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TECHNICAL NOTE

Short Term Ration Restriction and Re-Alimentation: Effect on Compensatory Growth, Body Composition and Insulin Like Growth Factor Gene Expression in *Cyprinus carpio*

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Abstract

Advanced fry of *Cyprinus carpio* (mean weight 1.73±0.02g) were fed at 10%, 25%, 50% and 75% of satiation as different treatment groups and one control group (6 weeks) for first phase (restriction feeding) and a second phase (8 weeks) of satiation feeding for all the treatments. At the end of first phase, there was significant difference in mean weight among the treatment groups (P<0.05) but, after second phase (8 weeks), treatment group fed at 75% of satiation obtained significantly higher body weight than control, while 50% satiation fed group obtained similar weight as of control. FCR was found to be better at moderately restricted fed group (50% and 75% satiation). Crude protein and crude lipid content decreased in severely restricted groups (10% and 25% of satiation fed), compared to others after 14 weeks. IGF-I and IGF-2a gene expression in the liver was observed as down regulated during ration restriction and up regulated after re alimentation. However, after re-alimentation for 8 weeks, IGF-2a gene expression was recorded to be increasing and was higher than control. The study concludes that moderate levels of ration restriction (50% to 75% of satiation levels) is sufficient for effective growth and feed utilization.

Introduction

Feeding costs in aquaculture typically accounts for 60 % of production cost. Different means of cost effective feeding along with water quality maintenance through reduced organic load to the system is the major challenge in modern aquaculture. One of the simplest and widely adopted practices in aquaculture is restricting the feed for a short period of time for cultured animals. The amount of feed given to fish is drastically reduced for days to months before the fish are re-fed to satiation levels (Robb, 2008; Jobling, 2012). By imposing ration restrictions, it will not only reduce the operating costs, but also reduce the organic load in to the culture system (Abdel-Hakim, Abo State, Al-Azab and El-Kholy, 2009; Jobling, 2012).

Fasting or ration restriction may be applied in commercial fish farming to induce growth compensation. Imposing ration restrictions naturally suppress fish growth for the entire period of restricted feeding. But once the restrictions are over and the fishes are re-fed at satiation levels, they have a remarkable ability to bounce back via compensatory and/or catch-up growth responses (Hayward, Noltie and Wang, 1997; Ali, Nicieza and Wootton, 2003; Weirich, Groat, Reigh, Chesney and Malone, 2006; Jobling, 2009). The degree of compensatory growth depends on species, duration of starvation, levels of food deprivation, different body

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size and different experimental protocols (Ali *et al.,* 2003; Foss, *et al.,* 2009).

Fish growth is controlled by the growth hormone (GH) / insulin-like growth factor-I (IGF-I) axis and muscle growth rate which depends on feeding regimes or environmental conditions (Johnston, 2001b; Wood, Duan, and Bern*,* 2005). Under anabolic conditions growth hormone stimulates the production and subsequent release of insulin-like growth factor-I into the circulation and IGF-I act on target tissues to proliferate and differentiate and will lead to body growth of the fishes (Wood *et al.,* 2005). Growth hormone also act directly on target tissues such as skeletal muscle to stimulate IGF-I production to promote tissue growth. It is widely accepted that fasting or reduced feeding decreases concentrations of IGF-I in channel catfish (*Ictalurus punctatus*) and other teleosts (Peterson and Waldbieser, 2009), whereas the effects of nutritional status on IGF-II are less defined. The purpose of the present study was to investigate the influence of different degree of restricted feeding on compensatory growth, body composition, IGF-I and IGF-2a gene expression in *Cyprinus carpio* (Linnaeus, 1758).

Materials and Methods

Animals and Experimental Design

The experiment was carried out at the wet laboratory of Aquaculture division, CIFE, Mumbai. The advanced fry of *Cyprinus carpio* (1.55±0.03g), were procured from Maa Tara packing centre, Bijoynagar, Naihati, 24-Pgs (North), West Bengal, India and were acclimatized to the experimental conditions for one month. Experiment was carried out in two phases; first phase (6 weeks) of ration restriction at various ration levels and second phase (8 weeks) of feeding up to satiation levels.

Fishes (n=20) were stocked in 15 circular tanks (300L) and were aerated well. The treatment groups T1, T2, T3 and T4 were fed at the rate of 10%, 25%, 50% and 75%, respectively of the satiation level of controls respectively for the first 6 weeks and the fishes of all the treatment groups were fed to the satiation level for the next 8 weeks. The control fishes were fed up to the satiation level daily and was offered in two equal portions once at morning (0100 h) and another at evening (1700 h) to ensure optimum feed intake and to minimize wastage of feed. The control group fishes were fed @ 5% body weight and later on they were fed at the level of apparent satiation. The amount of feed given to various treatment groups was adjusted by daily evaluation of satiation feeding for control.

The uneaten feed was collected after 60 min of feeding and then oven-dried at 70°C. The amount of uneaten feed was reduced during calculating FCR. The diet was formulated with 35% crude protein containing fish meal (27.65%), soybean meal (15.75%), groundnut oil cake (17%), rice powder (9%), wheat flour (16%), corn flour (8%), vegetable oil (5%), vitamin mineral premix (1%) and BHT (0.6%) (Table 1).

The water quality parameters like temperature (23.4- 28.1°C), pH (7.6-8.1), dissolved Oxygen (6.6-7.3mgL^{-1}), alkalinity (96-120 mgL^{-1}), Ammonia-Nitrogen (0.04-0.09mgL^{-1}), Nitrite- Nitrogen (0.002-0.005 mgL^{-1}) were recorded at biweekly intervals following the standard methods (APHA,1998). The water quality was maintained by regular siphoning of faecal matter, water exchange and aeration.

Sampling and Analysis

Sampling was carried out at bi-weekly intervals to assess the body weight of the fishes. Fishes were starved overnight and weighed in an electronic balance. Five fishes were taken from each tank for analysing the

Table 1. Ingredient and proximate composition of the experimental diet

Ingredient	% Inclusion
Fish meal	27.65%
Soybean meal	15.75%
Groundnut oil cake	17%
Rice powder	9%
Wheat flour	16%
Corn flour	8%
Vegetable oil	5%
Vitamin mineral premix	1%
BHT	0.6%
Proximate composition %	
Moisture	9.88%
Protein	34.27%
Lipid	7.70%
Ash	9.50%

*****Diet was formulated using software Win Feed Version 2.8.

growth of fishes. The growth performance was measured as final body weight and Feed conversion ratio (FCR): feed given (dw)/weight gain (ww) where, dw- dry weight, ww- wet weight. FCR was analysed only after second phase of experiment. IGF-I and IGF2a gene expression were measured after both the phases of the experiment.

Body Composition

At the end of second phase (14 weeks), three fishes were collected randomly from each of the experimental tank and the biomass of the fishes was noted down. Samples were stored at -20^{0}C and protein, lipid and ash contents were measured using prescribed method (AOAC, 1995). Moisture content was analysed by drying the samples to constant weight at 100-105^{0}C, crude protein (CP) by Kjeltec semi-automatic system (2200 Kjeltec Auto Distillation, Foss Tecator, Sweden) and ether extract by Soxhlet Apparatus using petroleum ether (Boiling point 40-60^{0}C) as solvent. Total carbohydrate was calculated by difference as 100-(moisture + Crude protein + Lipid + ash).

Quantification of IGF-I and IGF2a gene expression by qRT- PCR

Two fishes were randomly taken from each experiment after completion of each phase of the experiment (first phase 6 weeks and second phase 8 weeks) and were anesthetized using clove oil. Liver tissue was collected from these fishes and preserved in Qiagen's RNA later RNA stabilizing reagent (10µl 100 mg tissue^{-1}) and stored under -80^{0}C until RNA extraction.

RNA Isolation and Processing

Total RNA was isolated using Trizol® method (Sambrook and Russell, 2001). RNA was quantified by using Nanodrop (Thermo scientific, USA) with DEPC treated water or nuclease free water as blank and measured concentration in ng µl^{-1}. The purity of RNA was also checked at 1% agarose gel.

Total RNA was purified by treating with DNAse enzyme. The purification reaction mixture was prepared for a volume of 10µl by adding 1ug of total RNA, 1ug of buffer (10X), 1ul DNAse enzyme and nuclease free water. The reaction mixture was incubated at 37^{0}C for 30 min followed by addition of 2µl of 20mM EDTA. Later the reaction was terminated by incubating at 65^{0}C for 10min. The purified total RNA was used for the subsequent cDNA synthesis.

Complementary DNA (cDNA) Synthesis

The mRNA from DNAse treated total RNA pool was reverse transcribed to first strand cDNA using Maxima First Strand cDNA synthesis Kit RT-qPCR (Thermo Scientific, USA). Briefly, 1µg of total RNA, 2µl 5x reaction mix (2µl) and about 1µl maxima enzyme mix were taken in 0.5ml microfuge tube and total reaction mixture volume was made up to 10µl with nuclease free water. The tube was incubated in PCR machine for 10 min at 25°C followed by 15 min at 50°C. The reaction was terminated at 85°C for 5min.

Specific primers were designed for Insulin like Growth Factor-I (IGF-I), Insulin like Growth Factor 2a (IGF2a) and one housekeeping gene (GAPDH) for real Time PCR (Table 2).

Real Time PCR

Real Time PCR amplifications were conducted in Roche Light Cycler Real Time PCR detection system (Roche system). The 12.5µl of reaction mixture volume consist of 12.5µl of Maxima^{TM} SYBR Green qPCR Master mix (Thermo scientific, USA), 0.5µl of (0.3µM) each gene specific primer and 2µl (20ng) of cDNA. The default thermal profile was used for amplification and it consisted of initial denaturation at 95 ºC for 10 min followed by 40 cycles of denaturation at 95 ºC for 15 sec, annealing and extension at 60º C for 1 min. Melting curve analysis of amplification products were performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Comparative C_T method was used to estimate the relative expression of mRNA. The ΔC_T was calculated by subtracting C_T value of internal control from target gene and then mean ΔC_T was calculated from this normalized ΔC_T value. $\Delta\Delta C_T$ was calculated with respect to control by subtracting mean C_T of control treatment from mean ΔC_T of target gene. Fold change at various ration restriction and re-alimentation periods were calculated by $2^{-\Delta\Delta C_T}$. The house keeping gene, glyceraldehyde 3- phosphate dehydrogenase was utilized as an internal control for normalization (Su. *et al*., 2008).

Statistical Analysis

Difference between feeding treatments in terms of mean weight, FCR and IGF-I and IGF2a gene expression were tested with one-way analysis of variance (ANOVA). Significant differences between treatment means ($P<0.05$) were determined by Duncan's multiple range test (Duncan1955) using PROC GLM procedure (SAS 9.3)

Results

Initial mean weight of the fish did not vary significantly among the treatment groups (Table 3). At the end of first phase of the experiment, highest mean weight was observed in control followed by T4 (75% satiation fed). At the end of second phase, treatment T4 (75% satiation fed) attained significantly higher weight gain than control group and T3 (50% satiation fed group) obtained significantly similar weight as of control ($P<0.05$).

The better FCR was found in T3 (50% satiation fed) compared to other treatment groups but severely ration

Table 2. Primers used in the gene expression studies

Gene	Primers	Primer sequence	Annealing Temperature (ºC)	Amplicon size	Gene bank accession no
	IGFI-F	AACGGCACACAGACAGTCCCAG	58.6	157	D83271.1
IGF-I	IGFI-R	TCTCTCTCAGCCATTCGCCCTAC	58.5	157	D83271.1
	IGF2a-F	ATCAAACAGCCGCCGTCCTCAG	62.1	109	HM641129.1
IGF2a	IGF2a-R	TCGCTCGGACTTCACAGGCTTTG	62.3	109	HM641129.1
	GAPDH-F	TGATTCATTCTGCGTACCTCTGGG	59	126	JX244278.1
GAPDH	GAPDH-R	CAGCCGCAGCCTTAACCACTTTC	60.9	126	JX244278.1

Table 3. Effect of ration restriction (6th week) and re-alimentation (8th week) on body weight of *C. Carpio* after restricted feeding phase (first phase) and satiation feeding phase (second phase)

Parameters	Controls	Treatments			
		T1	T2	T3	T4
First phase (n=20)					
Initial body weight	1.72±0.09^{a}	1.74±0.08^{a}	1.75±0.10^{a}	1.73±0.08^{a}	1.73±0.09^{a}
Final body weight	3.13±0.15^{a}	1.41±0.06^{d}	1.75±0.07^{d}	2.15±0.14^{c}	2.70±0.18^{b}
Second phase (n=16)					
Initial body weight	3.13±0.03^{a}	1.40±0.21^{d}	1.75±0.19^{d}	2.14±0.29^{c}	2.70±0.07^{b}
Final body weight	4.33±0.27^{a}	2.47±0.24^{c}	3.32±0.25^{b}	3.74±0.25^{ab}	4.41±0.31^{a}
Feed conversion ratio (FCR)	2.10±0.04^{a}	1.98±0.10^{a}	1.74±0.04^{b}	1.67±0.01^{b}	1.69±0.05^{b}

The different superscripts in the same row indicate significant difference in mean values among the treatment groups (P<0.05). Data are expressed as mean ± SE, (n=4). First phase, control: satiation level fed T1: 10% of satiation fed, T2: 25% of satiation fed, T3: 50% of satiation fed, T4: 75% of satiation fed. Second phase (n=3), all the treatment groups fed up to the satiation levels during re-alimentation phase (8th week).

restricted groups (T1 and T2) obtained higher FCR (2.1±0.04 and 1.98±0.10) (Table 3).

Percentage body compositions of different treatment groups at end of second phase of experiment are shown in Table 4. There was no significant difference in ash and total carbohydrate content among various treatment groups. Severely restricted groups (T1 and T2) recorded significantly lower content of crude protein and crude lipid. But there was significantly higher moisture content in severely restricted groups (T1 and T2) compared to others.

IGF-I and IGF2a gene expressions were observed as down regulated in all the treatment groups at the end of first phase (Figure 1). IGF-I gene expression was significantly lower in treatment group T1 (10% satiation fed) followed by T2 compared to control. But, after second phase, there was no significant difference in IGF-I gene expression levels in T2, T3 and T4 (25%, 50% and 75% satiation fed respectively).

IGF-2a gene expression level was significantly similar in control and T1 (10% satiation fed) at end of first phase. However, after second phase, all the treatment groups obtained drastic increase in IGF-2a gene expression level and the highest expression was observed in T3 (50% satiation fed) and lowest was in T1 (10% satiation fed).

Discussions

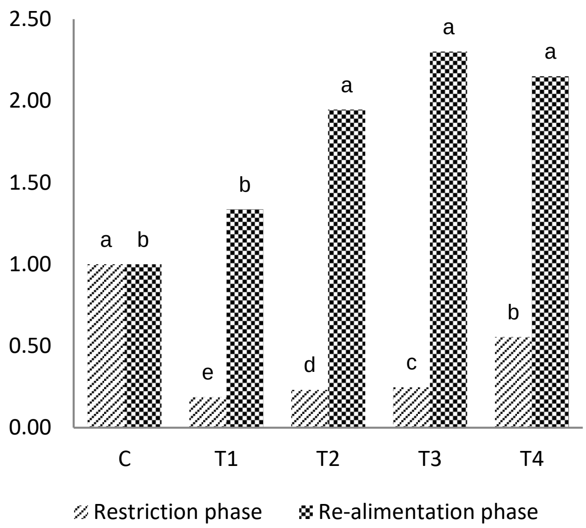
Inducing compensatory growth in fishes has the possibility of improving fish growth, feed utilization and reduced feed wastage in modern aquaculture (Johnston, Ritar and Thomas, 2004; Berrill, Porter and Bromage, 2006). The rate of fish growth depends on feeding regimes and environmental factors (Peres and Olive-Teles, 2005). In the present study, at the end of first phase, all the treatment groups recorded lower weight compared to control as supported by Peres and Olive-Teles, (2005), who reported that growth rate is linearly correlated with food intake. In the present study, severe restriction at 10% satiation had caused lowering of body weight from weight at stocking. While, moderately restricted (50-75% satiation fed) groups registered an increase in body weight after first phase, which was significantly lower than control.

In the present study, at the end of second phase, moderately restricted groups (50% and 75% satiation fed) attained significantly similar body weight as of control (satiation fed). The result indicates that the feeding can be efficiently restricted upto 50% of satiation for a period of 6 weeks to trigger compensatory growth in common carp fry. On the other hand, severe restrictions will lead to partial compensation as indicated by lower body weight obtained by T1 and T2 (10% and 25% satiation fed). Tian and Qin, (2004) observed that a moderate feed restriction (50% or 75%of satiation) for 2 weeks could fully compensate, while 0% or 25% satiation for 2 weeks did not help full compensation in Barramundi (*Latescalcarifer*). Srijila, Rani, Babu& Tiwari, (2014) also observed that moderate feed restriction (50% - 75%) for

Table 4. Effect of ration restriction (6th week) and re-alimentation (8th week) on body composition of *C. carpio* after 14th week (wet weight basis)

Treatment	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)	Total carbohydrate (%)
C	71.00±0.80^{b}	17.47±0.51^{a}	3.22±0.23^{a}	2.61±0.12^{a}	5.51±0.17^{a}
T1	74.04±0.40^{a}	15.26±0.39^{b}	2.49±0.08^{c}	2.76±0.13^{a}	6.06±0.17^{a}
T2	72.86±0.15^{ab}	15.65±0.17^{b}	2.66±0.05^{bc}	2.71±0.06^{a}	6.01±0.13^{a}
T3	71.42±0.76^{b}	17.01±0.38^{a}	2.99±0.10^{ab}	2.57±0.15^{a}	6.00±0.29^{a}
T4	71.22±0.65^{b}	17.48±0.32^{a}	3.24±0.03^{a}	2.56±0.09^{a}	5.48±0.22^{a}

Means with different superscripts in the same column are significantly different among treatment groups (P<0.05). Data expressed as Mean ± SE, (n=4). Values in percentages were arsine transformed and analysed. First phase, control: satiation level fed T1: 10% of satiation fed, T2: 25% of satiation fed, T3: 50% of satiation fed, T4: 75% of satiation fed. Second phase, all the treatment groups fed up to the satiation levels during re-alimentation phase (8th week).



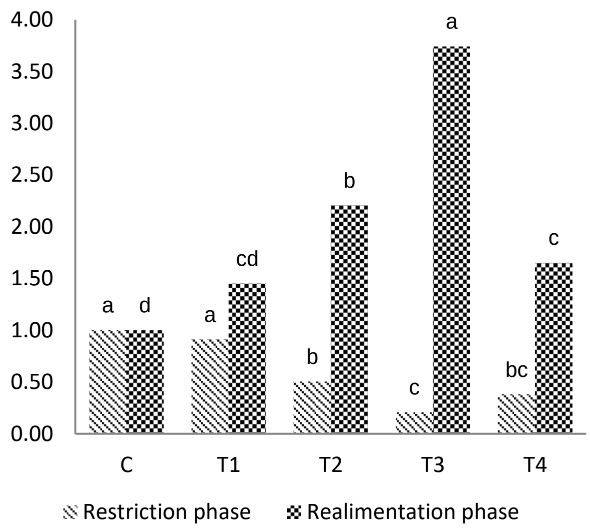


Figure 1. Relative IGF-I (left) and IGF2a (right) gene expression by Real Time PCR during ration restriction (6th week) and re-alimentation phases (8th week). The different superscripts in the same column are significant difference in mean values among different treatment groups (P<0.05). Date expressed as mean ± S.E, (n=4). Control fish fed to satiation levels though out the experiment, T1,T2,T3 and T4 treatment groups fed at the rate of 10%, 25% 50% and 75% of satiation, respectively during ration restriction (6th week) and all the treatment groups fed up to the satiation levels during re-alimentation phase (8th week).

a period of 6 weeks and realimentation helped to compensate the retarded growth in *Labeorohita.*

In the present study, improved FCR of 1.67 ± 0.01 and 1.69 ± 0.05 was registered in 50% and 75% of satiation fed group respectively. Moderate ration restriction led to improved FCR in *L. rohita* (Srijila*et al.,* 2014) and also there are reports on improved feed efficiency ratio in fishes which undergone compensatory growth (Van Dijk, Staaks and Hardewig, 2002). These findings can be attributed to increased efficiency in feed utilisation during growth compensation. Manipulating feeding regimes had improved feed efficiency of different fish species, reduced feed consumption and showed better growth effects (Johnston *et al*., 2004; Khan, Ahmed and Abidi, 2004; Berrill*et al*., 2006).

Body composition of fish is often used as an indicator of nutritional quality of the fish. In the present study, moisture, crude lipid, ash and total carbohydrate content were significantly affected by ration restriction at 10% and 25 % satiation level. Restricted feeding at moderate level (50-75% satiation fed) did not alter the body composition of the fishes as indicated by the present results. Hence it can be concluded that moderate restriction for a short term (6 weeks) will not alter the nutritional quality of fish flesh. Fasting or ration restriction generally leads to a reduction in crude lipid, crude protein content and increase in the moisture, ash and total carbohydrate content of fish tissues (Weatherley and Gill, 1987; Collins and Anderson, 1995). But according to the current results, the severe restriction for short term led to reduction in crude protein and crude lipid levels in the tissue.

Insulin-like growth factor I (IGF-I) is one of the chief anabolic agent responsible for tissue growth (Duan, 1998; Thissen, Underwood and Ketelslegers, 1999). In the present study, at the end of 1st phase, IGF-I gene expression had declined significantly with degree of restriction. But, those treatment groups restricted from 25-75% of satiation indicated significantly higher IGF-I gene expression after second phase in comparison to others. Reductions in liver IGF-I gene expression level due to fasting and subsequent recovery during re-feeding have been observed in many fishes (Gabillard, Kamangar and Montserrat, 2006a; Montserrat, Gabillard, Capilla, Navarro and Gutierrez, 2007a; Fox, Breves, Pierce, Hirano, Grau, 2010). It was previously reported that IGF-I gene expression levels decline in liver, after 2 to 4 weeks of fasting (Uchida *et al*.,2003; Fox, Riley, Hirano and Grau, 2006). Enhanced liver IGF-I expression can be related to the growth compensation in fishes and it varies with degree of feed restriction. Growth hormone acts directly on target tissue by stimulating mitosis and other energy metabolism and indirectly by initiating the production and release of IGF-I in the liver (Duan 1997; Wood *et al*.,2005). The reduction and recovery of IGF-I gene expression levels during ration restriction and re-alimentation in the present study indicated the importance of nutritional axis for regulation of growth in fish. Further, IGF genes play an important role in somatic growth, reproduction, osmoregulation and immune system (McCormick, 1996; Maestro, Mendez, Parrizas and Gutierrez, 1997; Norbeck, Kittilson and Sheridan, 2007; Yada, 2007). Similarly, Picha, Turano, Tipsmark and Borski, (2008b) reported that a decrease in plasma IGF-I is driven by a decrease in liver IGF-I production capacity due to a fasting-induced reduction in liver size. Therefore, a minimum intake of essential nutrients may be necessary to maintain liver IGF-I gene expression.

The present study indicated an elevated expression of IGF-2a gene in control compared to all the treatment groups which were fed restricted ration. But after re-feeding, all the feed restricted groups indicated higher expression of IGF-2a gene compared to control, of which 50% satiation fed groups recorded the highest expression. The highest IGF-2a gene expression obtained in 50% satiation fed group can be correlated with the higher rate of compensatory growth as evidenced in final weight gain. The present study supports the earlier finding (Yuan et al., 2011), which reported a reduced IGF-2a gene expression level in grass carp during starvation (6 days) and rebounding after re-feeding (6 days). Similarly, there is clear evidence that insulin-like growth factor II (IGF-II) is related to local paracrine/autocrine regulation of muscle tissue growth in teleost fishes (Vong, Chan and Cheng, 2003; Hevrøy*et al.* 2007). The present study emphasised nutritional effect on the IGF-I and IGF-2a gene expression levels in the liver of *Cyprinus carpio*. In other teleost fish species, the liver IGF-2 gene were also affected by nutritional status (Gabillard*et al.,* 2006; Ayson, de Jesus-Ayson and Takemura, 2007; Terova, *et al.,* 2007).

Conclusion

Compensatory growth after moderate ration restriction improves growth, feed utilization and feed conversion without affecting nutritional quality of the flesh. In Aquaculture management inducing compensatory growth improve the growth rates, feed utilisation and reduce feed wastage and the feed cost in the culture system. There is up regulation of expression of genes such as IGF-I and IGF-2a in fishes compensating for their restricted growth.

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