Genetically Engineered Fish: Potential Impacts on Aquaculture, Biodiversity, and the Environment

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Abstract Studies on transgenic fish for the aquaculture industry have focused on improving growth rates, enhancing disease resistance, altering body composition, acting as biological factories for medical proteins, and even altering temperature tolerance and coloration. The future impact of transgenesis will likely be quite large. Growth hormone-transgenic salmon has been approved for human consumption and has been introduced to the market in Canada and soon to the USA. This is the first human consumption of approved transgenic meat. Transgene insertion has many pleiotropic effects. Several studies have projected the fitness of transgenic fish to be low, in general, compared to non-transgenic and wild fish; thus, their environmental risk is likely low and they would have minimal, if any, long-term impact on ecosystems or biodiversity. However, there have been no actual escapements; thus, only projections of risk are available based on small-scale experiments and the characteristics of transgenic fish compared to controls. An active area of research is repressible transgenic sterilization and sterilization using gene editing, both of which would allow application of transgenic fish with only short-term consequences for ecosystems in the worst-case scenario. Transgenic technology could also be potentially used to reduce or eliminate populations of nuisance species.

Keywords Genetic engineering · Biodiversity · Aquaculture · Environmental risk · Transgenic · Fitness · Transgenic sterilization · Genetic enhancement

Aquaculture and Selective Breeding

Most agricultural crops are genetically modified (traditional approaches) products that have been bred for hundreds or even thousands of years. Natural selection and conventional breeding drove the phenotypic and genetic changes in food organisms,

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such as corn, watermelon, and peach as well as animals, including fish. For example, corn was revolutionized from small cobs (19 mm in length), hard and a meager kernel (5–10 in number), into a larger (1000-fold larger) and tastier modern version (rich in carbohydrates, oil, and proteins), easy to peel and cook through domestication and breeding activities. The compositions changed with 2% less juice and 2.5-fold more sugars (Beadle 1980; VOX 2014) in modern corn. Although these changes utilizing selective breeding were dramatic, the genetic enhancement and phenotypic change is usually slow. In some cases, phenotypic variance or lack of additive genetic variation prevents genetic enhancement from selective breeding.

Selective breeding in aquatic organisms has yielded similar results. Selection has been used to double growth rates and increase disease resistance as well as improve other production traits (Dunham 2011) over multiple generations.

Transgenic Technology

With the development of molecular biology theory and technology, transgenic technology emerged (Palmiter et al. 1982), providing a new method for breeding and genetic enhancement. By 1985, fish scientists were adapting transgenic technology with the goal of improving productivity of aquaculture. After a large initial thrust in this area, research slowed as it became clear public concern and regulation would make application difficult and slow. In addition, genomics emerged as an alternative area to practice molecular skills as well as generate resources for future genetic enhancement. A tremendous amount of research in aquatic genomics has been conducted during the last 25 years (Abdelrahman et al. 2017), although very little application in selective breeding has yet to occur. However, the Japanese flounder (*Paralichthys olivaceus*) industry has been transformed by marker-assisted selection (Fuji et al. 2007), while research on quantitative trait loci and genome-wide associated study has greatly expanded (Abdelrahman et al. 2017; Liu et al. 2018; Wang et al. 2019).

Growth

The greatest amount of transgenic fish work, especially early work, focused on transfer of growth hormone (GH) genes. This was especially true in the 1980s and 1990s, but a greater variety of transgenes are explored today. Maclean and Talwar (1984) working at the University of Southampton, UK, were the first to inject cloned genes into fish eggs (rainbow trout, *Oncorhynchus mykiss*). However, Zhu et al. (1985) and his team at the Institute of Hydrobiology in China were the first to report production of a transgenic fish, goldfish (*Carassius auratus*), using the hGH gene and resulting in a 4.6× increase in growth, although they did not report any integration data.

Growth (size and rate) improvement has ranged from 0% to, in some cases, an amazing 3000%. Several species, including loach (*Misgurnus mizolepis*), common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), Atlantic salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*), Nile tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), and northern pike (*Esox lucius*), containing either human, bovine, or salmonid GH genes, grew 10–80% faster than non-transgenic fish. Du et al. (1992) used an "all-fish" GH gene construct to make transgenic Atlantic salmon that grew 2–6× faster than non-transgenic controls.

Zhu followed up the goldfish work by making transgenic GH Yellow River common carp. An "all-fish" growth hormone (GH) chimeric gene construct, pCAgGH, using a promoter β-actin gene from Yellow River common carp linked to the growth hormone gene from the grass carp (*Ctenopharyngodon idellus*) was developed and transferred to fertilized embryos of Yellow River common carp to produce the "all-fish" growth-transgenic Yellow River carp (Zhu 1992). The growth rate of transgenic carp was 42–114.92% faster than the control. The control Yellow River carp needed 2 years to reach market size, while the transgenic carp needed only 1 year. Since the growth hormone gene had an inhibitory function on reproductive development, the weight of the gonads was reduced, and the edible portion of the fish was correspondingly increased.

Mori and Devlin (1999) examined the expression of the sockeye salmon (*Oncorhynchus nerka*) metallothionein-B (MTB)-sockeye GH1 gene in transgenic coho salmon (*Oncorhynchus kisutch*), resulting in 40× elevated circulating GH levels, and, in some cases, inducing 5–11-fold increases in weight after 1 year of growth. GH expression was greater in younger, smaller, transgenic coho salmon (20–21 g) compared with older, larger, transgenic salmon (400–500 g).

Response to transgene insertion can vary based on transgene, promoter, position effect, copy number, epigenetics, species, family, and genetic background. Heterozygous F_1 and F_2 lines of transgenic Nile tilapia possessing one copy of an eel (ocean pout) promoter-chinook salmon GH fusion grew 2.5–4-fold faster and converted feed 20% better than control siblings (Rahman et al. 1998, 2001; Rahman and Maclean 1999). However, F_1 fish transgenic for the sockeye salmon MT promoter-sockeye salmon GH gene exhibited no growth enhancement (Rahman et al. 1998), although salmon transgenic for this construct had greatly accelerated growth.

Similarly, sockeye salmon-sockeye GH cDNA1 introduced into coho salmon increased growth from 11-fold to 37-fold (Devlin et al. 2001). Results with Atlantic salmon are not as dramatic as with coho salmon. Transgenic Atlantic salmon containing the opAFP-chinook salmon GH cDNA1 gene construct had a three- to six-fold increased growth rate compared to non-transgenic salmon (Du et al. 1992; Cook et al. 2000), and insertion of sockeye MTB-sockeye GH cDNA1 (Devlin 1997) produced a similar result, fivefold growth enhancement.

Dramatic Growth of Transgenic Fish: Explanations and Limitations

As indicated above, growth enhancement varies greatly among different transgenic fish systems when GH transgenes are integrated (Devlin 1997; Dunham and Devlin 1998). Family effects, position effects, and others have been presented as potential explanations of this variable response. Family effect was observed for growth hormone-transgenic coho salmon produced from a wild strain. Promoters of sockeye salmon metallothionein-B or histone 3 were fused to a growth hormone-1 coding region from the same species (OnMTGH1 and OnH3GH1 constructs, respectively) and were used for evaluation of the growth rate. Salmon transgenic for the OnMTGH1 construct had consistently higher weight than those containing the OnH3GH1 construct, and both transgenic groups had greatly enhanced growth over non-transgenic fish. However, strong family effects were observed as some OnH3GH1 families had similar weight to OnMTGH1 families while others did not (Leggatt et al. 2012).

Domestication effects, innate growth rate, and life-history traits have been put forward as potential factors responsible for the tremendous range of GH transgene responses, with one of the first hypotheses revolving around domestication. Domestication in vertebrates may use the same genetic and physiological pathways in GH endocrine axis to regulate growth rate. Microarray analysis confirmed that transgene insertion and domestication affect the gene expression in concordant ways and implied that the two genetic processes modified the same regulatory pathway for growth (Devlin et al. 2009, 2013). Strains or species that have been selected to near maximum growth rates may have many of their metabolic and physiological processes optimized, and further growth enhancement might be more difficult to obtain by insertion of GH or other growth-related genes.

Insertion of salmon metallothionein growth hormone (OnMTGH1) transgenes increased growth 17× in slow-growing, wild rainbow trout strains (with naturally low growth rates), while the transgene did not stimulate growth (4.4% increase) in fast-growing, non-transgenic, domestic rainbow trout (Devlin et al. 2001). However, these P_1 domestic rainbow trout were mosaic, and very few families were evaluated.

However, additional data on transgenic rainbow trout (Devlin et al. 2001) are not consistent with the hypothesis that wild fish when made GH-transgenic immediately reach a growth plateau already existing for selected domestic lines. When OnMTGH1 was transferred to another wild rainbow trout strain, F77, growth was enhanced sevenfold, which exceeded by fourfold the growth exhibited by a non-transgenic domestic rainbow trout. In this case, the wild transgenic rainbow trout is actually superior to the domestic selected strain, indicating that genetic engineering can have a greater effect than, rather than an equivalent effect to, domestication and selection. When F77 was crossbred with the domestic strain, growth of the crossbreed was intermediate to the parent strains (Devlin et al. 2001). However, the transgenic wild X domestic crossbreed was 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 parent (Devlin et al. 2001). The combined effects of transgenesis and crossbreeding had a much greater growth enhancement than crossbreeding or transgenesis alone (Dunham 2011). A transgenic rainbow with 50% of its heritage from a domestic genome was much larger than a transgenic with a wild genome. Strain effects, in general, epistasis, and genetic background may be more significant in regard to affecting transgene response, rather than the domestic or wild nature of the fish (Dunham 2011).

An alternative explanation for the hyper growth response of GH-transgenic salmonids is that growth of non-transgenic salmonids is normally relatively slow prior to sexual maturity, and is extremely low when water temperatures are low and food resources in nature are scarce (Dunham 2011; Leggatt et al. 2017b), and transgenic individuals are less affected by these factors. The other amazingly dramatic example of growth enhancement is the 30× size attained by GH-transgenic mud loach, *Misgurnus mizolepis*, above that of the non-transgenic biological maximum (Nam et al. 2001). A pattern appears to be emerging that GH transgenesis has the most profound effects on slow-growing species. However, the very small model species, medaka and zebrafish, only grew 75% (Howard et al. 2004) and three times (Silva et al. 2015) faster than controls, respectively, when GH constructs were introduced; thus, the hypothesis that GH transgenesis is more effective in slow-growing species, strains, and lines is not universal.

Salmon may represent a unique case with their life-history and physiology making them especially amenable for growth enhancement via GH transgenesis. Genetic advantages could lead to further magnification of differences due to environmental advantages (Moav and Wohlfarth 1974). Even prior to first feeding, transgenic progeny were 21.2% heavier and 11.9% longer than their non-transgenic full-siblings, suggesting that the expression of GH in early development affected the rate and/or efficiency of conversion of yolk energy reserves (Devlin et al. 1995a, b). GH expression increased by 40-fold in cold temperatures, when GH expression is normally low (Mori and Devlin 1999). Parr-smolt transformation occurred 6 months early in the transgenic fish compared to the control fish. This becomes another advantage that can be further magnified genetically and environmentally as smolts are naturally in a faster-growing life stage.

Other Growth Genes

Overexpressing a growth hormone gene is not the only strategy to increase growth through transgenesis in fish. Jiang et al. (2017) introduced the grass carp follistatin gene into blunt nose bream (*Megalobrama amblycephala*). F_2 fish exhibited double muscling, increased size, body depth, and body width. The follistatin expression resulted in hypertrophic muscle growth.

Disease Resistance

Disease resistance is one of the most important aquaculture traits. Genetic gain is possible through traditional selective breeding, but the rate of genetic improvement and the likelihood of attaining genetic improvement for disease resistance will probably be better via transgenesis. Several successful examples of significant disease improvement using genetic engineering have been accomplished.

Bacterial disease resistance may be improved up to fourfold through gene transfer of antibacterial peptide genes. Cytomegalovirus (CMV)-cecropin-transgenic channel catfish had higher survival rate (100%) than non-transgenic channel catfish (27.3%) during an epizootic of *Flavobacterium columnare* in an earthen pond (Dunham et al. 2002d). Transfer of cecropin genes to Japanese rice fish (*Oryzias latipes*) resulted in an increased resistance to *Pseudomonas fluorescens* and *Vibrio anguillarum*, which killed about 40% of the control fish in both cases (Sarmasik et al. 2002), while only 0–10% of the F_2-cecropin-transgenic medaka were killed by *P. fluorescens* and about 10–30% killed by *V. anguillarum*. Cecropin-transgenic rainbow trout exhibited not only increased bacterial, but also increased viral disease resistance (Chiou et al. 2014).

Grass carp transfected with a carp β-actin/human lactoferrin gene resulted in P_1 individuals that were more resistant to *Aeromonas hybrophila* and showed enhanced phagocytosis and more viral resistance than controls (Mao et al. 2004). F_2-transgenic zebrafish containing the Japanese flounder keratin promoter linked to the hen egg white (HEW) lysozyme gene exhibited 1.75× higher lytic activity from liver protein extracts than that in the wild-type zebrafish (Yazawa et al. 2006). This translated to increased disease resistance as 65% of the F_2-transgenic fish survived an infection of *Flavobacterium columnare* and 60% survived an infection of *Edwardsiella tarda* (likely *Pfiesteria pisicida*), whereas 100% of the control fish were killed by both pathogens. Gao et al. (2012) confirmed the lytic activity of tilapia C-3 lysozyme against Gram-positive bacteria *Streptococcus agalactiae* along with other Gram-positive bacteria and Gram-negative bacteria by comparing the activities of recombinant lysozymes in a bacterial challenge test. The same research team reported that expression of Hsp70 in the liver of Nile tilapia was induced after *S. agalactiae* infection and Hsp70 could drive expression of GFP in zebrafish (Zhang et al. 2014). They further fused tilapia Hsp70 promoter with tilapia lysozyme-C3 and a reporter gene, GFP, using the goldfish Tgf2 transposon system to produce transgenic zebrafish (Sun et al. 2017). The turbidimetric assay of extracted protein from liver of transgenic zebrafish were 1.6 times higher than that of wild-type zebrafish, indicating that tilapia C-type lysozyme 3 gives transgenic zebrafish more resistance to *S. agalactiae* infection than wild-type zebrafish.

Cold Tolerance

Antifreeze proteins (AFPs) were first found in Arctic (Scholander et al. 1957) and Antarctic (DeVries and Wohlschlag 1969) fishes. These discoveries were key to understanding how these species survive in water colder than the freezing point of their blood, which gave early fish genetic engineers ideas on how to produce transgenic fish that could be farmed under Arctic conditions. Early transgenic research in this area involved the transfer of the antifreeze protein gene of the winter flounder (Fletcher et al. 1988; Shears et al. 1991; Hobbs and Fletcher 2008) into Atlantic salmon, but expression levels obtained have been inadequate for increasing the cold tolerance of salmon. However, preliminary results with goldfish showed some promise for increasing survival within the normal cold temperature range. Goldfish transgenic for ocean pout (*Macrozoarces americanus*) type III antifreeze protein (AFP) gene had significantly higher survival at the lower end of their normal temperature tolerance than controls (Wang et al. 1995). Similar to results observed with the goldfish, ovarian and testicular tissues of F_3 generation mice transgenic for ocean pout type III antifreeze protein gene driven by chicken β-actin promoter maintained normal morphology at 4 °C as compared to non-transgenic control tissues (Bagis et al. 2006). These studies indicate that the antifreeze protein has functional roles slightly above freezing point temperature.

Body Composition

Transgenic technology can be used to improve body composition and nutrient content and produce bioactive substances in fish. Zebrafish transfected with β-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. 2007). Cheng et al. (2014) inserted a masu salmon (*Oncorhynchus masou*) Δ5-desaturase gene driven by the β-actin gene of carp into common carp, which increased unsaturated fatty acids. However, genotype–environment interactions occurred, and feed needed to contain sufficient precursors to allow the transgenic common carp to produce elevated levels of *n*-3 fatty acids. Pang et al. (2014) performed a codon optimization of the Δ12-desaturase gene (fat1) and the Δ15-desaturase gene (fat2) from the nematode, and then produced a transgenic fat1 and fat2 zebrafish. These fish had a doubling to tripling of *n*-3 fatty acids compared to controls. Kabeya et al. (2016) produced transgenic Nibe croaker (*Nibea mitsukurii*), a marine species, containing elongation of very long-chain fatty acids protein 2 (Elovl2) gene isolated from masu salmon, and the transgenic fry were able to produce increased omega-3 fatty acids. Thus, de novo synthesis of long-chain polyunsaturated fatty acids in fish has been achieved using transgenesis.

Flavor

Flavor and texture are high priority traits in all, but especially Chinese cultures. Transgenic technology has greater potential to genetically alter these traits than traditional selective breeding. Although large quantities of carp are consumed, the Chinese would like to have the flavor and umami taste of these fish enhanced and even prefer the flavor of slow-growing goldfish to that of traditional carp. Yan et al. (2011) successfully microinjected total DNA extracted from Chinese shrimp (*Fenneropenaeus chinensis*) to the zygote of common carp with the goal of enhancing flavor. Analysis of AFLP markers indicated that prawn DNA was integrated into the carp and transmitted to the F_2 generation. Muscle-nutrient analysis of the transgenic carp showed that the protein content and total amino acids were higher in the transgenics than the control group, including the four kinds of umami taste from aspartic acid, glutamic acid, glycine, and alanine.

Transgenic Fish as Bioreactors

Transgenic mammals have been used as biological factories to produce biomedical proteins such as clotting factors. Such technology is especially important in the current millennium since human extracted products have the potential to be contaminated with HIV, hepatitis viruses, Ebola, prions, and other human pathogens (Dunham 2019). Transgenically produced biomedical compounds should be free of human pathogens, be less expensive, and more widely available than those extracted from people. Several examples are now available demonstrating the potential of fish as bioreactors for medical products as well as compounds that can be used in fish spawning (Dunham 2011). However, quality control and regulation have as yet prevented commercialization of this technology.

CMV-human coagulation factor VII was microinjected into fertilized eggs of zebrafish, African walking catfish (*Clarias gariepinus*), and Nile tilapia (Hwang et al. 2004). Clotting activity was detected, indicating proper post-translational modifications. Proteins could be collected in eggs, serum, or possibly different proteins in different tissues for other types of genes, which demonstrated the possiblity of application of trangenic fish as bioractors. Transgenic Nile tilapia secreted human insulin in Brockmann Bodies (Pohajdak et al. 2004). Goldfish follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene were respectively constructed, driven by medaka β-actin promoter/enhancer, and microinjected into rainbow trout eggs (Morita et al. 2003). At 4 days, goldfish LH and FSH were isolated from the transgenic rainbow trout embryos. In vitro bioassay showed that single chain gold fish (scgf) FSH and scgfLH were expressed in rainbow trout embryos, and significantly elevated testosterone levels about three times compared to negative controls in testis. Hu et al. (2011) used the oocyte-specific promoter zp3 to initiate expression of tilapia-like, insulin-like growth factors (IGFs [plasmid: ZP: tIGFs: hrGFP]) in fertilized zebrafish eggs to produce recombinant proteins.

Pleiotropy

Growth Hormone Gene

Pleiotropic effects are common and numerous in transgenic fish. This is not surprising as insertion of transgenes usually affect the expression of a multitude of other genes. The pleiotropic effects can be positive, negative, or neutral.

Lo et al. (2014) observed 478 differentially expressed genes in cecropin-transgenic rainbow trout. Roberts et al. (2004) found that insertion of GH transgenes in salmon altered expression of some, but not all, myostatin-related genes. Hepatic gene expression was also altered in transgenic coho salmon and transgenic amago salmon (*Oncorhynchus masou*; Rise et al. 2006; Mori et al. 2007). Gene expressions of appetite-regulating, gastric-regulating, muscle immune function genes were altered in GH-transgenic coho salmon (Kim et al. 2015, 2018; Alzaid et al. 2018), common carp (Zhong et al. 2013), and zebrafish (Dalmolin et al. 2015). However, the alteration in gene expression for GH-transgenics varied from one species to another.

The transfer of GH genes has pleiotropic effects on body composition, body shape, feed-conversion efficiency, disease resistance, reproduction, tolerance of low oxygen, carcass yield, swimming ability, and predator avoidance. How the transgene affects overall phenotype and performance dictates whether or not a transgenic genotype has commercial potential as well as its fitness and potential impact on the environment. Delayed reproductive development was reported in GH-transgenic common carp (Chen et al. 2018). Silva et al. (2015) found that GH-transgenic zebrafish had greatly impaired reproduction that was corrected by making a double transgenic with both GH and GH receptor genes.

Improved feed-conversion efficiency is usually a component of fast-growing, transgenic GH fish including common carp, channel catfish, Nile tilapia, and salmon (Chatakondi 1995; Stevens and Devlin 2000a; Rahman et al. 2001; Dunham and Liu 2002). In most of these cases, the feed conversion was improved by approximately 20%. Transgenic tilapia expressing the hCMV-tiGH cDNA had an amazing feed-conversion efficiency that was 290% better for the transgenic tilapia (Martínez et al. 2000), which was similar to results obtained with mud loach (Nam et al. 2001).

The surface area of the intestine of GH-transgenic coho salmon, *Oncorhynchus kisutch*, was 2.2 times that of control salmon and the growth rate was about twice that of controls (Stevens and Devlin 2000a). The relative intestinal length was the same in transgenic and control salmon, but the surface area was greater for transgenics as a result of an increased number of folds. These differences could be related to the level of food consumption or GH may have a direct effect on intestinal growth (Stevens and Devlin 2000a). This phenomenon occurred in both GH Atlantic and GH coho salmon. This change in intestinal surface area could be a partial explanation for the increased feed-conversion efficiency of transgenic salmon. Regarding a related efficiency issue, GH-transgenic salmon grew better on high plant protein diets (Ganga et al. 2015) and high carbohydrate diets (Higgs et al. 2009; Leggatt

et al. 2009) than controls, which could result in more profitable and more environmentally friendly aquaculture.

Integration of the rtGH gene alters the survival of common carp (Chatakondi 1995). Transgenic individuals had higher survival than controls when subjected to a series of stressors and pathogens, such as low oxygen, anchor worms, *Lernaea*, *Aeromonas*, and dropsy. GH-transgenic common carp had higher lysozyme activity in the serum compared to age-matched, non-transgenic control fish (Wang et al. 2006). The serum bactericidal activity in the transgenics was 20% higher than in the controls. Values for leukocrit and phagocytic percent of macrophages in head kidney were higher in transgenics than controls, but the phagocytic indices and relative spleen weights in the transgenics and the controls were not different. GH transgene expression apparently not only stimulated growth, but also the non-specific immune functions of common carp.

GH gene transfer does not always confer increased disease resistance as GH-transgenic salmon were more sensitive to *Vibrio* compared to controls (Jhingan et al. 2003). Again, family effects were important as survival among GH salmon families sometimes improved, sometimes decreased, and sometimes remained unchanged relative to controls. Alzaid et al. (2018) found that mimicked viral infection suppressed muscle immune response in GH salmon.

Pleiotropic effects of GH gene insertion on oxygen tolerance characteristics vary from one species to another (Dunham 2011). GH tilapia 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 well as controls despite higher demand for oxygen. GH-transgenic salmon have an increased need for dissolved oxygen; however, after 4 days of starvation, GH individuals had the same oxygen uptake as controls. After feeding, GH-transgenics had 40–70% increased O_2 demand even when controls consumed equivalent amounts of feed. Adult GH-transgenic salmon had higher oxygen demand, poorer swimming ability, and longer recovery time compared to ocean ranched salmon (Lee et al. 2003; Dunham 2011; Leggatt et al. 2017a).

The definition of survival and how survival traits are measured can alter the outcome and conclusions. When exposed to low dissolved oxygen, survival was the same for GH-transgenic and control common carp. However, when mean survival time was calculated for all fish, dead or alive, the transgenic individuals had longer mean survival time than the non-transgenic full-siblings (Dunham et al. 2002a). Family effects were important as transgenic common carp in some families had higher and longer survival than control common carp when subjected to low oxygen, but in some cases control full-siblings were more tolerant. Transgenic channel catfish with the same rtGH construct as the common carp have a lower ventilation rate when subjected to low dissolved oxygen, compared with controls.

GH transgenesis has a dramatic effect on body composition in mammals (Ebert et al. 1988), with a drastic reduction in fat deposition in transgenic mice (Pomp et al. 1992; Knapp et al. 1994), pigs (Ebert et al. 1988; Pursel et al. 1990; Wieghart et al. 1990), and lamb (Nancarrow et al. 1991). Transgenic mammals possessing recombinant GH genes also show elevated levels of protein. GH-transgenic fish also

exhibit similar changes in the fat-to-protein ratios in the muscle, but they are not as dramatic as those observed in mammals. F_1 and F_2 rtGH-transgenic common carp had more protein, less fat, and less moisture than non-transgenic full-siblings (about a 10% change; Dunham et al. 2002b). 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). Amino acid ratios can be altered. For example, hCMV-tiGH cDNA-transgenic tilapia, *Oreochromis urolepis hornorum*, had lower levels of cholesterol, free alanine, and aspartic acid in the muscle compared with controls (Martínez et al. 2000).

GH transgenesis also affects muscle-cell characteristics and activity. GH-transgenic channel catfish had increased numbers of mitochondria in the cell, increased numbers of glycogen globules, and increased numbers of muscle fibers, but a reduced number of fat globules (Dunham 2011). Muscle-fiber size was unchanged. Perhaps due to these changes in amino acid levels and ratio, changes in fat and ultrastructure of the muscle, the flavor and texture of transgenic catfish flesh were slightly better than those of non-transgenic controls. Heterozygous GH-transgenic coho salmon also had higher numbers of small-diameter fibers in somite muscles (Hill et al. 2000) or a doubling of muscle-fiber recruitment (Johnston et al. 2014).

Morphological changes from GH gene transfer is common in transgenic fish. GH-transgenic common carp were slightly more truncated than full-sibling controls (Chatakondi et al. 1994; Chatakondi 1995; Dunham et al. 2002b). Similar changes are seen in GH-transgenic Nile tilapia, as the head:total length ratio was higher in transgenic fish relative to controls (Rahman et al. 2001). However, GH-transgenic rainbow trout derived from a wild strain had a slender body shape similar to that of controls (Devlin et al. 2001). Family or strain effects had a role in pleiotropy for body shape as domestic transgenic rainbow trout derived from a deep-bodied strain had an even deeper body depth than the controls caused by either increased muscle or tremendous visceral fat deposits or both.

Some families in the P_1 generation of transgenic Pacific salmon containing chinook salmon GH gene had excessive levels of GH resulting in morphological abnormalities in head, fin, jaw, and operculum as a result of excessive cartilage and bone growth of the fastest-growing transgenic fish (Devlin et al. 1995a). Insertion of an *Oncorhynchus* metallothionein GH1 plasmid (pOnMTGH1) gene construct into coho salmon altered centroid size, and the dorsal caudal peduncle and abdominal regions were also distinctly enhanced in transgenic fish when compared with controls (Ostenfeld et al. 1998). Morphological changes in both whole body and syncranium were prominent. The endocrine stimulation had been elevated to pathological levels in these GH-transgenic salmon, and excessive, deleterious deposition of cartilage was observed (Devlin et al. 1995a, b) analogous to the mammalian acromegaly syndrome. This effect can be sufficiently severe for impaired feeding and respiration to 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 stimulated (Devlin et al. 1995a, b).

Despite their minimal growth enhancement, domestic transgenic rainbow trout exhibited cranial deformities (Devlin et al. 2001), and Devlin et al. (2001) suggested that this is evidence that transgenesis affects growth pathways outside the range supported by the homoeostatic processes that maintain normal morphology and viability. This hypothesis is supported by the data of Maclean et al. (1987), as domestic rainbow trout receiving exogenous GH not only showed modest increases in growth (9%), but also had cranial abnormalities and silver body coloration, whereas controls did not have these characteristics. However, no abnormalities were observed in rapidly growing, GH-transgenic Nile tilapia, although minor changes to skull shape were observed in some fish (Rahman et al. 1998).

The altered body shape of rtGHcDNA-transgenic common carp resulted in improved dressout percentage in the F_2 generation, and a similar result was obtained for transgenic channel catfish containing the same GH construct. Dressout percentage was higher in almost all transgenic families. pCAgGH Yellow River common carp had higher dressout percentage than controls (Zhu 1992) because the weight of the gonads was reduced due to the inhibitory effect from the transgene, and the edible flesh of the fish was correspondingly increased.

GH gene transgenesis can also affect gill morphology in salmonids. Transgenic Atlantic salmon (Stevens and Sutterlin 1999) and Pacific salmon (Stevens and Devlin 2000b) had altered gill morphology compared to controls, but the difference was expressed in different ways in the two species. Pacific transgenic salmon had gill filaments similar to those of controls in length but had smaller lamellar spacing. Atlantic transgenics had longer gill filaments than controls, but with similar lamellar spacing to controls. Pleiotropic effects can be dissimilar for even closely related species.

Phenotypic variation is a key and underappreciated aquaculture trait with both beneficial and harmful effects dependent upon the situation. GH-transgenic channel catfish had more uniform growth than controls (Dunham et al. 1992a). When the mean body weight of an F_1-transgenic GH common carp family was greater than that of the control full-siblings, the coefficient of variation for body weight was smaller for transgenic fish than for non-transgenic fish (Zhang et al. 1990).

Growth hormone gene insertion can affect tolerance of various physical parameters. GH-transgenic channel catfish can survive water temperatures of −0.5 °C dependent upon the salinity, which was lethal to non-transgenic full-siblings (Abass et al. 2016). Additionally, these transgenic catfish are more tolerant of high salinity as fry (Youssef 2017), which is not surprising since GH has a role in osmoregulation (Tang et al. 1993). Similarly, GH-transgenic coho salmon also had slightly expanded temperature tolerance as Arrhenius breakpoint temperature was higher than that for controls (Chen et al. 2015).

Cecropin

A vast amount of information has been generated on pleiotropic effects of GH gene, but little for other transgenes. Cecropin-transgenic channel catfish and cecropin-transgenic rainbow trout, although, theoretically, healthier, had identical growth rate compared to controls.

GFP

Reporter genes are commonly used in some exploratory transgenic research as markers coupled with the transgene of interest. However, results obtained from such experiments may have limited applicability to predict aquaculture performance or fitness in natural environments as expression of green fluorescent protein reduces cardiac function and aerobic performance in transgenic zebrafish (Avey et al. 2018).

Food Safety

Food safety of transgenic aquatic organisms is beyond the scope of this chapter, but is a critical issue to address for transgenic fish to be commercialized. WHO/FAO, the U.S. National Academy of Sciences, the Royal Society of London, and the European Food Safety Authority have all reviewed the science of transgenic technology and concluded that in the vast majority of cases, except for potential allergenicity and perhaps specific cases, transgenic meat should be a safe product (FAO/WHO 2004).

Environmental Risk and Evaluation

Ecological impacts are a significant concern of the public regarding transgenic aquatic organisms. The ecological impact evaluation of genetically modified fish includes two main aspects, perturbances to ecological balance through altering the food web and habitat destruction, and population genetic changes by matings with wild conspecifics, potentially altering biodiversity and genetic biodiversity. Intentional or accidental stocking of transgenic fish has not occurred in the natural environment, and, of course, such an event is being tried to be prevented. Potential risk is being evaluated with various confined, laboratory-scale experiments.

The published scientific literature generally considers the escape and establishment of transgenic aquatic organisms as a negative event. Establishment of a transgenic fish could have negative, neutral, or positive impacts on ecosystems. Societal values as well as science dictate what is desirable and what is undesirable. Both science and society define what is a natural environment and a natural ecosystem. For example, the State of Alabama has more miles of stream per land area than any other state in the USA except for Tennessee. However, dams have been erected that impact all but 11 miles of Alabama rivers and many miles of river are now reservoirs. All over the world, habitat has been altered and destroyed. These altered environments are now considered "natural." Prior to erection of the dams, ictalurid catfish were 60% of fish biomass in Alabama. Now centrarchids are the dominant species with catfish constituting only 11% of the fish biomass. One could argue that establishment of a genetically altered catfish resulting in an increase in catfish biomass would also result in a more "natural state" based on the population distributions 70–80 years ago.

This is likely an unpopular line of thought. Even if it were correct, thorough and careful scientific evaluation of environmental risk and potential impact on ecosystems should be, and is being, conducted prior to use of transgenic fish in potentially inappropriate environments.

The key factor determining the environmental risk of a transgenic fish is fitness. If the fitness of a transgenic fish is less than that of a wild conspecific, the transgenic genotype should be selected against in the natural environment. The key components of fitness are reproduction, predator avoidance, foraging ability, and swimming ability, which affects the first three traits.

Fitness is not easy to measure in a small research environment or mesocosm trying to mimic a river, lake, or ocean. Other difficulties include identifying whether or not effects are caused by age, size, culture, or transgene. Also, results and conclusions can vary depending upon the length of the experiment.

Transgenic Exotic

Exotic species are the most likely type of fish to cause disturbance to ecosystems once they are established (Dunham 2011). Introducing transgenes that can alter the geographic range of a species would be of high environmental risk. The escaped transgenics could enter an area where there were no wild conspecifics to compete against since it would have a larger geographic range than the wild conspecific. A hypothetical example would have been the successful decrease in lower lethal temperature in salmon by introducing antifreeze constructs. At the time this would have allowed expansion of salmon aquaculture, but at great environmental risk.

Domestication and Incumbent Wild Types

In general, but not always, it is difficult for even stocked wild conspecifics to have a genetic impact when moved from one watershed to another based on data generated on freshwater sportfish in the Southeastern USA (Norgren et al. 1986; Dunham et al. 1992b, 2002c; Dunham 2011). In most of these examples, strains from other watersheds were stocked repeatedly in a body of water with the purpose of changing allele frequencies, but this often failed. The resident genetic type appears to have some reproductive advantage, which may or may not persist with climate change, and should also translate to a transgenic type also having difficulty in becoming established. Factors dictating whether or not the allele frequencies could be altered were the number of stockings, the stocking densities, the years of stocking, and water quality parameters.

In general, domesticated fish appear to have reduced fitness compared to wild conspecifics (although this is very controversial in the case of salmon) and, since most transgenic fish would be produced from domestic founders, this should add to the reduced fitness of transgenic fish, further decreasing their risk. In the case of channel catfish, DNA analysis indicated no detectable genetic impact of domestic populations on wild populations after three decades of likely escapements (Simmons et al. 2006).

Weir and Grant (2005) examined multiple studies on the mixing of domestic and wild Atlantic salmon. Seven studies indicated the fitness of the wild fish was higher in regard to survival and reproduction, 13 documented significant phenotypic differences, and 10 found distinct genetic differences between the wild and domestic populations, but the authors indicated that no conclusion could be made on population impact, so caution should be practiced in regard to the domestic escapes.

Erkinaro et al. (2010) found repeat domestic spawners of Atlantic salmon in the River Teno, but introgression determination was complicated as the microsatellite markers indicated that escapees came from a multitude of locations. Their review of the literature indicated that domestic Atlantic salmon have inferior reproduction in the wild, but can mate with wild-type Atlantic salmon. However, Karlsson et al. (2016) and Glover et al. (2017) found widespread introgression of domestic Atlantic salmon into wild populations in Norway with the lowest levels found in national protected areas. In Newfoundland, Wringe et al. (2018) found extensive crossing of domestic and wild Atlantic salmon. Over time the percentage of F_1 and feral individuals decreased, but introgression persisted as domestic alleles in backcross individuals in both directions.

Data to date indicate lower fitness of domestic fish. However, under the right conditions such as massive or repeat escapes or stockings, domestic alleles can be established at least in the short term (Glover et al. 2017).

Fitness of Transgenic Fish

Reproduction

Female GH-transgenic Nile tilapia had a lower gonadosomatic index than non-transgenic siblings in both mixed and separate culture conditions (Rahman et al. 1998, 2001). The transgenic male gonadosomatic index was higher in mixed culture and lower in separate culture than that of their non-transgenic siblings. However, GH-transgenic male tilapia had reduced sperm production (Rahman et al. 1998).

Fecundity was not affected by insertion of rtGH in common carp, and precocious sexual development was not observed in these carp (Dunham 2011). Similarly, transgenic channel catfish harboring rainbow trout or coho growth hormone gene had normal reproductive ability under artificial conditions (Dunham et al. 1992a).

Liu et al. (2011) studied the effects of starvation on the growth and gonadal development of *Oncorhynchus keta* growth hormone-transgenic common carp. When fed sufficiently or short fed, the salmon growth hormone-transgenic common carp had a faster growth rate than the control and a slightly better gonad development than the control. Guan and Liang (2013) compared the microstructure and gonadal development of testis between *O. keta* growth hormone-transgenic common carp and non-transgenic common carp. The structures of the testis in transgenic and non-transgenic males were similar, developmental degree of testis had no significant difference, and both genetic groups were able to reach sexual maturity. However, in another study, fast-growing, GH-transgenic common carp exhibited delayed sexual maturation and decreased gonad size (Cao et al. 2014). In this case, overexpression of GH depressed reproduction by directly inhibiting luteinizing hormone (LH) production and release through GH receptors in the pituitary gonadotrophs. Chen et al. (2018) conducted follow-up gene expression analysis with these fish and further found that pituitary gonadotropin inhibitory hormone (*gnih*), dopamine receptor D1 (*drd1*), dopamine receptor D3 (*drd3*), and dopamine receptor D4 (*drd4*) had increased expression in the fast-growing, GH common carp, and this expression profile was associated with the retarded reproductive development. Additional alterations in neuroendocrine factor gene expression and reduced hepatic leptin signaling to the pituitary were likely part of the response cascade to overexpression of GH, resulting in delayed sexual maturation.

Several examples of reduced reproduction in GH-transgenic fish exist. Bessey et al. (2004) and Fitzpatrick et al. (2011) observed reduced courtship and spawning in GH-transgenic coho salmon, and wild salmon outcompeted them reproductively in a semi-natural environment. GH-transgenic salmon put more energy into somatic growth and had reduced gonad size (Bessey et al. 2004) as did GH Nile tilapia (Rahman et al. 1998). Male GH-transgenic salmon displayed reduced nest loyalty, quivering frequency, and spawning participation (Moreau et al. 2011).

Predator Avoidance and Foraging Ability

Fitness of F_2- and F_3-transgenic, RSVLTR-rtGH1, and RSVLTR-csDNA (salmonid growth hormone genes) channel catfish and non-transgenic channel catfish fingerlings (BW: 3.45~4.31 g) and fry (BW: 0.37~0.85 g) were evaluated under natural conditions without supplemental feeding in confined earthen ponds (Dunham et al. 1999). Transgenic fish were more vulnerable to predators, largemouth bass *Micropterus salmoides* and green sunfish *Lepomis cyanellus*, than non-transgenic channel catfish (Dunham et al. 1999). An alternative explanation could be starvation due to higher metabolism of the transgenic fry. GH-transgenic coho salmon lost weight faster than non-transgenic coho salmon when starved (Abernathy et al. 2015). When supplemental feed was applied, the same groups of transgenic channel catfish grew 33–50% faster than non-transgenic individuals; while with natural food, no difference in growth was found between the two genetic groups (Dunham et al. 1999).

Marnis et al. (2016) conducted similar experiments with GH-transgenic African walking catfish fry, which had the potential to grow 19% faster than non-transgenic controls, but evaluated in aquaria. They conducted a unique experiment that addressed foraging ability and predator avoidance and behavior in a single experiment in which older, larger GH-transgenic and control African walking catfish were used as the predators. The transgenic fry were ten times more vulnerable to being cannibalized than the non-transgenic controls. All of the cannibalism came from the non-transgenic predators as the GH-transgenic African walking catfish exhibited minimal or no cannibalism. Under the restricted feeding regime, there was no growth difference between the transgenic and control fry. GH transgenesis totally altered the normal cannibalistic behavior. The opposite phenomenon occurred for GH coho salmon as under low food conditions some transgenic individuals turned cannibalistic (Devlin et al. 2004).

GH salmon are more vulnerable to predators than controls. GH-transgenic Atlantic salmon do not show the appropriate fear response in the presence of predators (Abrahams and Sutterlin 1999), and GH coho salmon fry are more easily preyed upon than controls (Sundström et al. 2004).

Several papers indicate that GH-transgenic fish have greater foraging ability. In most of these studies, appetite and foraging ability are being confused as pellets were the food. Foraging is the ability to obtain natural food items as one would not find pellets in the natural environment, and this is essentially spoon feeding and requires no food searching behavior. Foraging ability can be different at different life stages and the type of food or prey presented. Zhu et al. (2017) conducted two experiments providing GH-transgenic common carp with gastropods as prey, one providing a single species of gastropod and second providing four species of gastropods. In the first experiment with a single species of gastropod, transgenic and control common carp had the same minimal consumption, and gastropod biomass was not affected as apparently this species was not a preferred food. However, this prey item was mostly shunned in the second experiment, and the other three gastropod

populations were strongly affected with the foraging of the transgenic genotype almost three times that of the control.

Models

Ecological and mathematical models have been explored to predict the risks of bio-engineered fish. The ability of a transgene to establish and spread will depend on the fitness of the transgenic fish. Muir and Howard (1999, 2001, 2002) and Howard et al. (2004) used a GH transgenic medaka to model the potential effects of growth hormone gene transgenesis for environmental risk, and observed the "Trojan gene effect." Control females preferentially chose GH-transgenic males as mates because of their larger size. However, the GH-transgenic progeny had lower survival than controls, and the model indicates that this genetic load could, in some cases, lead to extinction of the population.

However, there are many flaws in the model. The fish were fed artificial feed, which would not occur in the natural environment; thus, foraging ability was not considered and genotype–environment interactions were not in the model. There was no predation or habitat present. Most species of fish do not preferentially choose large males for mates. Medaka have very low fecundity. It is doubtful that this experiment and model yielded realistic results.

Realistic models are important for identifying additional factors that impact behavior and genotype–environment interactions. When predators were present in the aquatic environment, female fish of various species decreased selectiveness with regard to male size (Forsgren 1992; Bierbach et al. 2011; Pennington and Kapuscinski 2011) and coloration (Godin and Dugatkin 1996). Furthermore, Atlantic molly (*Poecilia mexicana*) females (lab-reared) chose small males in the presence of the cichlid *Cichlasoma salvini*, a natural predator of *P. mexicana* in dichotomous choice tests. Wild-caught females did not respond to the same extent to the presence of a predator, and could alter their mate choice only when a natural predator was present, most likely due to a learned ability to evaluate their predators' motivation to prey (Bierbach et al. 2011). Genetic background (wild or domesticated) of fish impacted their mate choice, and wild fish have evolved visual predator recognition mechanisms; thus, risk evaluation should take this point into consideration.

A likely invasion case model using growth hormone-transgenic Atlantic salmon showed reduced breeding performance, such as fertilization success, compared to controls. However, the transgenic genotype had the capability to mate with their non-transgenic rivals, leading to gene flow to wild salmon populations (Moreau et al. 2011).

Semi-Natural Risk Experiments

Semi-natural environments, where conditions are kept as close as possible to natural in a closed system, are under evaluation to estimate potential ecological risks of transgenic fish. Interaction among strengths of promoter and transgene, strain and environment should gain attention during determining risk assessments (Leggatt et al. 2012, 2017b). Performance of wild-type coho salmon (*Oncorhynchus kisutch*) fry was evaluated for a fast-growing, GH-transgenic strain containing a sockeye salmon metallothionein promoter (MT, OnMTGH1), and three coho salmon lines/strains containing a reportedly weaker sockeye salmon histone-3 promoter (H3, OnH3GH1, H3-A,B,C) in hatchery conditions and semi-natural stream tanks, and they had varied growth and survival demonstrating the importance of these interactions and genotype–environment interactions in environmental risk evaluation.

Growth hormone-transgenic rainbow trout having the same OnMTGH1 were assessed in naturalized stream mesocosms in the presence of predators and without predators for two life stages, first-feeding fry and 60-day post-first-feeding (Crossin et al. 2015). For the first experiment with first-feeding fry, they found that in the late summer, the transgenic rainbow trout had lower survival rate either in the presence of predator, potentially due to the additive effect of the transgene that negatively decreased their foraging ability and risk of being predated, or in the absence of predator, potentially due to the transgene requiring a great metabolic demand in a food-limited environment. In regard to the growth rates, transgenic rainbow trout had lower growth rates than their sibling control in these two environments.

Results in the second experiment were much different. When the rainbow trout were 60 days old and past the critical mortality bottleneck period (2–3 weeks after emergence) in winter, effects of transgene on wild and domestic X wild genetic background were evaluated. Two genetic backgrounds, transgenic with wild genetic and transgenic with wild X domestic genetic background, had similar survival rate to their control siblings that had the same genetic backgrounds. Transgenic types grew faster than non-transgenic control siblings in the predator and predator-free environments. Thus, risk results can be life-stage dependent and vary from one life stage to another. Once again, genotype–environment interactions were important in environmental risk evaluation of transgenic fish.

The largest-scale and most complex “mesocosm” experiment was conducted in China. Hu et al. (2007b) constructed a 6.7-ha artificial lake containing mollusks, shrimps, rye grass, and other fish species (12 families and 23 genera with majority carp for approximately 65.2% of the total species), mimicking the carp habitat of the Yangtze River in China to evaluate the ecological risk of “all-fish” transgenic carp. A follow-up study (Lian et al. 2013) showed that in a natural aquatic environment, “all-fish” transgenic common carp (carp β-actin gene driving grass carp GH gene) had identical mating competitiveness to wild common carp, large fish did not have advantage in fertility, and juvenile viability of transgenic common carp was low. Additionally, the swimming speed of the GH-transgenic common carp was slower (Li et al. 2009). The authors concluded that fitness of the “all-fish” growth-transgenic carp was lower than the control carp.

Behavior could affect the key fitness traits. GH-transgenic salmon were more active and had a higher average swimming speed in a simulated ocean mesocosm (Hollo et al. 2017). Genotype–environment interactions related to abnormal behavior under laboratory conditions are key for environmental risk assessment and can confound risk evaluation. GH-transgenic coho salmon exhibited similar predator avoidance behavior regardless of environment as wild coho salmon raised in natural settings (Sundström et al. 2016). However, when wild coho salmon were reared in hatchery conditions, they exhibited extreme adverse predator avoidance behavior.

Taken in total, the results of environmental risk evaluation are quite variable. Often similar transgenes had different effects on different species. Although there are exceptions for the results for each fitness trait, there are some trends. In general, transgenic fish are less fit for reproductive traits and foraging for natural food, which usually eliminates the growth advantage of most GH-transgenic types. Early fry survival of GH-transgenics is greatly reduced compared to controls, GH-transgenics are more vulnerable to predators, swimming speed is reduced, requirements for oxygen are increased, and major behavior changes occur. In combination, it appears that GH-transgenic fish are less fit than non-transgenic and wild fish, and transgenes would likely be selected against in the natural environment. However, at this time confinement should be our major goal for prevention of transgenes making their way to the environment, even though these genotypes would likely, but not certainly, be eliminated.

Containment and Confinement

Risk-based frameworks or platforms need to be developed to evaluate individual transgenes and transgenic fish species under the actual (best simulation of) ecosystem on a case-by-case basis. Regardless of risk, several options exist for containment of transgenic fish. Simultaneous application of multiple containment strategies was recommended by the National Research Council (NRC 2004). Wong and Van Eenennaam (2008) reviewed and compared different containment strategies, and grouped them into different categories including physical, biological, and genetic containment. Physical containment and biological containment have various advantages and disadvantages. Physical containment and most other forms of containment cannot be 100% effective.

Genetic confinement is one strategy. One option for genetic containment is triploid induction, and that is one of the confinement aspects of the *AquAdvantage* salmon. However, these fish are 99% + triploid, so risk has not been totally eliminated. Triploid induction rates vary between 10% and 100%, depending on the species, shock conditions, and egg batch quality. Triploidy can decrease performance in fish (Dunham 2011), and is not feasible on a commercial scale in the case of catfish, tilapia, and many other species of fish. Triploid induction reduced performance of GH-transgenic fish, negating about half of the enhancement from the transgenesis in salmon and loach (Dunham 2011; Nam et al. 2004). AquaBounty

reported that family variation exists for this phenomenon, and the problem can be solved with family selection (Xu et al. 2013). Triploidy also has the disadvantage that it requires fertile diploid brood stock; so again, the possibility of escape and risk can be reduced, but not eliminated. Repressible transgenic sterilization and gene editing have great promise as effective confinement strategies.

Transgenic Sterilization

The ultimate means of preventing environmental or ecological impact of transgenic, domestic hybrid, or exotic fish are repressible transgenic sterilization or gene editing coupled with hormone therapy. In this case, escaped fish are incapable of breeding or their progeny are incapable of breeding, resulting in absolute reproductive confinement and the prevention of introgression of transgenes into wild populations and any potential associated impacts. Several potential transgenic approaches have been evaluated including antisense, shRNAi, and overexpression of cDNAs. Alternative systems have shown promise, but they require fertile individuals at some point in the process. In this case, long-term environmental risk cannot be eliminated. If repressible systems are used and all individuals in the population are homozygous for the sterilization construct at all life stages, environmental risk can only be short-term in the worst-case scenario.

One option is the disruption of translation of reproductive genes with antisense followed by hormone therapy when reproduction is needed. Uzbekova et al. (2000) used antisense Atlantic salmon sGnRH cDNA driven by the sGnRH Pab promoter in production of transgenic rainbow trout. They had positive results in their initial studies, but did not follow-up. Hu et al. (2007a) reported that a recombinant construct using carp β-actin driving antisense Atlantic salmon GnRH gene microinjected into fertilized common carp eggs resulted in 30% of the founders having no gonads. They also reported that the fertility could be restored by exogenous hormone administration.

Wu et al. (2010) also modified a similar system to control reproduction of transgenic zebrafish. The construct was driven by an ovary-specific and a testis-specific promoter. The transgene of interest was a suicide gene, consisting of a reductase and a photosensitizer, while the reductase gene was linked to a reporter gene. The novelty of this concept is that infertility is induced if transgenic fish expressing the reductase are treated with an effective amount of reductase-activated cytotoxic prodrug or if the transgenic fish expressing photosensitizer are treated with light irradiation. They reported 100% reliable infertility in zebrafish. However, to effectively produce a transgenic male with three copies of fusion transgene is unclear and doubtful, because the need for high copy number of the transgene prevents high transfection rate of the cell, and integration sites are random events. A piece of Simian virus 40 sequence, a polyomavirus, was used for design of the construct, which would be disadvantage when considering commercialization. The system is too complicated for practical use, and fertile transgenic fish are part of the system, thus, risk cannot be eliminated.

One form of transgenic sterilization, Sterile Feral (SF) technology, has shown promise for achieving transgenic reproductive confinement. Components of these constructs are fused so that a specific promoter is coupled to a repressible element that in turn drives expression of a blocker gene, antisense RNA, dsRNA, sense RNA, or ribozyme to an early key developmental gene (Dunham 2004; Thresher et al. 2005, 2009).

This strategy has two parts: one is to suppress the expression of critical gene related to embryonic development, gonad development, or sexual maturity via a knockdown construct in the absence of a repressible element. The second step is to reverse the sterility of transgenic fish by administrating exogenous compound to shut off expression of the blocker gene allowing rescue of the embryos and/or to produce brood stock. This knockdown strategy could be used to generate reversibly sterile transgenic fish. This is essentially a Tet-off or "Tet-off-like" system. Proof of principle of the sterile feral system has been demonstrated with the repression of the knockout function demonstrated in zebrafish, oysters, channel catfish, and common carp for disruption of embryonic development (Templeton 2005; Thresher et al. 2005; Chaimongkol 2009; Thresher et al. 2009). "Sterile feral technology" based on the Tet-off system was successfully evaluated in zebrafish and channel catfish (Thresher et al. 2009; Chaimongkol 2009). Mortality rates of integrated transgenic lines of channel catfish overexpressing a dorsolateral gene (BMP2) were less than 50% and were not significantly different from the control in the presence of 100 ppm of doxycycline in the hatching water. Without doxycycline, 95.6% of embryos died that carried BMP2, which was being up-regulated (Chaimongkol 2009).

The SF approach could be utilized to disrupt gamete production by preventing primordial germ cell (PGC) migration during embryogenesis. PGCs migrate far from the site of developing gonads to the genital ridge where they differentiate into gametes. A number of genetic markers are associated with and expressed in PGCs, such as *vasa*, *nanos*, *dead end*, *cxcr4b*, and *dazl* (Raz 2004). Tet-off systems for transgenic sterilization for knocking out PGCs have shown great promise as repressible systems. The primary problem with these systems is that the transgene contains small viral sequences. This does not pose any food safety or biological risk; however, public perception and negative advertising could prevent the marketing of such aquatic organisms.

Wong and Collodi (2013) used HSP70 promoter to initiate expression of stromal-derived factor 1a (SDF 1a) and control zebrafish fertility by controlling migration of primordial stem cells. Expression of the transgenic SDF 1a requires induction of fertilized eggs at 34.5 °C for 18 hours, thereby producing 100% sterile male zebrafish. Such a strategy has its potential risks. For commercialization, it will require large-scale, high-temperature incubation equipment. Many species cannot be incubated at this temperature. Fertile brood stock are required, so risk remains.

Zhang et al. (2015) used the GAL4/UAS transgenic technology system to inhibit endogenous dead end expression by transcription of the antisense dead end (primary germ stem cell marker). The advantages of this system are that fertile brood stock are effectively produced, and the sterile offspring are easily and simply produced by crossbreeding. However, the use of CMV sequences in plasmids as

promoters limits the application of commercialization in this system. Additionally, the creation and maintenance of two fully fertile brood stock lines does not eliminate risk. Another potential problem with this system is that GAL4 is temperature sensitive (Fortier and Belote 2000).

"Tet-off-like" systems have been recently developed, which contain no viral sequences (Su 2012; Li 2016). Modified Tet-off approaches were applied with a number of different combinations of promoters, target genes, and repressible elements in channel catfish and common carp. Su et al. (2014, 2015a, 2015b) and Li et al. (2017, 2018) modified the Tet-off system to induce expression of shRNAi and overexpression of cDNA knockdown constructs for nanos and dead end. Chemicals such as copper sulfate and sodium chloride used at high dosage turned off the construct in developing embryos and restored the gonadal development to allow production of brood stock that can produce sterile progeny.

One potential drawback of transgenic sterilization is potential negative pleiotropic effects, as knocking out reproduction can affect other traits similar to what might be observed with triploidy. For example, knockout of some primordial germ cell genes resulted in channel catfish with no gonads, and these fish had a 25% reduction in growth rate and survival (Li et al. 2018). Gene editing of reproductive genes followed by hormone therapy to restore fertility (Qin et al. 2016) may be one option to overcome this problem.

Transgenic Technology to Control Invasive Species

Invasive, exotic, or feral species/populations commonly damage biodiversity once established in their new habitat or geographic range. In Australia, 90% of the freshwater fish biomass is feral common carp (Zhang 2016). However, one option to reduce or eliminate these populations requires a willingness to intentionally stock transgenic conspecifics of the invaders into the environment.

Autocidal technologies have been proposed as a mechanism and strategy to reduce or eliminate invasive species populations. Eight autocidal approaches have been explored to control invasive species (Thresher et al. 2013). Autocidal refers to controlling or eradicating populations of noxious organisms (such as the screwworm) by reducing their capacity to produce viable or fertile offspring. This concept covers a wide array of strategies, including "lethal construct," "sex or stage-specific lethality/sterility," "inducible mortality," "Trojan gene," "mutual incompatibility," "engineered under dominance," and "Daughterless" (Thresher 2008; Thresher et al. 2009; Thresher et al. 2013). The main weaknesses in the strategy are the requirements for stocking large numbers of carriers and the high numbers of independently segregating copies in each carrier. Other pros and cons were also compared and discussed (Table 2 in Thresher et al. 2013).

"Trojan Y Chromosome," a non-transgenic genetic strategy, is the most promising technique that would likely have public acceptance (Teem et al. 2014; Teem and Gutierrez 2014). YY females are stocked to shift sex-ratio of a target population

toward males. The advantages are: (1) it is relatively inexpensive to develop; (2) it is likely to be publicly acceptable as any organism having YY is acceptable; (3) it may not require legislative changes; and (4) as female carriers outperform wild-type females that are decreasing over time, relative stocking rates increase. However, the internal potential disadvantage within itself is limitation to use in only fish and amphibians (Thresher et al. 2013). In reality, sex determination of fish could be plastic, and two typical sex-determination mechanisms exist, that is, genetic-sex determination and temperature-sex determination. Other environmental factors, such light, pH, dissolved oxygen, and water pressure also influence the sex determination and differentiation (Yan et al. 2017). This strategy requires ten generations under optimal conditions and could take longer. This strategy remains to be tested and documented (Thresher et al. 2013). Additionally, not all genetic-sex determination is XY, and YY females are not viable in all species of fish (Dunham 2011).

Teem and Gutierrez (2014) proposed that combining the Trojan Y chromosome and daughterless carp (a transgenic approach) eradication strategies could be more effective than both strategies working independently, modeling a rapid decline of females in the population and a shorter time to extinction. Theoretically, these strategies, the preliminary data, and the modeling of the results appear promising. However, none of these approaches have been fully developed or field tested.

Status of Commercialization

Eighteen years after the first transgenic fish was produced, this technology began to impact commercial aquaculture, not as a food organism, but as ornamental fish. In the spring of 2003, the first transgenic fish, green fluorescent protein medaka, TK-1, was sold in Taiwan (Wikipedia 2019). In December of 2003, the US Food and Drug Administration (FDA) approved the sale of red fluorescent protein zebrafish, GloFish, in the USA, but they continued to be banned in California. Even California decided there were no food safety or environmental risks and legalized GloFish in 2015. Now a multitude of transgenic aquarium species fluorescing with a wide array of colors from several source species are sold in the aquaria trade in the USA, Canada, and other countries. These fish are still banned in the European Union, but Dutch authorities found 1400 transgenic fluorescent fish that originated from several aquaria shops in 2006.

In 2013, 28 years after the first transgenic fish was produced, a transgenic food fish was approved by Canada making the first impact of transgenic fish food technology. In November of 2013, the Canadian government became the first to approve a transgenic food fish, growth hormone-transgenic Atlantic salmon, for commercial sale, however, with the stipulation that they should be triploid embryos for export only to countries with both containment and approval of transgenic flesh (DFO 2013). History was made when the flesh of these triploid salmon produced by AquaBounty was approved by the US Food and Drug Agency for consumption in the USA (US Food and Drug Administration 2015). This was the first known

approval of the consumption of transgenic animal meat in the world, but approval was not granted for the culture of transgenic salmon in the USA. Then on November 19, 2015, Canada also approved the consumption of GH-transgenic salmon, *AquAdvantage Salmon* (Ledford 2015). On May 19, 2016, the Health Canada and the Canadian Food Inspection Agency approved the sale of *AquAdvantage Salmon* into the market. The first 4.5 tons were placed in the Canadian market on August 4, 2017, and were all quickly sold (Waltz 2017). This is the first government-approved consumption of any transgenic animal. Now, more than 18 tons have been sold in Canada.

After first approval in the USA, an import ban was triggered preventing importation and sale of transgenic salmon in any form because of a lack of labeling laws in the USA. Anticipating that this would be resolved, AquaBounty purchased an indoor recirculating aquaculture system (RAS) in Indiana, and this is where the first US grown genetically engineered fish were to be produced (IntraFish Media 2017). On December 20, 2018, the National Bioengineered Food Disclosure Standard was announced, which requires food manufacturers, importers, and certain retailers to label foods containing genetically modified or bioengineered ingredients (Federal Register 2018). This lifted the import ban and AquaBounty is now raising transgenic salmon in Indiana. First sale of GH-transgenic salmon in the USA is anticipated in the next few months. Production of GH-transgenic salmon has also now been approved in Canada (CBC 2019).

Summary and Conclusions

Transgenic fish have been produced that have traits that can impact aquaculture in major ways allowing increased production, efficiency, profitability, and, if used properly, increased environmental friendliness of aquaculture. A concern is how these fish could impact the natural environment, ecosystems, biodiversity, and genetic biodiversity if these were to escape from an aquaculture facility. Evaluation of fitness traits indicates that transgenic genotypes are, in general, less fit than non-transgenic and wild fish when evaluated for fitness traits in simulated natural systems. However, in some cases, there is great variability for some fitness traits. It is likely that if transgenic fish were to escape into the natural environment, the transgene would be selected against, and there would be no long-term effect on ecosystems or biodiversity. However, it is better to be cautious, and provide strong confinement to prevent transgenic fish from entering the natural environment. Repressible transgenic sterilization and related genetic strategies are likely the best strategy to provide absolute biological confinement. In this case, the fish can only reproduce with the intervention of man. Any environmental impacts would be short term. There are no physical containment systems that can guarantee 100% confinement.

In the future, there may be strategies employing transgenic technology to control feral populations, which could actually have a positive impact on biodiversity by

controlling these damaging organisms. Much of our world has been altered, and genetic biodiversity has been lost. As of today, it could be considered heresy, but there may be times and locations in the future where transgenesis might be used to create positive and increased biodiversity.

The generation of a multitude of species and varieties of GloFish has certainly added biodiversity by strict definition to the world. However, there is no ecological value or detriment from these fish. Their value is primarily to aquaria enthusiasts who enjoy the unusual, strange, and colorful. In actuality, from a pure ecological point of view, these are analogous to the large number of "varieties" of ornamental goldfish developed by selective breeding of mutants over hundreds and thousands of years, but are of no environmental consequence because of their extremely low fitness. One segment of society deems these as beautiful useful pets, and another segment of society deems them ugly, bizarre, and useless.

Application of transgenic fish in the future will likely be common in aquaculture and be of great benefit. Used properly, it should reduce pressure on the natural environment and natural resources, and in that way more efficiently use our food-producing footprint. The relief for natural ecosystems should impact biodiversity in a positive manner. More traditional genetic approaches have already started this process, as in the case of catfish for which production per surface area has increased 5× during the last 30 years, and transgenesis will assist in making food production increasingly efficient.

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