RESEARCH ARTICLE Indian Journal of Animal Research, Volume 57 Issue 7: 860-867 (July 2023)

Effect of Different Dietary Starch Inclusion Levels on Growth Performance, Glucose Metabolism, Histological Alterations and Modulation of Hepatic Gene Expressions in Genetically Improved *Labeo rohita* (Jayanti Rohu)

Kiran D. Rasal[1,2], Manohar Vasam[1], Sangita Dixit[1,3], Jitendra Kumar Sundaray[1], Avinash Rasal[1], Kanta Das Mahapatra[1], Lakshman Sahoo[1], Khuntia Murmu[1], Mir Asif Iquebal[4], Dinesh Kumar[4] **10.18805/IJAR.B-4937**

ABSTRACT

Background: Feed cost mainly constitutes 40-60% of total aquaculture production expenses, due to the inclusion of expensive animal-derived ingredients to the feed industry. Studies suggested that herbivore and omnivore fish species can consume higher level of carbohydrate (150-300 g/kg starch) as compared to carnivore fish. However, lower carbohydrate utilization in fishes is less understood till date. In the present work, It was attempted to decipher the physiological changes in genetically improved rohu (*Jayanti Rohu*) upon inclusion of different levels of starch in the feed regime.

Methods: Three isonitrogenous (24% crude protein), isolipidic (10% ether extract) experimental diets *viz*. TS20 (@20% starch), TS30 (@30% starch) and TS40 (@40% starch) were formulated and fed to *Jayanti Rohu* fingerlings (180 number with average weight of 45±3.5 g) twice daily up to satiation level for 45 days experimental period. The growth performance, nutrient utilization, histological changes and modulation of hepatic gene expressions were evaluated after completion of experiment.

Result: Results indicated significantly ($p<0.05$) increased weight gain percentage, SGR and condition factor in TS30 and TS40 as compared to TS20. The histoarchitecture of hepatic cells depicted greater cell damage with increasing levels of dietary carbohydrates. The glucose membrane transporter 2 gene and carbohydrate metabolism involved genes showed a higher expression in TS40 and TS30 compared to TS20. Therefore, the present study concluded that inclusion level of 30% starch as optimal level in the diet of genetically improved Jayanti rohu.

Key words: Carbohydrate metabolism, Glycogenesis, Glycolysis, Growth, Jayanti rohu (*Labeo rohita*).

INTRODUCTION

The contribution of aquaculture has been growing immensely to provide food security, nutrition and economic wellbeing across the globe (FAO, 2018). To address the growing fish demand for human consumption, intensification of aquaculture practices are the need of hour. However, there are several concerns, such as feed cost, diseases and climate change, which affect the sustainability of aquaculture production. Feed cost mainly constitutes 40-60% of total aquaculture production expenses, due to the inclusion of expensive animal-derived ingredients to the feed industry. Carbohydrates enhance fish growth using energy and supply for some amino acids and nucleic acids precursors (NRC, 2011). The growth and survival of aquaculture species can be affected due to incorporation of formulated feed regime as evidenced by several studies (Kamalam *et al*., 2017). However, lower carbohydrate utilization in fishes is less understood till date. Studies suggested that herbivore and omnivore fish species can consume higher levels of carbohydrate (150-300 g/kg starch) as compared to carnivore fish (Tan *et al*., 2009).

Farmed rohu, *Labeo rohita* is a vital carp species in South Asian continent and contributes considerably in the aquaculture production. Jayanti Rohu, a Genetically improved *L. rohita* variety developed by selective breeding at ICAR-Central Institute of Freshwater Aquaculture in Collaboration with Institute of Aquaculture, Research Norway, with 18% genetic gain after 8th generation (Mahapatra *et al*., 2016)

[1]ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar-751 002, Odisha, India.
[2]ICAR-Central Institute of Fisheries Education, Mumbai-400 061, Maharashtra, India.
[3]Siksha 'O' Anusandhan University, Bhubaneswar-751 002, Odisha, India.
[4]ICAR-Indian Agricultural Statistics Research Institute, New Delhi-110 012, India.

Corresponding Author: Jitendra Kumar Sundaray, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar-751 002, Odisha, India. Email: jsundaray@gmail.com

How to cite this article: Rasal, K.D., Vasam, M., Dixit, S., Sundaray, J.K., Rasal, A., Mahapatra, K.D., Sahoo, L., Murmu, K., Iquebal, M.A. and Kumar, D. (2023). Effect of Different Dietary Starch Inclusion Levels on Growth Performance, Glucose Metabolism, Histological Alterations and Modulation of Hepatic Gene Expressions in Genetically Improved *Labeo rohita* (Jayanti Rohu). Indian Journal of Animal Research. 57(7): 860-867. doi: 10.18805/IJAR.B-4937.

Submitted: 18-05-2022 **Accepted:** 09-03-2023 **Online:** 12-06-2023

(Rasal and Sundaray, 2020). In the present work, an attempt was made to decipher the physiological changes in genetically improved rohu (*Jayanti Rohu*) upon inclusion of different levels of starch in the feed regime by reducing the protein content in feed to reduce feed cost. The glucose tolerance level of rohu was examined using physiological as well as molecular approaches. In the present study, investigation was carried out to know the hepatic glucose metabolism in fingerlings of rohu and suggested to include optimal starch level in the diet for improving glucose utilization efficiency. This work will encourage and provide an important lead for undertaking research in other carp species of aquaculture industry for sustainable and economically viable fish production sytems.

MATERIALS AND METHODS

Experiment and sampling

A total of 180 fingerlings (45±3.5 g) of the genetically improved rohu, Jayanti rohu were collected from the nursery ponds of ICAR-Central Institute of Freshwater aquaculture, Bhubaneswar, Odisha, India (20°18′61.20″N, 85°85′72.53″ E) in 2018. The collected fingerlings were acclimatized at laboratory condition by maintaining in one communal cemented tank (1000 L) for seven days. Three isonitrogenous (24% crude protein), isolipidic (10% ether extract) experimental diets *viz*. TS20 (@ 20% starch), TS30 (@ 30% starch) and TS40 (@ 40% starch) were formulated and prepared (Table 1), where fish meal, casein were protein sources, gelatinized starch and cellulose provided carbohydrate and fish oil and vegetable oil were lipid sources. The experimental diets were subjected to proximate analysis (Table 1), according to AOAC (2006).

After acclimatization, 30 fingerlings were randomly distributed into six number of 1000 L cemented tanks keeping an experimental replicate for each treatment. The feeding experiment was conducted for 45 days and fish were fed twice a day (9:00 h and 16:00 h) with the formulated feed (@ 3% of body weight daily). The water quality parameters during the experiment were maintained, such as water temperature, 29.5±0.5°C, dissolved oxygen, 8.69±0.37 mg/L, pH 7-8 and ammonia respectively. After 0.17 completion of experiment, fish were harvested 0.17±0.05 mg/L and growth parameters (length and weight) were measured. The five fish for each treatment were randomly collected from either of the treatment replicate and were anaesthetized using MS-222 at 100 mg/L (Sigma) and sacrificed; liver tissues were collected and preserved in 10% NBF (neutral buffered formalin) and LN_2 (liquid nitrogen) for histology and RNA extraction, respectively.

Estimation of growth parameters and condition factor

At the end of feeding trial, fish from each treatment were measured and weighed for recording length and weight. Weight gain (%), specific growth rate (SGR) and condition factor were determined using length-weight data following below-mentioned equations.

$$\text{Weight gain (\%)} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{SGR (\% day}^{-1}\text{)} = \frac{\text{Ln final weight - Ln initial weight}}{\text{No. of days}} \times 100$$

$$\text{Condition factor} = \frac{\text{Weight in g}}{\text{Length}^3 \text{ in cm}} \times 100$$

Liver histological examination

The collected liver tissues were stored in 10% NBF (neutral buffered formalin) for histological examination. The fixed liver tissues were placed for dehydration in a graded ethanol series solution and further cleared with xylene. Further tissues were infiltrated and blocked in paraffin. Tissue sectioning was done using a microtome (Microm D-6900; GmbH) and tissue sections (5 mm thick) were stained with haematoxylin-eosin Y 0.25% w/v (Chaplin, 1985). Finally, images were obtained by taking photographs using a standard camera (Olympus U-CMAD3, Tokyo, Japan) mounted on the microscope.

RNA isolation and first strand cDNA synthesis

Total RNAs from the liver tissue were extracted with RNA-iso Plus reagent (DSS Takara Bio India Pvt., New Delhi, India) according to the manufacturer's instructions. The RNA quality and quantity were analyzed using 1% agarose gel and NanoDrop (Eppendorf, Germany). For this study, sequences of genes/transcripts involved in glycolysis (*insulin receptor, glucose membrane transporter 2, glucose 6 phosphatase, 6 phospofructo kinase*), gluconeogenesis (*glycogen synthase, hexokinase, pyruvate kinase*) and lipogenesis (*fatty acid synthase* and *acetyl co-*α) and *HSP70* were taken from transcriptome data generated in our project and primers were designed using FastPCR 6.6.99 to flank in the exon region with an amplicon size of around 200 bp (Table 2).

From the total RNA obtained, 1 mg was used for DNase I (RNase-free) treatment followed by cDNA synthesis using the 1st strand cDNA synthesis kit (DSS Takara Bio India Pvt) according to the manufacturer's instructions. Optimization of the PCR condition for maximum amplification of the target genes was carried out in a 20 µl reaction mixture containing 10 µl of 2 × PCR master mix (Genei, India), 1 µl of cDNA or PCR grade water as negative control, 5 µl of PCR grade water and 2 µl of primers each (Final concentration 20 picomole). Amplification was performed by denaturation at 95°C for 1 min, followed by 40 amplification cycles at 95°C for 30 sec, gradient at 50, 55, or 60°C for 45 sec and 72°C for 30 sec. A maximum amplification was observed at the annealing temperature of 55°C when 5 µl each of all the PCR amplified products were run on a 1% agarose gel at 60 V (Fig 1).

Quantitative real time PCR analysis

The qPCR was performed in the Life-Cycler (LC) 480 real-time PCR system (Roche, USA) as per the manufacturer's instructions. *β-actin* known as a housekeeping gene, serves as the internal control for normalization of expression.

Reaction of qPCR consisted of 20 µl reaction mixture having 10 µl of 2 × Quanti-Fast SYBR mix from RT-Kit (Qiagen), 1 µl of DNase treated diluted RNA (Final concentration 100 ng) or PCR- grade water as negative control, 4.8 µl of PCR-grade water, 0.2 µl Quanti-Fast RT mix and 2 µl of each 10 pmol primer. cDNA was synthesized at 50°C for 10 min, followed by reverse transcriptase inactivation and DNA polymerase activation at 95°C for 5 min. Quantitative PCR was carried out with the optimized PCR conditions, *i.e.*, denaturation at 95°C (1 min), with 40 cycles of amplification at 95°C (30 sec), 55°C (45 sec) and 72°C (30 sec) and then 95°C (10 sec), 50°C (60 sec) and 97°C (15 sec) to get the melt curve. The relative gene expression analysis was assessed using the $2^{-\Delta\Delta Ct}$ method as reported by (Livak and Schmittgen, 2001).

Statistical analysis

All the data are shown as mean±standard error of mean (SEM). The data were analyzed and Kolmogorov-Smirnov test (one-sample) and Levene's test were used to check for the normality of distribution and homogeneity of variances, respectively. The SAS package version 9.3 (SAS Institute, Cary, NC, USA) was used to analyze the data using one-way ANOVA and Tukey's multiple tests at a minimal significant level of 0.05 (P<0.05).

RESULTS AND DISCUSSION

Growth performance of experimental fish

A 100% survival rate was observed for fish in all treatments of the feeding experiment. The box plot for SGR, weight gain and condition factor was evaluated using SAS statistical package (V9.3). It was observed that fish fed with 30% starch level *i.e.*, TS30 exhibited an increase in mean body weight ($p<0.05$) by 24 g compared to TS20. On the contrary, fish from TS40 fed with high CHO diet showed a decrease in body weight ($p<0.05$) by 23 g. Thus, in TS30 treatment in which fish fed with 30% starch had a greater gain in body weight (150%), higher SGR (150% day^{-1}) and better condition factor compared to that of other treatments (Fig 2). The growth performance was observed significantly better in TS30 and TS40 as compared to TS20. Seenappa and Devaraj (1995) has been reported that fingerling of catla, *Catla catla*, showed better growth on diets containing 35% protein and 35% carbohydrate. The dietary protein could be reduced from 30 to 25% in the diet by increasing carbohydrate from 26 to 34% without compromising growth of silver barb, *Puntius gonionotus*, fry (Mohanta *et al*., 2007). As per literature, condition factor is more than 1 indicates the fish is in appropriate average weight (Kumar *et al*., 1979). In the present study, fish fed with 30% starch inclusion level

Table 1: Formulation and proximate composition of experimental diets fed to Jayanti rohu (*Labeo rohita*).

Ingredients composition	Experimental groups		
(%)	TS20	TS30	TS40
Fish meal[a]	20	20	20
Casein	10.2	10.2	10.2
Gelatinized starch	20	30	40
Cellulose	40	30	20
Fish oil	4	4	4
Vegetable oil	3	3	3
CMC binder	0.5	0.5	0.5
Vitamin and mineral premix[b]	2	2	2
Vitamin C	0.2	0.2	0.2
BHT	0.1	0.1	0.1
Total	100	100	100
Proximate composition (on dry matter basis)			
Moisture	7.8±0.3	8.1±0.2	6.9±0.32
Crude protein	24.18±0.2	24.08±0.3	24.98±0.2
Crude lipid	10.01±0.4	10.30±0.3	10.23±0.5
Ash	6±0.1	6±0.1	6±0.1
Starch	20.8±0.12	31.4±0.5	41.7±0.3

Proximate composition are expressed as Mean±SD.

Abbreviation: BHT-Butylated hydroxytoluene; CMC-Carboxymethyl cellulose.

Experimental diets *viz*. TS20 (@20% starch), TS30 (@30% starch) and TS40 (@40% starch).

[a]Fish meal was purchased from local market having all kind of dried fish available at Odisha.

[b]Vitamin and mineral premix (IU or mg/kg diet): Vitamin-A, 700000 IU; Vitamin-D3, 70000 IU; Vitamin-E, 250 mg; Cobalt, 150 mg; Copper, 1200 mg; Iodine, 325 mg; Magnesium, 6000 mg; Potassium, 100 mg; Sodium, 5.9 mg; Manganese, 1500 mg; Sulphur, 0.72%; Zinc, 9600 mg; DL-Methionine, 1000 mg; Calcium, 25.5%; Phosphorus, 12.75%.

had better condition compared to other groups, as the condition factor was 1.2 which indicated the superior wellbeing of fish. Similarly, rohu fingerlings fed with *Azolla* incorporated (@25%) diets recorded condition factor of 1.23 in a 150-day rearing trial (Datta, 2011).

Higher level of CHO in the diet damages the hepatic cells

The histological examinations of stained liver tissues (Hematoxylin) from all the treatments are shown in (Fig 3). An enlarged hepatocyte, lipid vacuolation and damaged sinusoids were observed in the liver cells of fish from TS30 and TS40 treatment groups. Our study indicated occurrence of significant damage to the hepatocytes with increasing concentrations of starch (Fig 3C). The hepatocytes of fish in TS20 dietary treatments were in normal size, but enlarged hepatocytes were observed in TS30 and TS40 where fish received 30% and 40% dietary starch in fish diet. It was displayed that a degree of lipid vacuolization occurred in

Table 2: List of primers used to study the carbohydrate metabolism involved genes.

Gene name	Primer	Sequence (5′-3′)
Insulin receptor	Forward	GAGCTCGGTCAGGGATCGTTTG
	Reverse	ACCAAGCAACCGGACCACTG
Glucose transporter Membrane 2	Forward	GTCCGTCTGCCCGCTCTGTC
	Reverse	GTCCATCCTGCGATTGCTTCG
Glucose 6 phospatase translocase	Forward	CGTCAGTGGCGGTGTTCTCAG
	Reverse	GCAGGTCAGGCCGGAGATG
6 phospofructokinase	Forward	ATGGCCTTCTGCACCTCTTGAG
	Reverse	AATCTGGTGGTTCAGCGTCTGG
Glycogen synthase	Forward	TAACATTGCACCGACACAAGGC
	Reverse	TGCTCTTACCCTCAGGCTCTGC
Hexokinase	Forward	TTTGCTAACATGGACGAAACGC
	Reverse	TGCCGGTACCAATAATAATGCC
Pyruvate kinase	Forward	TGCGGCAGGCTGGGATAATC
	Reverse	TGAGGCGGTGGAGACGTTGAC
Fatty acid synthase	Forward	CCGCTCTAAATGAAGCCACATC
	Reverse	CGCTATCTCAATCCTCAGCTCG
Acetyl Co A	Forward	GGATGGACGATGCCCTCTGG
	Reverse	GGCTGGCTGATGCACAGAGC
β-Actin	Forward	GTATGTGGCCATCCAGGC
	Reverse	TAGCCACGCTCGGTCAGG
HSP 70	Forward	GGCAGAAGGTGACGAATGCAG
	Reverse	TCCGCCCAGGTCAAAGATCAG

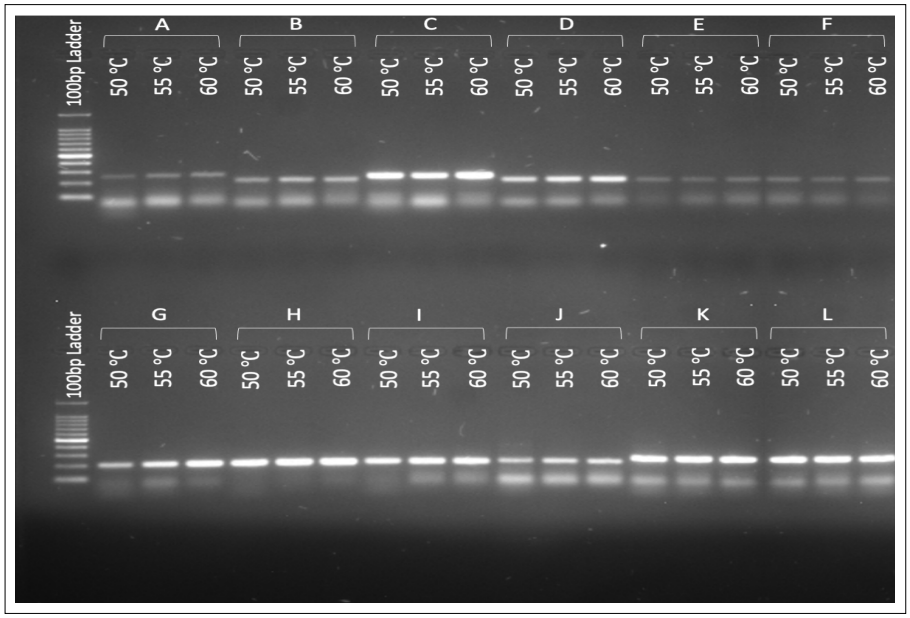

Fig 1: 5 µl of PCR product at gradient annealing temperatures 50°C, 55°C and 60°C on an 1% Agarose gel.

TS30 and TS40 treatment compared to TS20. The sinusoid breakage was also observed in the hepatocytes of fish from TS30 and TS40. An increased level of starch in the diet was found to damage the hepatocytes. It was reported that lipid vacuolization and hepatocytes enlargement were observed in *Tor tambroides,* when fed with 20-25% dietary carbohydrate (Ishak *et al*., 2016). Previous studies have demonstrated the changes in hepatic cells and sinusoid due to increased level of gelatinized starch (@50%) in the feed of *L. rohita* fry (Mohapatra *et al*., 2003) and *Catla catla* fingerlings (>40% carbohydrate in diet) (Yengkokpam *et al*., 2006). The damages in hepatocytes may be due to deposition of glycogen (*de no* lipogenesis) in presence of excess glucose load. Similarly, damages in the hepatocytes and vacuolations were observed in largemouth bass fed with dietary starch above 100 g/kg (Zhang *et al*., 2020).

Metabolic gene expression profile

The expression data generated using qRT-PCR of those selected genes are presented in (Fig 4). The genes/ transcripts, such as *GLUT2, ACC, FAS, GYS, G6P, PK, HK, HSP70, 6PfP* and *IRS* exhibited differentially expressed pattern among different treatments. *IRS* revealed a 3-fold level expression in the liver tissues of rohu from TS30 group, while 2-fold expression level was observed in TS40. The genes involved in glucose transport, *Solute carrier family* 2 (facilitates glucose transporter)*, member 8* (*GLUT2*) displayed significant (2 to 3- fold) up-regulation (P<*0.05*) in liver tissues of both TS30 and TS40 treatment groups in comparison TS20. Selected genes such as *hk, pfk* and *pk* associated with glycolysis, showed significant (P<*0.05*) changes in expression level between TS30, TS40 to TS20 group. *Hexokinase* (*HK*) was observed up-regulated (3-fold). *Fatty acid synthase* (*FAS*) and *Acetyl-coenzyme-A-synthetase* (*ACSS*) linked with *de novo* lipogenesis showed a significant (P<*0.05*) alteration in expression, 1.8 to 2- fold in the liver of rohu from TS40, while 1.5-fold expression level was detected in TS30 in comparison TS20. Stress associated *HSP70* showed a significant up-regulation (2.3-fold) in the liver of rohu from TS40 and 1.2-fold expression level was detected in TS30 as compared to TS20. Further, G6P, *6-phospofructokinase* (*6PFK*) and *pyruvate kinase* (*PK*) exhibited neutral regulation in liver of both TS30 and TS40 treatment groups in comparison TS20. *Glycogen synthase* (*GYS*) associated with glycogen synthesis in liver, displayed 1.3 to 1.75-fold increase in expression, *i.e*. significant up-regulation (P<*0.05*) was observed in liver tissues of both TS30 and TS40 treatment groups in comparison TS20.

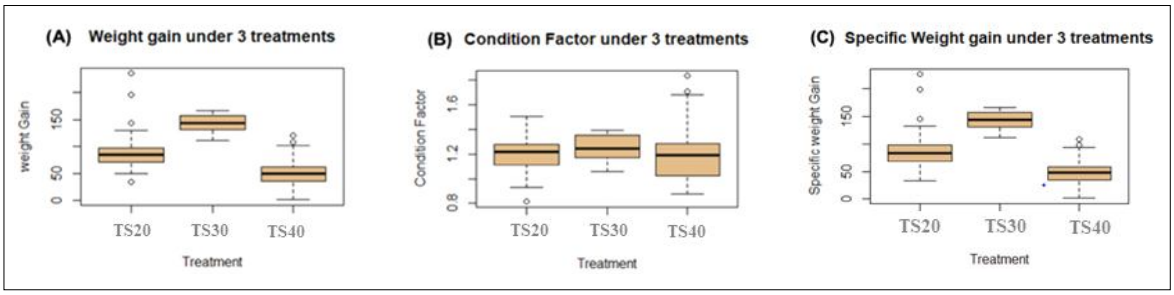

Fig 2: Box plot representing A) Weight gain, B) Condition factor and C) Specific weight gain in 45 days under treatment conditions.

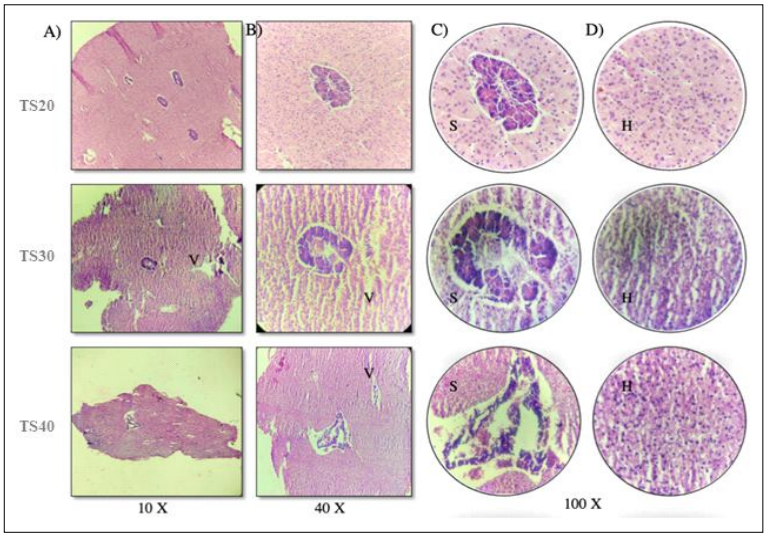

Fig 3: H&E stained liver tissue from treatment conditions; Where row A) Liver histology at 10X magnification; B) Liver histology at 40× magnification; C) Liver sinusoids at 100X magnification; D) Liver hepatocytes at 100× magnification.

The earlier studies also revealed elevated expression of hepatic *GT1* in grass carp and *GT2* in rainbow trout due to excess glucose in the diet (Chen *et al*., 2017). Contrarily, *GLUT1* and *GLUT2* were dysregulated in liver tissues of the tilapia during first 1-3 hr of digestion upon inclusion of high carbohydrate in the regime, further increase in the plasma glucose level was observed (Chen *et al*., 2017). Interestingly, *GT2* expression was found to be down-regulated during supplementation of excess dietary starch in largemouth bass and suggested the induction of glycaemia (Zhang *et al*., 2020). Other genes, *glucose 6 phosphatase* (*G6P*), *6 phospofructo kinase* (*PFK*) also had an elevated level of expression in TS30 and TS40 as compared to control. It was reported that *PFK* plays a pivotal role in glycolysis in fish as indicated in tilapia (Chen *et al*., 2017), *Epinephelus lanceolatus* (Lu *et al*., 2018), *Sparus aurata* (Meton *et al*., 2004), *Oncorhynchus mykiss* (Enes *et al*., 2008) and *M. salmoides* (Zhang *et al*., 2020).

Subsequently, in the present study expression of genes/ transcripts linked with gluconeogenesis (*glycogen synthase, hexokinase, pyruvate kinase*) in liver tissues of Jayanti rohu with enhanced profile due to excess glucose in the regime was observed. Previous studies deciphered the role of *hexokinase* (*HK*) and *pyruvate kinase (PK*) in fishes, such as grass carp (Yuan and Wood, 2013) during different percentages of carbohydrate level in their diet. Interestingly, our work depicted higher expression of *HK* gene in Jayanti rohu fed with high carbohydrate groups. Increase in carbohydrate percentage in the diet significantly increased *PK* expression activities in grass carp liver tissues (Yuan and Wood, 2013). However, in the present study, a neutral expression level of *PK* gene was observed among all the treatments.

As the excess glucose will be converted into fatty acid *via de novo* lipogenesis (NRC, 2011), we have also analysed the expression patterns of key genes, such as fatty acid synthase (FAS) and acetyl co-synthase (ACC) were analysed. FAS is a vital gene having crucial role in the *de novo* lipid synthesis in the liver. Several studies reported that induction of lipogenesis process occurs due to incorporation of higher level of dietary digestible carbohydrates in fish, such as blunt snout bream, *Megalobrama amblycephala,* along with *peroxisome proliferator-activated receptor* γ (*PPARγ*) pathway induction (Wang *et al*., 2017). In tilapia and rainbow trout (*O. mykiss*), hepatic transcripts, such as *accα, fas* and *dgat2* were up-regulated in liver tissues when high starch levels (more than 45% dietary starch) were included in the diet (Chen *et al*., 2017). Furthermore, higher expression level of *insulin receptor substrate* (*IRS*) was observed in TS40 group, while lower expression level was found at TS30 as compared to TS20. This gene plays an essential role in the insulin signaling pathway. In the study an elevated expression level of *HSP70* in 40% starch fed treatment group, which indicated an increase in stress in hepatocytes due to high level of glucose uptake (Rasal *et al*., 2020) was observed.

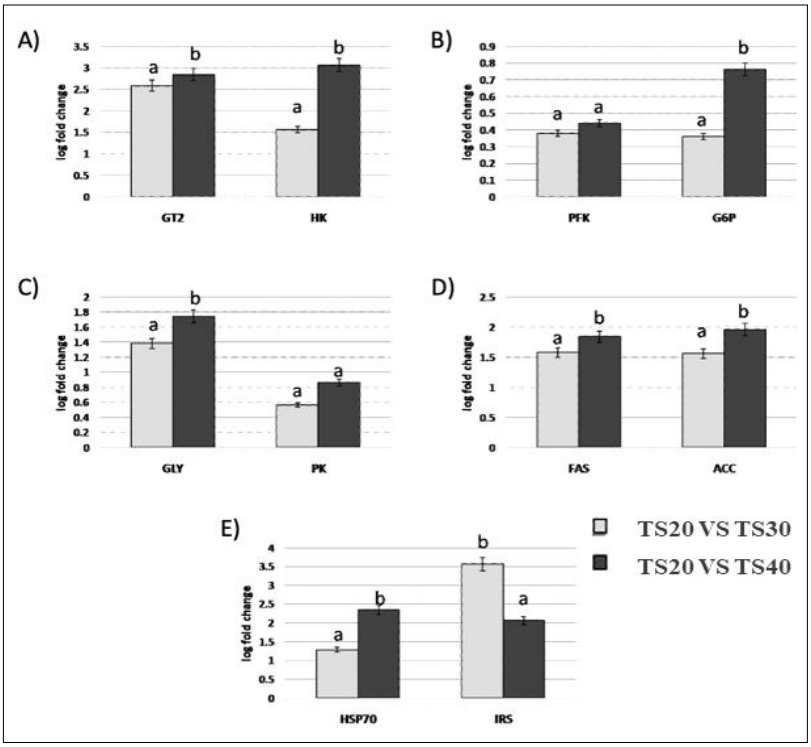

Fig 4: Relative quantification of carbohydrate metabolism involved genes under treatments represented in Log fold change of genes. A) GT2; HK, B) PFK; G6P, C) GLY; PK, D) FAS; ACC, E) HSP70; IRS under treatment conditions (TS20 Vs TS30 and TS20 Vs TS40).

CONCLUSION

The present work highlighted the requirement of carbohydrate level in the diet of Jayanti rohu based on physiological and molecular studies. The growth performance and liver histology indicated that excess glucose lead to damage of hepatic cells significantly. It was also observed that higher expression of glucose membrane transporter 2, possibly promotes glucose uptake in liver cells. Subsequently, modulation in expression profile of hepatic genes, such as *G6P, PFK, FAS, GYS, HK, PK, FAS* and *ACC* revealed that excess glucose induced the glycogenesis and *de novo* lipogenesis process in the liver. Thus, carbohydrate in the diet enhances the glycolytic process mediated by IRS-insulin signalling pathway. The present work indicated to maintain correct balance between dietary carbohydrates originated from plant sources to utilize available energy sources for sustainable production of Jayanti rohu. It can be concluded that the inclusion level of starch @30% in the diet of *Jayanti rohu* would be a better solution to enhance overall performance by reducing feed cost at farm level. This result will be helpful to carry out nutritional programming in Jayanti rohu to adapt them for assimilation of more carbohydrate in the regime.

ACKNOWLEDGEMENT

We are thankful to the Director, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar for providing facility to undertake this work and the Director, ICAR-Indian Agricultural Statistics Research Institute, New Delhi for assisting in data analysis.

Ethics statement

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The research undertaken complies with the current animal welfare laws in India. The care and treatment of animals used in this study were in accordance with the ethical guidelines of ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India.

Funding information

The financial assistance to carry out this work was provided in the form of CABin (Centre of Agricultural Bioinformatics) grant (F. no. Agril. Edn.4-1/2013-A&P), Indian Council of Agricultural Research, Department Agricultural Research and Education, Ministry of Agriculture and Farmers' Welfare, Govt. of India.

Data availability statement

All data will be available upon request to the corresponding author.

Conflict of interest: None.

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