a number of methods for gene transfer were investigated, including electroporation, microinjection, spermatophore microinjection, and particle bombardment (Li and Tsai 2000; Preston and others 2000; Tseng and others 2000). According to the results reported by Preston and others (2000), microinjection was found to be a more effective technique for introducing DNA into Kuruma prawn (*Penaeus japonicus*) embryos than electroporation and particle bombardment. However, all studies showed poor efficiency, with low survival rates of transfected embryos, indicating a need for further development of gene transfer methods in shrimp. Another necessity for successful gene expression in prawns/shrimp involves identification of appropriate promoter sequences. To this effect, promoters from infectious hypodermal and haematopoietic virus (IHHNV) were recently reported to be functional for use in shrimp transgenics, and efforts are currently under way to develop expression vectors using these promoters that allow for successful transgenic protein production in shrimp (Dhar and others 2005, 2006). In addition to viral promoters, ubiquitous promoters of housekeeping genes found in the target species also have strong potential for use in transgenics. In a recent study, the promoter of the EF-1 $\alpha$  housekeeping gene in Kuruma prawns was linked to 1 of 2 indicator genes (green fluorescence protein [GFP] gene or chloramphenicol acetyl transferase [CAT] gene) (Yazawa and others 2005b). Constructs containing the genes for GFP and EF-1 $\alpha$  were then introduced into black tiger shrimp (*Penaeus monodon*) embryos by microinjection or particle gun bombardment, and GFP expression was measured. Although a higher percentage of the microinjected embryos was found to be GFP positive, these embryos were damaged and failed to progress through embryogenesis. A lower percentage of GFP positive embryos was recovered with the particle bombardment method; however, these embryos successfully progressed through embryogenesis and showed ubiquitous expression of GFP. Therefore, the particle bombardment method was reported to be more suitable for use in shrimp, and the optimized conditions for this method were used to carry out introduction of the EF-1 $\alpha$ /CAT constructs, which showed CAT activity from 1 to 7 d following fertilization.

A major threat to aquaculture of commercial shrimp has been disease outbreaks, specifically in the case of highly pathogenic viruses such as Taura syndrome virus (TSV) (Lu and Sun 2005). These viruses can cause considerable setbacks to shrimp farmers, and there is currently a lack of effective methods for their control. In a recent study, the gene coding for an antisense TSV-coat protein (TSV-CP) driven by a shrimp  $\beta$ -actin promoter was successfully expressed in shrimp (*Litopenaeus vannamei*) zygotes using a jetPEI-based transfection method (Lu and Sun 2005). Transgenic shrimp did not show any biological abnormalities in the parameters tested, with similar weight gain, appearance, morphology, swimming, and feeding as compared to nontransgenic controls. However, when challenged with infectious TSV, the transgenic shrimp showed a survival rate of 83%, which was significantly higher than the survival rate of the controls (43%). The authors suggested that the protective effect of the antisense TSV-CP was due to its ability to target and degrade specific sequences of

TSV RNA. Transgenic methods such as this one for combating pathogens could substantially improve worldwide cultivation of commercial shrimp by reducing losses due to disease outbreaks.

Another approach toward maximizing profits from cultivation of prawns has been in the area of sexual differentiation, with a specific study on the freshwater prawn *Macrobrachium rosenbergii* (De Man) (Sagi and Aflalo 2005). Males of this species of prawn have been found to grow at faster rates and therefore achieve a larger size at harvest than their female counterparts. In addition, cultivation of sterile, all-male populations could be advantageous because energy that normally would be used in reproductive efforts would be diverted toward somatic growth, and concerns over escapement would be reduced, as the cultured prawns would not be able to breed with wild populations. Therefore, cultivation of sterile, all-male populations could prove to be economically and environmentally profitable. However, the cost of manual sexing often outweighs the benefits of all-male populations (Sagi and Aflalo 2005). Use of biotechnological tools could provide a more efficient means of producing monosex populations by utilizing knowledge of certain reproductive properties. Sexual differentiation in crustaceans is determined by hormones that are released from the androgenic gland and promote male differentiation while inhibiting female differentiation. Although the androgenic hormone in crustaceans has yet to be purified, Sagi and Aflalo (2005) have suggested the possibility of altering the sex of prawns by manipulation of the androgenic gland. Three proposed lines of future aquacultural and biotechnological research and development are (1) cultivation of monosex populations by manual segregation along with selective harvesting (separation of larger males from smaller ones resulting in increased growth rates among the small males) and claw ablation, (2) microsurgical manipulation of the androgenic gland to develop neofemales that could be mated with normal males to produce all-male progeny, and (3) determination of androgenic gland bioactive products that could enable biochemical or molecular alteration of sex differentiation (Sagi and Aflalo 2005).

### **Environmental and human health concerns**

Although transgenic applications in aquaculture have the potential to greatly advance the industry and help supply a growing global food demand, there is concern over the potentially negative effects of introducing genetically altered fish into the food chain. Prior to introduction of transgenic fish into commercial aquaculture, appropriate risk analyses must be carried out in order to evaluate possible detrimental effects on both the environment and human health (Kapuscinski 2005).

**Environmental concerns.** A major cause of concern regarding aquaculture is the escapement of farmed organisms into the wild and subsequent interaction with native populations, possibly leading to significant alterations in the properties of the natural ecosystem (Maclean and Laight 2000; Ramirez and Morrissey 2003). Even when fish are in a contained aquaculture environment, there is a high probability that some will escape. For example, in Norway, escaped farmed salmon account for around 30% of the salmon in rivers (FAO 2000). These escapees can be particularly harmful to wild populations, especially when farming occurs in the native habitat of the escaped fish, when there is a proportionally high number of farmed fish compared to the wild stock, or when the wild population is exposed to pathogens occurring in farmed fish (Naylor and others 2005). Also, it has been shown that escaped farmed salmon can undergo freshwater spawning, although not as successfully as wild stocks (Fleming and others 2000). When farmed salmon breed with wild populations, not only is the natural genetic diversity altered but also the resulting offspring show reduced fitness compared to their par-

ents and wild cousins (McGinnity and others 2003; Roberge and others 2006).

Introduction of transgenic fish into aquaculture and their possible escapement poses a number of substantial risks to consider. Escaped transgenic fish could disrupt the natural biodiversity of an environment by breeding with wild species and altering the gene pool, or by increased predation or competition, resulting in improper balances of native species and possibly leading to extinction (FAO 2000; Naylor and others 2005). When transgenic fish breed with wild populations, the resulting fish may acquire transgenes that could alter natural behavior in areas such as reproduction, antipredator response, and feeding (Galli 2002). Most marine organisms have high mobility and fecundity, and, therefore, newly introduced genetic material has the potential not only to affect local populations of fish but also to spread to neighboring fish populations. On a large scale, these events could permanently alter the dynamics of fish populations and seriously damage the survivability of a species.

Proponents of transgenic aquaculture argue that GMOs will have decreased fitness in the wild and will not be able to successfully compete with native fish populations (FAO 2000). However, the effects of particular transgenes on behavior of fish in the wild are difficult to predict, in part due to the possibility of pleiotropic gene expression patterns, in which changes in 1 trait affect the expression of genes related to other traits (Galli 2002). Fish containing artificial genes such as those coding for AFPs, increased growth, or increased disease resistance have the potential to outcompete native populations. Studies involving comparisons of GH-transgenic and nontransgenic coho salmon have shown contradictory results, with reports of GH-transgenic salmon showing increased competitive feeding abilities (Devlin and others 1999), greater mortality in the fry (Sundstrom and others 2004), and equal competitive feeding abilities without increased mortality rates (Tymchuk and others 2005). GH-enhanced tilapia were reported to have lower feeding motivation and dominance status compared to wild tilapia, but higher competitive feeding ability than their nontransgenic siblings (Guillen and others 1999).

The creation of sterile GMOs by chromosome or gene manipulation might reduce some of the apprehension over transgenics breeding with natural populations (FAO 2000). However, the methods that are currently available for creating sterile organisms are not 100% effective, and the possibility exists that some of these GMOs will be able to reproduce (Wang and others 2003; Dunham 2004). In addition, escaped transgenic fish, although sterile, may still be able to outcompete native fish populations for habitat resources or interfere with the natural behavior of the population (Galli 2002). Besides the possibility of increased survivability, disease resistant transgenic fish also have the potential of carrying certain bacteria, parasites, or viruses that may be harmful to natural populations. Although advocates of transgenesis argue that GMOs are not too different from species that have been genetically altered by breeding techniques, the general population and many environmental groups remain wary of the concept of artificial gene insertion (FAO 2000).

**Human health concerns.** As with any emerging food technology, use of transgenic fish is accompanied by a number of human health concerns. The effects of long-term consumption of GM foods are unknown. Also insertion of foreign genes into species might result in production of toxins or allergens that were not present previously (Galli 2002; Kelly 2005). Another potential area of concern is that increased disease resistance of transgenic fish might make them better hosts for new pathogens, which could then be passed on to humans through consumption (FAO 2000). Worldwide, the health concerns of GM foods have been met with considerable controversy. Although countries such as the United States and Canada have been relatively quick to adopt a number

of GM foods, progress in Europe has been slow due to widespread public resistance and the enactment of a number of anti-GMO regulations based on the "precautionary principle" favored there (Lapan 2004).

Allergens or toxins may be produced as a result of gene transfer if the transgene codes for an allergenic protein or a protein that induces expression of a previously inactive toxin (Galli 2002; Kelly 2005). For example, if the gene coding for a shellfish protein were used to create transgenic fish, consumers that are allergic to shellfish could be at risk for an anaphylactic reaction. Also, the pleiotropic effect mentioned earlier could result in a series of events leading to unintended results such as the production of a toxin or allergen that is not normally expressed. However, the expression of previously inactive toxin genes as a result of transgene introduction in food animals has been deemed to be highly unlikely, as animals used for food are generally known to be safe for human consumption and rarely produce toxic compounds (Berkowitz and Kryspin-Sorensen 1994; NAS 2002; Kelly 2005). In addition to production of allergens and toxins, there is also some concern over the expression of bioactive proteins such as GH and cecropins, which may continue to possess bioactive properties following consumption. For example, the antimicrobial properties of cecropins have the potential to alter the normal intestinal flora in humans and/or selectively promote the development of human pathogens with increased resistance (NAS 2002; Kelly 2005).

However, according to the Food and Agriculture Organization of the United Nations (FAO), the risks associated with the current use of biotechnology in aquaculture are "clearly circumscribed and minor" (FAO 2000). Unlike plant transgenics, which involve the introduction of genes that code for general antibiotics, possibly allergenic compounds, and resistance to common pests and weeds, many of the GMOs being tested for use in fish aquaculture are gene constructs containing fish-derived GHs (FAO 2000). Initial tests on the safety of consuming all-fish GH-transgenic common carp showed no apparent negative health impacts on mice (Zhang and others 2000; Wu and others 2003). The mice were fed transgenic carp at doses of 5 or 10 g/kg body weight twice daily over a 6-wk period and were compared to a control group fed nontransgenic carp in terms of a variety of tests, including growth performance, histochemical assays of 12 organs, and biochemical analysis of blood (Zhang and others 2000). Although this study reported no adverse effects of transgenic fish consumption, many more studies are warranted examining the effects of a variety of factors, including long-term and high-level consumption, before significant conclusions can be formed concerning this topic.

Food safety trials with tilapia GH and transgenic GH tilapia meat have been conducted in Cuba using both nonhuman primates and healthy human volunteers (Guillen and others 1999). Six nonhuman primates were intravenously administered recombinant tilapia GH daily for 30 d. Blood samples were examined before and after the treatment period for clinical and biochemical parameters, including hemoglobin, total serum proteins, glucose, creatinine, leukocytes, and erythrocytes. The animals were also evaluated daily for clinical indicators such as weight, heart rate, and rectal temperature. Following the experiment, the animals were killed and detailed autopsies were carried out. The results showed no biological effects of tilapia GH on primate biology, with no significant effects on blood sample profiles, somatic growth, or morphological characteristics of the organs and tissue (Guillen and others 1999).

To evaluate the safety of consuming transgenic tilapia meat, 22 human volunteers were randomly divided into 2 groups that received either transgenic or nontransgenic tilapia twice daily for 5 d (Guillen and others 1999). Blood samples were taken daily and evaluated for similar clinical and biochemical parameters

as in the nonhuman primate study. Following the experiment, the individuals were asked to evaluate the tilapia meat on the basis of flavor and quality. Transgenic tilapia meat was well accepted by consumers, with no reports of adverse effects and no changes in the clinical and biochemical parameters evaluated, as compared to individuals in the control group (Guillen and others 1999). To the authors' knowledge, this study represents the 1st evaluation of human consumption of transgenic fish. Although these studies have reported no adverse effects, the safety and acceptance of transgenic fish is currently in the hands of government-run food regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the Canadian Food Inspection Agency (CFIA), which are evaluating transgenic organisms according to the established food safety testing protocols (Fletcher and others 2004).

Even if transgenic fish become approved by federal regulators, consumers will be the ultimate determinants of the success of these products (Aerni 2004). Therefore, in addition to the evaluation of environmental and human health effects of GMOs, surveys into consumer acceptance of transgenic products are also important. Interestingly, in a study into the opinions of 1365 Canadian consumers regarding sales of transgenic animal products, the majority showed a favorable attitude toward the concept of GM salmon, while a lesser percentage revealed positive attitudes toward intent to purchase transgenic salmon products (Castle and others 2005). Consumer attitudes were found to be most favorable when the greatest amount of information was provided regarding the transgenic products, indicating the importance of keeping consumers informed.

### Polyploidy

A promising biotechnological tool for increased production of food from aquaculture and creation of sterile organisms is polyploidy. Polyploidy refers to a genetic state that can be produced artificially in fish and shellfish through manipulation of embryos. Polyploid individuals have extra sets of chromosomes beyond the normal 2, with triploids having 3 and tetraploids having 4. Although it is a lethal state for mammals and birds, polyploidy has shown some promising results in the field of aquaculture (Dunham 2004). Triploid fish and shellfish are viable and tend to be sterile due to a lack of gonadal development. This sterility allows for reproductive energy to be diverted toward somatic growth, resulting in higher growth rates for some triploid individuals. Although triploidy has been highly effective for enhancing growth in shellfish, results thus far in fish have shown variable, conflicting results, with reports of triploid fish growing slower, at the same rate, or faster than diploids (Dunham 2004).

The techniques for creating triploid bivalves were first developed in the early 1980s, with research beginning at the Univ. of Maine and then continuing at the Univ. of Washington. Stanley and others (1981) succeeded in creating the first triploids using the American (Eastern) oyster and, since then, shellfish polyploidy has been a research priority (Wang and others 2003). Polyploidy is highly regarded in China and has been studied in close to 30 shellfish species, including Pacific oysters, Chinese scallop (*Chlamys farreri*), pearl oyster, and abalone (*Haliotis discus hannai*) (Beaumont and Fairbrother 1991). The benefits of triploids vary with species, from larger adductor muscles in scallops to increased survival in the Chinese pearl oyster (*Pinctada martensi*) (Allen 1998). Use of triploids in aquaculture can be advantageous when reproductive efforts negatively affect growth, survival, or product quality. For example, during the spawning season, the majority of an oyster's energy is directed toward reproduction, and flesh palatability is reduced as gonad replaces stored glycogen (Shatkin and others 1997). Thanks to extensive research efforts in the field of polyploidy, triploid oyster breeding has developed

into an industry in coastal China, with triploid oysters exhibiting increased flesh quality and growth compared to diploids: triploid Pacific oysters are 17% larger than diploids prior to spawning and more than 30% larger after spawning (Wang and others 2003; Dunham 2004).

Besides advantages such as increased growth rates, use of sterile triploids in aquaculture can help protect the genetic diversity of native populations and prevent establishment of populations of escaped organisms. This could be particularly relevant in addressing concerns over use of transgenic organisms in the aquaculture industry and their possible escapement and subsequent mating with wild broodstocks. To prevent unauthorized breeding of farmed shrimp, research into shrimp polyploidy is currently under way, with successful production of all-female, sterile triploids with comparable growth to diploids (Sellars and others 2006). Aquaculture of sterile organisms can also be beneficial with species such as tilapia that tend to undergo excess reproduction in production ponds (Dunham 2004).

Although there is great potential for use of triploidy in aquaculture of fish and shellfish, several downsides remain. For one, triploidy induction is not a failsafe way of creating sterile broodstocks, as some triploids are capable of producing a few mature eggs and sperm, and some triploid oysters have been reported to revert a portion of their cells to the diploid state (Wang and others 2003; Dunham 2004). In addition, induction of polyploidy can cause reduced performance for some traits and negatively affect survival rates; generally speaking, triploid shellfish have increased mortality rates compared to their diploid counterparts (Dunham 2004). Although most triploids are sterile, they still exhibit sexual behavior, participating in mating rituals and thereby disrupting natural spawning processes within populations (Dunham 2004). And, finally, induction of polyploidy on a mass production scale may not be economically advantageous to those in the aquaculture industry.

A potential alternative to the oftentimes problematic and expensive production of triploids by mechanical induction is the crossing of tetraploids with diploids. Tetraploids are induced in a similar way as triploids, but during a more advanced stage of embryonic development. A tetraploid/diploid cross would be expected to produce all-triploid progeny that might be more viable than mechanically induced triploids, as triploid embryos would not have to undergo the same stress and damage that occurs during mechanical induction. Although tetraploid/diploid crossing remains a possible method for production of triploids, the optimal conditions to maximize tetraploid survival and growth rates need to be determined, as thus far tetraploid fish and shellfish have only been subviable and tend to grow more slowly than diploids (Dunham 2004). In 1 promising study, an increased tetraploid survival rate was achieved when triploid eggs were crossed with diploid sperm, followed by inhibition of the 1st polar body (Allen and Guo 1998). The enhanced viability of the tetraploid progeny was presumed to be due to the greater size of triploid eggs as compared with diploid eggs (Allen and Guo 1998).

## Conclusions

Although world capture fisheries have experienced slow or stagnant growth over the past several years, cultivation of aquatic species has been continually increasing. Since global food demand is predicted to increase in the years to come, food production from aquaculture may help compensate for the limited resources provided by capture fisheries. Recent work with gene transfer research has resulted in the production of aquatic species with enhanced abilities in areas such as growth, cold tolerance, disease resistance, and metabolism of plant-based diets. Research with transgenic GHs has made the most progress, with

the patented production of a line of Atlantic salmon capable of increased growth and feed conversion efficiency. This product has been licensed to a major biotechnology company and is currently awaiting regulatory approval for commercial use in the United States and in Canada. Although transgenic research with invertebrates is far behind that for vertebrates, there is much potential for genetic improvements among commercial bivalve species. Recent advances include development of successful, patented gene transfer methods and research into boosting disease resistance. Despite the potential for GMOs in aquaculture, a number of environmental and human health concerns remain. Major concerns include escapement of transgenic fish into the wild, where they could disrupt natural gene pools through breeding with wild species, and the possible detrimental effects of introducing transgenics into the human and aquatic food chains. One way to alleviate fears of escapement and breeding with natural populations has been the creation of sterile organisms. Recent work using the gene excision method has shown a potential way to produce sterile transgenic organisms by crossing 2 specific lines of transgenic parents. A more traditional method for inducing sterility has been the creation of triploid organisms through chromosome manipulation. Although fish have not responded very well to this technology, it has been very advantageous in the shellfish industry. Because of their sterility, triploid shellfish also have increased growth and flesh palatability. Given the recent advances in gene and chromosome manipulation, the production of sterile, transgenic organisms in aquaculture may become a reality in the near future. However, the success of these products will ultimately be determined by consumers and their perspectives on the advantages (such as price and availability) of aquatic GMOs versus environmental and human health concerns.

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