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**Expression and Inheritance of Growth Hormone
Gene Constructs and Selective Breeding of
Transgenic Farmed Fish**

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TABLE OF CONTENTS

Abstract	3
Objectives of the original research proposal	4
Relevant Data, Methodology, Results and Discussion	4
U.S.	5
Predator Avoidance	5
Combining Gene Transfer with Selection to Improve Body Weight of Channel Catfish	17
Israel	30
Construction of vectors for microinjection	30
Microinjection	31
NLS	32
Radioimmunoassay for GH in blood samples	33
Selection of transgenic fish	33
Large scale growth performance of transgenic common carp	34
Evaluation of the Research Achievements	46
Description of the Cooperation	46
Conclusion	46
List of publications	47

Abstract

Title: Expression and inheritance of growth hormone gene constructs and selective breeding of transgenic farmed fish

Objectives: To accomplish stable expression, inheritance of transgenes and growth improvement in transgenic channel catfish, Ictalurus punctatus, and common carp, Cyprinus carpio, containing growth hormone (GH) genes, develop transgenic fish with all fish constructs, determine the relationships between copy number, expression and growth, determine the combined affect of selective breeding and gene transfer and assess environmental risk of transgenic fish. To develop mechanisms of triploidization for transgenic carp.

Results: Performance of transgenic channel catfish was made uniform by selection. Growth of channel catfish and common carp was improved 40-50% more by combining gene transfer of GH genes with selection and crossbreeding than with either selection or crossbreeding. Growth improvement of transgenic catfish was not strongly correlated with copy number and expression levels. Progress was made in producing triploid transgenic common carp. Insertion of salmonid GH gene did not alter reproductive performance in channel catfish. Transgenic channel catfish grew no faster than controls when they had to forage on natural food and transgenic individuals were slightly more vulnerable to predation indicating that fitness of transgenic individuals in natural conditions is less than or equal to non-transgenic channel catfish.

Contribution to Agriculture: These experiments are the first to demonstrate that transgenic fish can increase aquaculture production in the aquaculture environment. This research also demonstrated that maximum benefit of gene transfer in farmed fish is attained when combined with traditional selective breeding.

U. S.

Objectives of the original research proposal:

To accomplish stable expression, inheritance of transgenes and growth improvement in transgenic channel catfish, Ictalurus punctatus, and common carp, Cyprinus carpio, containing GH genes, develop transgenic fish with all fish constructs, determine the relationships between copy number, expression and growth, determine the combined affect of selective breeding and gene transfer and assess environmental risk of transgenic fish to develop mechanisms of triploidization for transgenic carp.

Relevant Data, Methodology, Results, and Discussion

Several all-fish constructs were inserted into channel catfish, Ictalurus punctatus. These fish are being raised for performance evaluations and spawned to evaluate F₁'s.

Constructs inserted include:

<u>Promoter</u>		<u>GH gene</u>
trout metallothionein	-	seabream growth hormone
trout metallothionein	-	silver carp growth hormone
ocean pout antifreeze	-	salmon growth hormone
carp β -actin	-	rainbow trout growth hormone
carp β -actin	-	trout IGF

Predator Avoidance

Introduction

Transgenic channel catfish possessing foreign growth hormone genes have been produced (1,2,3). The transgenic catfish containing salmonid growth hormone gene produce salmonid growth hormone and transmit the recombinant gene to their progeny (3). Transgenic catfish can grow 25% faster than controls.

For the aquaculture industry to benefit from this research and future research, the transgenic fish need to be released and utilized by the private sector. Before this can be done the potential risk of transgenic catfish to natural populations and the natural environment must be determined. Our long term goals include the study of a variety of traits of transgenic fish, especially fitness traits such as spawning ability, foraging ability and predator avoidance which are critically related to survival and competitiveness in the natural environment.

Our laboratory has transferred salmonid growth hormone genes, RSVLTR-rtGH₁ cDNA, RSVLTR-rtGH₂ cDNA and RSVLTR-csGH gene, into channel catfish. One-nine copies of the foreign DNA were inserted in either head-to-tail tandem array at single insertion sites or single copies at multiple insertion sites. All P₁ transgenic catfish evaluated produced salmonid growth hormone regardless of the construct. Five P₁ X P₁ matings have been accomplished. The spawning rate and fertility of these P₁ transgenics in artificial spawning conditions was comparable to that of normal channel catfish. In two of three years, 100% spawning and 100% hatch were obtained. Percent transgenic progeny observed in the five matings were 20, 52, 7, 47 and 0% which was lower than the 75% inheritance expected assuming the P₁ brood stock had at least one copy of the foreign gene integrated and were not mosaics in the germ-line. At least

seven of ten P_1 were mosaics and a minimum of 2 of 10 P_1 did not possess the salmonid growth hormone gene in their germ-line.

P_1 transgenics grew at the same rate as their non-transgenic full-siblings which is not surprising since the P_1 were mosaics. F_1 transgenic progeny in two families possessing RSVLTR-csGH grew 26% faster to 40-50 g than their non-transgenic full-siblings when evaluated communally. One F_1 progeny group produced by a RSVLTR-rtGH₁ cDNA X RSVLTR-csGH mating and one F_1 progeny group (parents either RSVLTR-rtGH₁ cDNA or RSVLTR-csGH) grew at the same rate as normal full-siblings when grown communally to 25 g. In families where F_1 progeny grew faster than controls, the range in body weight and coefficient of variation for the transgenic full-siblings was less than that for controls. In families where F_1 progeny grew at the same rate as controls, range in body weight and coefficient of variation were similar for transgenic and normal individuals. The percent deformities observed in P_1 transgenics, 13.6%, was higher than in microinjected P_1 non-transgenics, 5.1%. Percent deformities in transgenic and control F_1 channel catfish was not different, 0.5 and 2.8%, respectively.

The RSV-LTR (Rous sarcoma virus-long terminal repeat) promoter, a constitutive promoter used in these experiments, appears to be effective in fish. Channel catfish and common carp express salmonid growth hormone genes driven by RSV-LTR. Northern pike, Esox lucius, and goldfish, Carassius auratus, also express recombinant genes driven by the RSV-LTR promoter. The mouse metallothionein promoter does not appear to consistently drive expression in fish (6, 7). The SV-40 promoter usually does not allow expression (8, 9) with the lone exception being the successful expression of catalase in transgenic tilapia (10).

Results we have obtained for F₁ transgenic common carp (11, 12) and channel catfish (2, 3) containing salmonid trout growth hormone gene were similar. The presence or absence of increased growth can vary among families, and may be related to family effects, genetic background, epistasis or dosage effects of the foreign growth hormone gene expression. Apparently, a combination of both family selection as well as gene transfer is needed to optimize growth increase from the insertion of salmonid growth hormone genes.

Factors affecting establishment of new genotypes in established natural populations

Several factors might affect the establishment of domestic populations by accidental or intentional release and their opportunity or ability to interact with and influence wild populations. These include size of fish, number of fish stocked, number of repeat stockings or releases, timing of stocking or release, selective value of the new genotype and other environmental variables. These variables have not been completely evaluated, but a growing base of data illustrates their importance and function (13-22). These studies indicate that it is difficult to establish a new genotype, even wild rather than domesticated, in an established natural population (16, 19, 20, 23-25).

One example illustrating this point is that of the massive stockings of Florida largemouth bass, Micropterus salmoides floridanus, into established northern or native largemouth bass, M. salmoides, populations (13, 15, 16). Genes from Florida largemouth bass were established in these populations at varying levels, and in some cases not all. Key factors in the establishment of Florida alleles were total numbers of fish stocked, number of years since initial stocking, number of repeat stockings, elevation, age of the lake and water clarity.

Size of fingerling or sub-adult fish stocked has an effect that is not well defined. Kulzer et al. (15) did not find a correlation between size of fingerling or sub-adult largemouth bass stocked and the success of the introduction in largemouth bass. However, the results were confounded by lack of replication and a multitude of additional variables. Studies on the success of stocking large and small sub-adult trout had contradictory results (17, 18, 26-28). The large sub-adults had greater survival when introduced in one study and lower survival in another. The main conclusion of these studies is that it is difficult to genetically impact established natural populations of fish.

Reproductively isolated sympatric populations

Another possible interaction between domestic and wild populations of fish is the establishment of sympatric, but reproductively isolated populations. Although strains of fish usually do not have reproductive isolating mechanisms preventing them from interbreeding, occasionally behavioral mating blocks prevent or decrease the rate of inter-strain matings. We have found that Marion channel catfish females preferentially mated with their own strain rather than Kansas males (29), and Ghana strain of Oreochromis niloticus was more likely to mate with its own strain than other strains (30). The existence of reproductively isolated, sympatric populations of trout (31-33), especially brown trout, Salmo trutta, is well-documented. Some strains of domestic and wild rainbow trout are sympatric, but reproductively or near to reproductive isolation. This occurs because of behavioral differences including temporal or spatial differences in spawning (30).

Fitness traits - reproduction, predator avoidance and foraging

Reproduction

In addition to other factors, size often plays an important role in reaching sexual maturity and reproductive success. Often the fastest growing individuals within a population will reach sexual maturity the earliest (35, 36), although early sexual maturity does not necessarily correspond to an increased growth rate alone (34, 35-39). Hulata et al. (40) reported a negative association between growth rate and onset of sexual maturity in Cyprinus carpio, and this same relationship between growth rate and sexual maturity has been documented in channel catfish (36, 39).

Egg production also is related to size in channel catfish, and it is likely that transgenic channel catfish may produce more eggs than a normal channel catfish of the same size. However, we have observed that the maximum relative size difference between the transgenic and non-transgenic carp occurs by the time they reach 200 g. When these fish are three years old and have reached a size of 2 kg, the relative size difference is the same or slightly less, and preliminary evidence in catfish indicates that relative size differences of transgenic and control fish occur early, and then remain unchanged. This growth rate relationship would help negate any size-related reproductive advantage of the transgenic catfish.

To gain a reproductive advantage in the natural environment, a specific genotype would need to be vastly superior to that of other genotypes. The ability to produce large numbers of offspring is not necessarily advantageous because of the tremendous mortality of young fish in the natural environment. In a typical Alabama reservoir, only 0.04% of the channel catfish larvae produced survive to be adults. Among the factors causing natural mortalities and affecting

the survival of catfish during their early life history stages are predation and availability of food. Unless a large reproductive advantage existed, the ability to forage and avoid predators are likely of more importance than reproduction for fitness in the natural environment.

Predator avoidance

Predator avoidance probably has both behavioral and growth components. Aggressiveness or lack of wariness can lead to mortality due to predation by insects, fish, animals, angling or netting. Additionally, increased growth can be a disadvantage making a fish susceptible to selective harvest (40, 41) by either animal predators or man. Conversely, rapid growth at certain life stages is probably important to reach a size that allows avoidance of small predators.

Foraging ability

The ability to forage and find feed is, of course, critical to survival in natural settings. The ability of a genotype well adapted or selected for feeding in the cultured environment is not necessarily well adapted for feeding in the natural environment since the genetic value or rank of a particular genotype may change as the environment changes. The best genotype for one environment may not be the best for another.

Genotype-environment interactions are prevalent in aquaculture experiments when different genotypes are compared utilizing either natural food organisms or artificial supplemental feeds (43-46). High performance triploid oysters have no advantage compared to normal oysters when food is limiting.

Catfish is the major aquaculture species in the United States accounting for over 50% of all U.S. aquaculture. Production in 1992 is estimated to be 500 million lbs. This crop is worth

1.5 billion dollars including value added. The benefit of utilization of high performance transgenic fish, if it can be demonstrated they do not pose risk to the natural environment, would be worth billions of dollars to the catfish and aquaculture industries.

The primary goal of the research is to determine the genetic risk of transgenic channel catfish, Ictalurus punctatus, which possess the rainbow trout growth hormone gene to wild populations of channel catfish. If transgenic channel catfish containing the rainbow trout growth hormone gene were to be accidentally released into the natural environment from either a research or commercial aquaculture facility, several possible scenarios exist. These transgenic fish may be eliminated by predation or starvation before they reproduce. They may not be able to reproduce or spawn effectively. They may reproduce but catfish possessing the rainbow trout growth hormone gene (or other growth hormone genes) would be eliminated by random genetic drift because of their low population numbers. These transgenic fish could spawn and the foreign gene be established at low frequency based upon initial gene frequencies resulting from the surviving and reproducing escaped fish. The transgenic fish could segregate, spawn with themselves and form a reproductively isolated, sympatric population. Lastly, the transgenic catfish could increase in numbers or proportion compared to non-transgenic catfish.

The last example has the greatest potential for genetic impact, be that positive or negative, on natural populations. The last scenario, increase in transgenic fish numbers in the wild, will only occur if the transgenic fish possess traits that allow them greater predator avoidance (therefore, increased ability to survive to sexual maturity), greater ability to forage on natural food, or a greater ability to spawn and produce young. These three traits are the key to fitness, the ability to transmit ones genes to the next generation.

To accomplish the primary goal, determine the genetic risk of transgenic channel catfish which have foreign growth hormone genes to natural populations, three secondary objectives must be achieved. The predator avoidance, foraging ability, and spawning ability of transgenic channel catfish are fitness traits that must be evaluated.

Several critical questions will be addressed and answered by the proposed research:

(1) What effect does the addition of a foreign (rainbow trout) growth hormone gene have on the spawning and reproductive ability of catfish (fish)? This is important for two reasons, to determine if the transgenic fish are reproductively fit in the natural environment and whether or not they can be spawned naturally (commercial conditions), which is a criteria for commercial application.

(2) What is the survival and growth as related to foraging ability on natural food of these transgenic catfish? Although we have demonstrated that transgenic catfish can grow faster than non-transgenic catfish in aquaculture settings where food is not limiting and provided in an accessible manner, we do not know if they have the ability to forage for limited natural food items, exhibit accelerated growth under natural conditions, find and consume enough food to support an increased metabolism and ultimately survive under these conditions. Predator avoidance under these conditions may also be directly related to growth.

(3) What ability do transgenic catfish have to avoid predators? This is of course a key to fitness and survival in the natural environment. The opportunity to reproduce only avails itself if the fish survive to sexual maturity.

The elucidation of these traits should allow the prediction with a high level of accuracy whether or not transgenic catfish possessing foreign growth hormone genes, specifically

salmonid growth hormone gene, have the potential to genetically impact natural populations of channel catfish.

Methodology

Management of experimental fish

Transgenic and non-transgenic channel catfish were artificially spawned by pairing male and female catfish in aquaria and intraperitoneally injecting the female catfish with carp pituitary extract. The channel catfish were allowed to spawn naturally in the aquaria. After spawning the fertilized egg masses were collected and placed in wire mesh baskets in paddle wheel troughs to hatch. The newly hatched fry were fed Artemia and fry starter diet and were reared in an indoor hatchery for 10-15 days. DNA samples isolated from each fertilized egg mass were analyzed to determine the presence of RSVLTR-rtGH, cDNA or RSVLTR-csGH cDNA by PCR and dot blot analysis.

Experimental ponds at the Fisheries Research Unit, Auburn University were dried and spread with 45 kilograms of lime prior to filling with water. One week before stocking the fish, approximately 23 kilograms of fish feed were spread on the pond surface to serve as fertilizer to establish a plankton bloom. Metal cans, brush and cement blocks were placed in each pond to provide cover for the fry.

Fifty fry per family were sacrificed to determine the percentage of transgenic fry before stocking in each pond. Fry weighing approximately 0.15 g were stocked communally in ten 0.04 ha earthen ponds. Stocking rates ranged from 5,000 - 32,500 fish/ha. One day after the fry were stocked the predators were added to the experimental ponds; seven ponds were each stocked with six green sunfish Lepomis cyanellus, three ponds were each stocked with six largemouth bass

Micropterus salmoides and one pond received 31 largemouth bass. The weight of green sunfish and largemouth bass ranged between 20-30 g.

After approximately two weeks of predation, all remaining fish were recovered by seining with 0.6-cm mesh and then draining the ponds. DNA samples isolated from fin tissue of individual survivors were analyzed by PCR and dot blot to determine the number of transgenic catfish remaining.

In 1995, four ponds were initially stocked at a rate of 8,000 larger channel catfish/ha (average weight = 3.72 g). One month later largemouth bass were stocked as predators at a rate of four hundred twenty-five largemouth bass/ha (average weight = 0.14 kg). The period of predation was approximately 3.5 months.

Results and Discussion

Predator avoidance of transgenic channel catfish

The stocked and recovered ratios of transgenic and non-transgenic channel catfish in ponds with green sunfish and largemouth bass during 1994 are summarized in Tables 1 and 2, respectively. When the experiments with green sunfish stocked as predators were combined, non-transgenic channel catfish had better predator avoidance ($P < 0.01$) in two trials (trials 6 and 7). Transgenic channel catfish exhibited better predator avoidance in two trials (trials 1 and 5). There was no difference in predator avoidance for trial 2 ($P > 0.05$). All catfish were eliminated in trials 3 and 4. Pooled data also showed non-transgenic channel catfish exhibited better predator avoidance when stocked with largemouth bass ($P < 0.01$). There was no difference in predator avoidance for trial 10. Non-transgenic fish had better predator avoidance in trial 8. Transgenic channel catfish exhibited better predator avoidance in two trials (trials 9 and 11).

The stocked and recovered ratios of transgenic and non-transgenic channel catfish stocked with 425 largemouth bass/ha in 1995 are presented in Table 3. No significant difference in predator avoidance between transgenic and non-transgenic individuals was found in trial 13 ($P > 0.01$), in which the survival of channel catfish was the lowest. The non-transgenic individuals had better predator avoidance in other trials ($P < 0.01$). When the pooled data were analyzed, the non-transgenic individuals had better predation avoidance.

During the first year there were some families that appeared to have superior predator avoidance. When some of these families were examined further during the second year, their predator avoidance was either average or poor indicating no strong family effects on predator avoidance.

Growth of transgenic and control channel catfish in earthen ponds with natural food

The mean weights of surviving fry from trials 1, 6 and 7 are presented in Table 4. The mean weights of transgenic channel catfish were not different from non-transgenic channel catfish ($P > 0.05$). Possibly, the limited natural food did not allow the transgenic channel catfish to express their growth potential.

Table 5 summarizes mean body weight and coefficient of variation of stocked and recovered F_2 transgenic channel catfish stocked in ponds with largemouth bass during the second year. There was a difference in stocking size between transgenic and non-transgenic fish ($P = 0.005$) observed in trial 13. Differences in initial body weight were also found in trial 15 ($P = 0.03$). No difference in stocking size between transgenic and non-transgenic channel catfish was observed in trial 12 and 14 ($P > 0.01$).

Growth of transgenic and non-transgenic catfish was not different after 3.5 months in ponds with only natural food items and no supplemental feeding. These results suggest that transgenic channel catfish would not exhibit increased growth under conditions of limited food availability. Initial size difference between groups did not become magnified as it is often found when supplemental feeding is used (48). In parallel experiments in aquaria and earthen ponds where supplemental feed was applied, the same groups of transgenic channel catfish used in this study grew 33% - 50% faster than non-transgenic individuals. Genotype-environment interactions for transgenic channel catfish were evident based on food source and availability.

Non-transgenic channel catfish had better predator avoidance than transgenic channel catfish. If transgenic channel catfish containing rainbow trout or coho salmon growth hormone gene were accidentally released into nature from either research or commercial aquaculture facilities, they would likely decrease in number because of their increased susceptibility to predators and eventually the transgene would be eliminated. In addition, there was no difference in growth between transgenic and non-transgenic channel catfish when there was no supplemental feeding. As a result, it seems likely that the ecological risk of transgenic channel catfish is low or non-existent.

Combining Gene Transfer with Selection to Improve Body Weight of Channel Catfish

We were able to combine selection and gene transfer to stabilize growth enhancement in transgenic channel catfish by the F₄ generation. Transgenic catfish containing pRSVrtGHcDNA, selected for fast growth had uniform growth performance and grew 41% faster than mass selected controls in earthen ponds. Transgenic individuals had lower fat, higher protein, better flavor and better texture than non-transgenic controls. Inheritance of the transgene was high and stable.

Long term: This demonstrates the commercial feasibility of growing transgenic catfish.

Table 1 The ratios of F₂ and F₃ transgenic channel catfish, *Ictalurus punctatus* containing either RSVLTR-rGH1 cDNA or RSVLTR-esGH1 cDNA and non-transgenic stocked with green sunfish, *Lepomis cyanellus* in 0.04 ha earthen ponds.

trial ¹	generation	fish/ha	stocked		days	recovered					
			positive			negative		positive		negative	
			#	%		#	%	#	%	#	%
1**	F ₂	16,250	80	12.31	570	87.69	21	40	40.40	59	59.60
2	F ₂	13,750	86	15.61	465	84.39	22	25	16.13	130	83.87
5**	F ₃	25,000	423	42.30	577	57.70	15	18	69.23	8	30.77
6**	F ₂ , F ₃	10,000	164	41.00	236	59.00	17	4	5.97	63	94.03
7**	F ₂	10,000	167	41.75	233	58.25	17	5	16.13	26	83.87
Total**			920	30.66	2081	73.95		92	24.34	286	75.66

** Stocked/ recovered ratios of transgenic and control were different (Chi-Square, P<0.01).

¹ Trials 3 and 4 were eliminated from the analysis because of 100% predation of both genotypes.

Table 2 The ratios of F₂ transgenic channel catfish, *Ictalurus punctatus* containing salmonid growth hormone gene and non-transgenic channel catfish stocked with largemouth bass, *Micropterus salmoides* in 0.04 ha earthen ponds

trial	generation	fish/ha	stocked		days	recovered					
			positive	negative		positive	negative				
			#	%	#	%	#	%			
8**	F ₂	22,500	804	89.33	97	10.67	15	50	46.30	58	53.70
9**	F ₂	32,750	270	20.55	1044	79.45	13	266	27.48	702	72.52
10	F ₃	5,000	46	23.00	154	77.00	29	42	27.81	109	72.19
11 ¹	F ₂	14,250	149	26.09	422	73.91	16	3	50.00	3	50.00
Total**			1269	42.51	1716	57.49		361	29.28	872	70.72

** Stocked/ recovered ratios of transgenic and control were different (P<0.01).

¹ Chi-square may not be a valid test because the sample was less than 5.

Table 3 The ratios of F₂ transgenic channel catfish, *Ictalurus punctatus* containing salmonid growth hormone gene and non-transgenic channel catfish stocked at a total of 8,000 per ha with 425 largemouth bass, *Micropterus salmoides* per ha in 0.04-ha earthen ponds for 3.5 months

trial	generation	stocked				recovered			
		positive		negative		positive		negative	
		#	%	#	%	#	%	#	%
12**	F ₂	103	31.99	219	68.01	10	6.67	140	93.33
13	F ₂	71	22.12	250	77.88	15	26.79	41	73.21
14*	F ₂	116	36.02	206	63.98	63	8.13	161	71.88
15**	F ₂	52	15.90	275	84.10	14	7.00	186	93.00
Total**		342	26.47	950	73.53	102	16.19	528	83.81

*** Stocked/ recovered ratios of transgenic and control were different (P<0.05, P < 0.01, respectively).

Table 4 Mean body weight (g) of F₂ transgenic channel catfish, *Ictalurus punctatus* containing either RSVLTR-rtGH₁ cDNA or RSVLTR-csGH cDNA and non-transgenic channel catfish recovered from 0.04-ha earthen ponds stocked with green sunfish, *Lepomis cyanellus*

Mean ± S.D.		
Trial	Transgenic	Non-transgenic
1 ^{NS}	0.37 ± 0.07	0.35 ± 0.08
6 ^{NS}	0.78 ± 0.36	0.85 ± 0.30
7 ^{NS}	0.55 ± 0.06	0.62 ± 0.11

^{NS} Transgenic and non-transgenic channel catfish mean weights were not different (t-test, P>0.01).

Table 5 Mean body weight (g) $\pm$ standard deviation and coefficient of variation (CV) of F₂ transgenic channel catfish, *Ictalurus punctatus* containing salmonid growth hormone gene and non-transgenic channel catfish stocked at 8,000 /ha with 425 largemouth bass, *Micropterus salmoides* per ha in 0.04-ha earthen ponds and provided only natural food.

trial	stocked		recovered	
	transgenic	non-transgenic	transgenic	non-transgenic
12	3.63 $\pm$ 0.46 (12.67)	3.61 $\pm$ 0.44 (12.19)	8.72 $\pm$ 3.33 (38.19)	9.02 $\pm$ 2.57 (28.49)
13**	4.11 $\pm$ 0.88 (21.41)	3.45 $\pm$ 0.58 (16.81)	13.48 $\pm$ 5.93 (43.99)	12.51 $\pm$ 3.28 (26.22)
14	3.92 $\pm$ 0.62 (15.82)	3.70 $\pm$ 0.54 (14.59)	10.21 $\pm$ 2.75 (26.93)	10.60 $\pm$ 3.14 (29.62)
15*	4.31 $\pm$ 0.86 (19.95)	3.75 $\pm$ 0.57 (15.20)	14.09 $\pm$ 3.88 (27.54)	15.25 $\pm$ 4.12 (27.01)

*, ** Transgenic and non-transgenic channel catfish means of stocked fish were significantly different (P<0.05 and P<0.01, respectively).

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ISRAEL

(1.) Construction of vectors for microinjection

In order to develop all-fish expression vectors for microinjection into fertilized eggs, we have prepared two constructs: rainbow trout metallothionein-a and common carp growth hormone cDNA (ptMTa-cGHcDNA), and carp β -actin common carp GH cDNA (pc β A-cGHcDNA). The inducible metallothionein-a promoter was cloned from rainbow trout, and the constitutive promoter was cloned by using the PCR technique, which contains 430 bp. The basic plasmids used were pBR322, PGEM-3Z and PUC18.

For the β -actin carp GH vector the construct 6 of Liu et al., 1990 was the source for the carp β -actin maximal promoter (Moav et al., 1992; Moav et al., 1993). The construct was sequentially cut with KpnI (the site is located between the β -actin promoter and the CAT gene), blunt-ended by removing the protruding 3'termini with T4 DNA polymerase, and digested with EcoRI to remove the CAT gene. It was then dephosphorylated and ligated to the 1.2 kb piece which was cut with EcoRI from the pBRcGH. The clones with the correct orientation were chosen by restriction analysis with HindIII and EcoRV. In the correct clone the combination of digestion by the two restriction enzymes resulted in 1.7 kb., 1.0 kb and 0.6 kb fragments.

For construction of the vector with the metallothionein-a promoter, the ptMT-a-scGH (rainbow trout metallothionein-a silver carp growth hormone) was used. The 5.9 kb plasmid was cut with EcoRI, dephosphorylated and then ligated to the 1.2 kb piece which was cut with EcoRI from the pBRcGH. The clones with the correct orientation were chosen by restriction analysis with HindIII. The correct clones were further verified by sequencing of 400 bp obtained by cutting with HindIII and XbaI; 300 bp were from the cGH and 100 bp from the metallothionein-a

promoter. Similarly, the promoters OnMT and OnH3 were cut from the original Devlin's constructs and ligated to the carp GH cDNA (obtained from V. Daniel, Koren et al., 1989).

Sparus aurata IGF-1 cDNA was obtained from Funkenstein at the IOLR and ligated to the rtMT-a promoter described above. The plasmid containing carp MT promoter fused to carp IGF-1 (Liang et al., 1996) was obtained from King M. Chan from CUHK.

(2.) Microinjection

Microinjection of carp zygotes took place in the two cooperating laboratories: in the Zoology Dept., Tel Aviv University (B. Moav) and in IOLR, Haifa (B. Z. Cavari). Once a week the Gan Shmuel Fish Breeding Centre (Dr. S. Rothbard) provided eggs and sperm of Dor70 selected carp or the Israeli commercial common mate, the "Yugoslavian" carp. The eggs and sperm were shipped immediately to the two laboratories and microinjection proceeded for 5-6 hours. Several hundred microinjected fingerlings were being grown in glass aquaria and plastic containers, to reach the size of 20-30 g - for individual marking and screening.

The following constructs were microinjected in Tel Aviv:

- 1) carp β -actin/ rtIGF-1 cDNA (from T. T. Chen)
- 2) carp β -actin/carp GH cDNA (from V. Daniel)
- 3) pOnMT/GH1 sockeye salmon construct (from R. Devlin)
- 4) pOnH3/GH1 sockeye salmon construct (from R. Devlin)
- 5) pOnH3/carp GH cDNA
- 6) pOnMT/carp GH cDNA
- 7) carpMT/carp IGF-1 cDNA (from K. M. Chan)

The following constructs were microinjected at the IOLR, Haifa:

- 1) rtMT-a/carp GH cDNA.
- 2) carp β -actin/carp GH cDNA.
- 3) rtMT-a/Sparus aurata IGF-1 cDNA

(3.) NLS

In some of the 1996 microinjections we used the SV40 large T antigen nuclear localization sequence (NLS; CGGPKKKRKVG-NH₂) peptide complexed to the plasmids in order to facilitate transfer and expression of the transgene into the embryo nuclei. This was done in a molar ratio of 100:1 (NLS:DNA), according to Collas et al., 1996; 1997.

A variety of constructs in various concentrations, linear and supercoiled, were microinjected in the presence of various concentrations of the NLS, at the Tel-Aviv University and at the IOLR. Comparative results from these experiments is still not conclusive and we could not make a statement whether the NLS treatment increased the uptake and/or integration of transgene into the carp genome. On the other hand, comparative studies on transient expression of a reporter gene construct (Luciferase gene) indicated that only at relatively low concentrations of the plasmid, two orders of magnitude less than the standard concentration of plasmid used for microinjection, the NLS treated plasmid have a comparatively better transient expression rate. However, the total expression levels under the standard conditions (without NLS) were significantly higher.

(4.) Radioimmunoassay for GH in blood samples

The levels of growth hormone in the plasma of the F_0 generation were determined by RIA. The fish were sampled when tagged and again on three more occasions during the year in order to eliminate any seasonal fluctuations. At the Tel-Aviv University the levels of GH in most of the fish were 0-2 ng/ml with very few exceptions that appear to be a seasonal effect. At the IOLR results of RIA ranged between 0.8-15.2 ng/ml. No correlation was found between levels of cGH and the transgenic status of the fish or its weight. It is important to note in regard to these results that only 13.5% of the fish were found to be transgenic and only a part of the fish were transgenic in their blood. Thus only the levels of GH in the fully transgenic F_1 would be significant for assessing the expression of the transgene.

(5.) Selection of transgenic fish (present status)

A. Tel-Aviv University

pOnMT/GH1 - 33 adult fish, average weight 400 g. All screened - No transgenic fish were found.

pOnH3/GH1 - 43 adult fish, average weight 400 g. All screened - 1 transgenic fish was found in sperm DNA.

carp β -actin/carp GH cDNA - 250 adult fish, average weight 300-800 g. 25% screened - 2 transgenic females (in caudal fin DNA) and 1 transgenic male (in sperm DNA) were found.

pOnH3/carp GH cDNA - 212 adult fish, average weight 500 g - 72 males screened, 14 F_0 transgenic in sperm. 13 crossed with a non-transgenic female. 13

F₁ groups in a screening process. Sperm of positive F₀ is frozen for further use to avoid loss of transgenic lines.

OnMT/carp GH cDNA - 64 adult fish, average weight 850 g. All were screened. 6 females were found positive in caudal fin DNA.

carp MT/carp IGF-1 cDNA - 172 adult fish, average weight 330 g., not screened yet. **β-actin/csGH constructs** (FV-1 and FV-2 promoters and chinook salmon cDNA) - Moav et al., 1995 and Hinitz et al., 1997. Transgenic fish were obtained during a previous BARD project #US-1517-88. 13 adult F₀ transgenic males and females and their F₁ offspring.

B. IOLR

All fish microinjected prior to February 1997 were lost due to massive predation by Kormorans at the Dor Experimental Station. Among the lost fish about 50 putative transgenic fish that were selected by PCR and Southern blots of fin DNA.

rtMT-a/carp GH cDNA - 380 adult fish, average weight 250 g., PCR screening in process.

rtMT-a/Sparus aurata IGF-1 cDNA - 100 adult fish, average weight 350 g., PCR screening in process.

(6.) Large scale growth performance of transgenic common carp

Summer 1994 experiment

In a previous BARD project (#US-1517-88; Moav et al., 1995) we injected the two “all-fish” gene constructs FV-1/csGH and FV-2/csGH into carp zygotes. A few of these transgenic F₀ carps were used to establish new F₁ generations. These F₁ generations had

different percentage of transgenic individuals due to different degrees of mosaicism in the F_0 transgenic parents. The sexually mature F_1 carp were screened by PCR in order to find the non-mosaic individuals suitable to establish a new F_2 generation in order to conduct a large scale growth performance experiment. At onset of the experiment we had only male carp that carried the FV-1/csGH construct. Three of these transgenic individuals originated from different integration events: #90522, #77E22 and #30066 were crossed with a Dor 70 female carp. As a control, a Yugoslavian male carp was crossed with a Dor 70 female carp. This crossbred carp is the commercial carp variant in Israel and was found to have better growth and improved qualities as compared to other carps (Wohlfarth and Moav, 1993). Each of the four progeny were stocked in separate earthen ponds of 400 m² at the Aquaculture Research Center in Dor, Israel. After one month of nursing in separate ponds, the fish were marked and co-stocked in four identical ponds for communal testing. Each pond was stocked as follows:

29 fish from crossing transgenic male #90522 with Dor 70 female.

41 fish from crossing transgenic male #77E22 with Dor 70 female.

60 fish from crossing transgenic male #30066 with Dor 70 female.

60 fish from crossing Yugoslavian male with Dor 70 female.

The average weights of each transgenic family from all four communal ponds during the experiment, average weight gain and % SGR/day are detailed in Table 1A and Fig. 1. The correction of observed weight gains for the differences in initial weights (including the calculation of the correction factor b , is detailed in Table 1B. After correcting the weight gains no significant difference in growth rate was found between the transgenic groups and the control.

Winter 1997 experiment

DNA screening of the F₀ generation

Ninety four of the F₀ generation injected with FV-2/cGH attained a size that allowed individual tagging with PIT tags. They were weighed and sampled (fin clips and blood were collected from all fish and sperm collected from the males). Only 59 of these had survived by the time PCR screening was performed. Of these fish, only 8 were found to contain the transgene (PCR positive) in one of the tissues tested (13.5%) and only one transgenic male was found to contain the transgene in its sperm and was therefore fit to establish the next transgenic generation (see Table 2).

Radioimmunoassay for GH in plasma

The levels of carp growth hormone in the plasma of the F₀ carps injected with FV-2/cGH were determined by RIA. The fish were sampled when tagged and on three additional occasions during the year in order to eliminate any seasonal fluctuations. The levels of GH in most of the fish were 0-2 ng/ml with very few exceptions of higher levels.

Communal testing experiments

The winter experiment tested three progenies. The first transgenic group was the progeny of the F₀ male carp carrying the FV-2/cGH transgene in its sperm (#76874) crossed with a Dor 70 female carp. The second transgenic group was the progeny of F₁ carp carrying the FV-1/csGH construct (#26E1A), as in the summer experiment, crossed with the same Dor 70 female carp. The control non-transgenic group was the progeny of Yugoslavian male carp also crossed with the same Dor 70 female carp. The three progenies were transferred to earthen ponds of 400 m² in the Aquaculture Research Center in Dor in mid October.

After six weeks of nursing in the separate ponds, the fish were marked and co-stocked in one communal pond for the communal testing (the number of fish in each group was too small for pond repetitions). The pond was stocked as follows:

51 fish from crossing transgenic male #76874 with Dor 70 female.

80 fish from crossing transgenic male #26E1A with Dor 70 female.

80 fish from crossing Yugoslavian male with Dor 70 female.

The procedure for weighing the fish during the experiment is described in materials and methods. The average weight of each transgenic family during the experiment was calculated and is given in Table 3 and Fig. 2. Comparison between the observed weight gains of the tested groups (without the need for correction, Wohlfarth and Moav, 1985) reveals a higher growth rate for the two transgenic groups as compared with the control group.

Table 1

a) Weights of transgenic carp during Summer experiment.

Average weight (g) of each group in the summer growth experiment as a function of days from co-stocking in communal ponds, average weight gain (g) and %SGR/day. The data is a mean of all four replica ponds.

^aObserved weight gains were compared by t-test. 90522xDor 70 was found significantly higher than the control group ($p = 0.026$). 77E22xDor 70 ($p = 0.128$) and 30066xDor 70 ($p = 0.125$) were not significant.

Days of experiment	Group			
	90522xDor70	77E22xDor70	30066xDor70	YugxDor70 (control)
0	91.5(±1.8)	104.2(±2.6)	69.9(±2.4)	51.9(±2.5)
13	165.9(±7.8)	181.1(±10.4)	153.9(±19.2)	123.6(±12.7)
27	259.4(±22.5)	255.3(±18.7)	229.7(±14.3)	169.9(±6.9)
39	334.6(±28.1)	323.8(±30.0)	284.1(±6.9)	235.6(±4.6)
53	392.5(±25.2)	395.4(±33.4)	359.5(±17.6)	299.2(±16.8)
67	459.1(±32.9)	481.0(±36.0)	441.1(±30.0)	349.3(±26.8)
80	546.1(±40.9)	520.8(±34.1)	480.4(±23.4)	430.1(±18.7)
Average	454.6(±41.2) ^{*(a)}	416.6(±33.0) ^(a)	410.5(±25.5) ^(a)	378.2(±18.3) ^(a)
weight gain				
%SGR/day	2.2	2.0	2.4	2.6

Table 1 (Continued)

b) Correction of the weight gain due to differences in the initial weight.

^aCorrected weight gains were compared by t-test. 90522xDor 70 ($p = 0.686$), 77E22xDor 70 ($p = 0.900$) and 30066xDor 70 ($p = 0.064$) were not significant compared to the control group (YugoslavianxDor 70).

$$Y' = Y - b(X - X.)$$

Y' = Corrected weight gain

Y = Observed weight gain (final weight-initial weight)

b = Correction factor

X = Initial weight of each genetic group

$X.$ = Mean initial weight of all groups

$Y.$ = Mean weight gain of all groups

Y = Mean observed weight gain of all groups

Prediction equation (according to Wohlfarth and Milstein, 1987):

$$b = 4.543 - 0.113 * X. + 0.00671 * Y.$$

$$X. = 79.4; Y. = 494.3; Y. + 494.3 - 79.4 = 414.9$$

$$b = -1.645$$

Genetic group	Initial weight	Deviation from average initial weight	Correction for the weight gain	Weight gain	Corrected weight gain
	X	$d = (X - X.)$	$-b * d$	Y	$Y' = Y - b * d$
90522	91.5	12.1	-19.9	454.6	434.7 ^(a)
77E22	104.2	24.8	-40.7	416.6	375.9 ^(a)
30066	69.9	-9.5	15.6	410.5	426.1 ^(a)
control	51.9	-27.5	45.3	378.2	423.5 ^(a)

Table 2

PCR screening for F₀ carp injected with FV-2/cGH

Results of PCR screening in different tissue samples of F₀ carp injected with FV-2/cGH. Data shown refers only to the fish found positive.

Fish Code	Sex	PCR Result for fin DNA	PCR Result for blood DNA	PCR Result for sperm DNA
27260	Female	+	+	
27270	Female	+	-	
30577	Female	+	+	
3092F	Male	+	+	-
76874	Male	-	-	+
77D2E	Male	+	-	-
83C5E	Male	+	-	-
8486B	Female	+	not tested	

Table 3

Weights of transgenic carp during Winter experiment.

Average weight (g) of each group in the winter growth experiment as a function of days from co-stocking in communal pond, average weight gain (g) and %SGR/day for the entire experimental period (96 days) and for the period until the second weighing (63 days).

^aGrowth rates were compared by F-test for difference between regression coefficients. Groups 76874xDor 70 and 26E1 AxDor 70 were each found significant ($p < 0.001$) when compared to the control group (YugoslavianxDor 70).

Days of experiment	Group		
	26E1AxDor70	76874xDor70	YugxDor70 (control)
0	16.7±14.8	18.6±11.8	21.8±17.4
29	24.3±20.7	24.2±23.4	21.9±21.1
63	35.4±40.2	32.2±34.1	25.4±16.2
96	44.5±45.5	53.4±58.6	31.3±22.3
Average weight gain	27.8 ^{***(a)}	34.8 ^{***(a)}	9.5
%SGR/day (63 days only)	1.0	0.9	0.4
%SGR/day (all 96 days)	1.2	1.1	0.2

Fig. 1
Growth performance of transgenic carp - Summer experiment
Average weight (g) per fish during the experiment (mean of all the four ponds).

Fig. 2
 Growth performance of transgenic carp - Winter experiment.
 Average weight (g) per fish during the winter growth experiment.

(7.) References

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Evaluation of the Research Achievements

The primary research objectives were achieved of demonstrating that gene transfer combined with selective breeding maximizes genetic improvement and makes transgenic response more uniform. Environmental risk assessment indicated transgenic channel catfish probably pose no ecological risk. Secondary objectives were also achieved.

Description of the Cooperation

Gene constructs were exchanged. Research plans were jointly planned. Results were discussed. Future collaboration and exchanges were planned.

Conclusion

Results clearly demonstrated that catfish and carp production can be increased by utilizing genetically enhanced fish developed by gene transfer and selective breeding.

List of Publications

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