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RESEARCH PAPER

Comparative Study of Metabolic Gene Regulation with Seasonal Fluctuation of Temperature in Rainbow Trout (*Onchorynchus mykiss)* Under Captive Conditions

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Abstract

Temperature is the most important abiotic factor which influences growth, reproduction, survival of fishes. The nutritional uptake its utilization and conversion by metabolic pathway enzymes are highly dependent on external water temperature. The present study was carried out for comparative analysis of expression of metabolic genes in Winter and Summer. The blood glucose estimation was highest in February 86.175 mg/dl >10°C and lowest in January 83.9 mg/dl >4°C during Winter period, whereas in Summer, highest was estimated in June 105.32mg/dl >17.8°C and lowest was in July 103.4 mg/dl >21°C. The triglyceride levels estimated highest in February 396.74mg/dl and least in January 382mg/dl where as in Summer highest was in June 481.92mg/dl and lowest in July 471.77. It was observed that mRNA transcript levels of GK and FBpase were extremely low and GLUT2 expression level was maintained, due to Q10 effect at temperature ≤4°C but at 8-10°C although GK gene expression was very low but FBpase and GLUT2 were maintained slightly higher. The effect of temperature on metabolic gene expression will be very important for understanding the differential seasonal growth pattern in fishes.

Introduction

The seasonal environmental changes are challenges for animal's especially aquatic animals for survival and growth. Availability of food, day length and temperature are changing during the course of the year. Aquatic animals in general and fishes in particular are strongly influenced by temperature of water body which have influence over physiological and metabolic activities. The range of temperature is species specific and dependents on geographical distribution of fish. In order to survive at very low temperature at which food intake capacity is very low, many aquatic animals enter in a state of low metabolic rate.

The optimal temperature for growth of most of the salmonids is close to 15°C (Jonsson and Jonsson 2009). The capability of growth in terms of feed uptake and its conversion is maximum at about 3°C lower than the temperature for optimum growth (Handeland, Imsland, & Stefansson, 2008). The adaptation of low temperature tolerance ranges for growth or feed conversion efficiency is very narrow in salmonid species and also phenotypic plasticity (Forseth, Larsson, Jensen, Jonsson, Naslund, & Berglund, 2009; Jonsson, Forseth, Jensen, Naesje, 2001). However, adaptations to colder environments exist in feed conversion efficiency of Arctic *charr* (Larsson and Berglund 2005) and also in Atlantic salmon (Jonsson et al. 2001). In nature (lakes, rivers and streams) fish tend to move regions of optimum temperature zones for survival and growth. Rainbow trout in nature move to warmer places where depth of water is deep to elude the extended harsh environmental conditions. The change in climate likely has strong impact on aquaculture practice, population

size of wild fish stock with serious economic and ecological consequences (Morgan, McDonald, & Wood, 2001; Pankhurst and King 2010).

The rainbow trout is one of the carnivorous fish and limited utilization of carbohydrates is characteristic feature thus considered as glucose intolerant. Administration of glucose either through oral or intravenously results in persistent hyperglycemia like many other fish species (Wilson, 1994; Hemre, Mommsen, & Krogdahl, 2002; Moon 2001; Del Sol Novoa, Capilla, Rojas, Baro, Gutierrez, & Navarro, 2004; legate, Bonen, & Moon, 2001). The glucose transport is facilitated by insulin regulated GLUT4 and GLUT2 membrane transporters as former reported in white muscle and fatty tissue and latter in liver of rainbow trout (Capilla, Diaz, Albalat, Navarro, Pessin, Keller, & Planas, 2002; Diaz, Capilla, & Planas, 2007a; Krasnov, Teerijoki, & Molsa, 2001). Most of the key regulating enzymes involved in catabolism of dietary carbohydrates have been studied and characterized which includes glucokinase (liver), phosphofructokinase (liver and muscle) and pyruvate kinase (liver and muscle) and their induction is expected that in mammalians (Fideu, Soler, & Ruiz-Amil, 1983; Panserat, Plagnes-juan, & Kaushik, 2001a; Panserat et al., 2000b). Studies have shown that diet rich in carbohydrates does not affect the gene expression of key enzymes like glucose-6-phosphatase, fructose-1,6-biphosphatase and phosphoenolpyruvate carboxykinase of metabolic pathways involved in synthesis of glucose from non carbohydrates such as lactate, glycerol and glucogenic amino acids (Panserat, Plagnes-Juan, Breque, & Kaushik 2001b; Panserat, Medale, Breque, Plagnes-Juan, & Kaushik, 2000a; Panserat et al., 2001c; Tranulis, Christophersen, Blom, & Borrebaek, 1991).

The primary site for glycerol production is liver due to the presence of enzymes complement and expression of genes both invivo and isolated hepatocytes. Glycerol is produced from glucose and amino acid metabolism obtained from diet or either reserved as indicated by radio-isotopic studies (Raymond 1995; Driedzic and Ewart 2004; Driedzic, Clow, Short, & Ewart, 2006; Liebscher et al., 2006). The main precursor of glycerol production is glycogen stored in liver and has been studied that 75 hours post capture fish held without feeding at freezing temperature left small amount of muscle glycogen than that of liver. The antifreeze protection is governed by conversion of dietary glucose, amino acids and glycogen in to glycerol production. In rainbow smelt glycerol content begins to pile up to a concentration of 500mmoles/L as water temperature turn down to 4°C (Lewis, Ewart, & Driedzic, 2004; Driedzic et al., 2006). The present research was carried out to investigate effect of seasonal temperature on the expression of metabolic gene in rainbow trout.

Material and Methods

Experimental Animal

In the presents study ten rainbow trout were sacrificed of average body weight 240±10 g of average length 25±0.8 cm (Mean±SE) of 18 month age group are collected from government trout fish farm located at higher altitudes of northern Himalaya of India (N: 34°,03.207, E:074°,16.157). The water source for the farm is glacier fed stream and inlet flow rate of water was maintained at 2-4l/min/m^3 for Winter and 6-7 l/min/m^3 for the Summer and mean pH of the water was ranged from 6.8-8.0. The fishes were fed on twice a day at the rate of 3% of body weight with commercially available feed which contains 67% fish meal, 9% soyabean meal, 8% whole wheat, 6% fish oil, 4% yeast, 1% minerals, 2% vitamin, 1% cholin choloride, 1% D.L methonine and 1% alginate. The fishes of a single genetic strain were dissected at the respective farm and excised liver tissue were immediately stored in Lab made RNA guard (data unpublished), transported in liquid nitrogen for laboratory based investigations. The health status of the fishes was done on behavioral characteristics and morpho-indicative features of the body.

Table1. Primers used in the present study

Genes	Primers sequences (5' to 3')
Glucokinase gene (GK) *Onchorhynchus mykiss*:AF053331.1 *Ctenopharyngodon idella*:GU065314.1 *Homo sapiens*:AC009042.5	F:TGAAGGATCAGAGGTGGGTGATT R:GAAGGTGAAACCCAGAGGAAGC
Fructose-2,6-biphosphatase (FBPase) *Onchorhynchus mykiss*:AF246148;AF33188.1;AY113693.1 *Rattus norvegiens*:M86240 *Caenorhabditis elegans*: AJ271466	F:GCCAATGAGAGGGACGCAC R:CCACCATAGGGGGCACTGCC
Glucose Transporter gene (GLUT-2) *Onchorhynchus mykiss*: NM001124289.1 *Salmo salar*:XM014185434.1 *Homo sapiens*: J03810 *Gallus gallus*:Z22932	F:ATGTATTGGTCTCTGTCTGTGC R:CCCTGGGAGAAGAGCTCTGCC

RT-PCR

The total RNA was extracted by Trizol (Invitrogen) method as well RNA extraction kit (Fermentas). The RNA preparation was quantified using spectrophotometer and pretreated RNase free DNase to eliminate potential DNA contaminations. The first strand cDNA preparation was done using first strand cDNA synthesis kit (Fermentas) by random haxamers as per manufactures protocol. The primers (Table 1) used for the study were designed by retrieving sequences of different fish species from NCBI and were compared by using Clustal-W multiple alignment algorithm (Higgins & Sharp, 1989) and both upstream and downstream sequences testified for thermodynamic properties using Primer3 software. Quantitative real time PCR was carried out using SyberGreen master mix (Thermofisher Scientific) as recommended by manufacturer. PCR was carried out using 25µl reaction volume containing 2 µl genomic DNA, µl (10pM) of each primer, 2.5 µl of dNTPs, 1.5 µl $MgCl_2$, 2.5 µl PCR buffer, 0.2 µl of Taq polymerase (Fermentas) and water. The PCR cycling conditions are hot start at 94°C for 10 min, followed by 35 cycles of denaturation 94°C for 30sec, annealing 55-60°C for 30 sec., elongation 72°C for 30 sec., and final elongation 72°C for 10 min. The PCR products were separated on 2 per cent of Agarose followed by sequencing.

Quantification Analysis of GK, FBPase & GLUT2 Genes

The SyberGreen assay was used for differential quantification of the relative gene expression between samples collected in Winter with those collected in Summer. The comparative C_T method which is also known as Δ_G method (Livack & Schmittgen, 2001) was used to achieve the results of relative quantification.

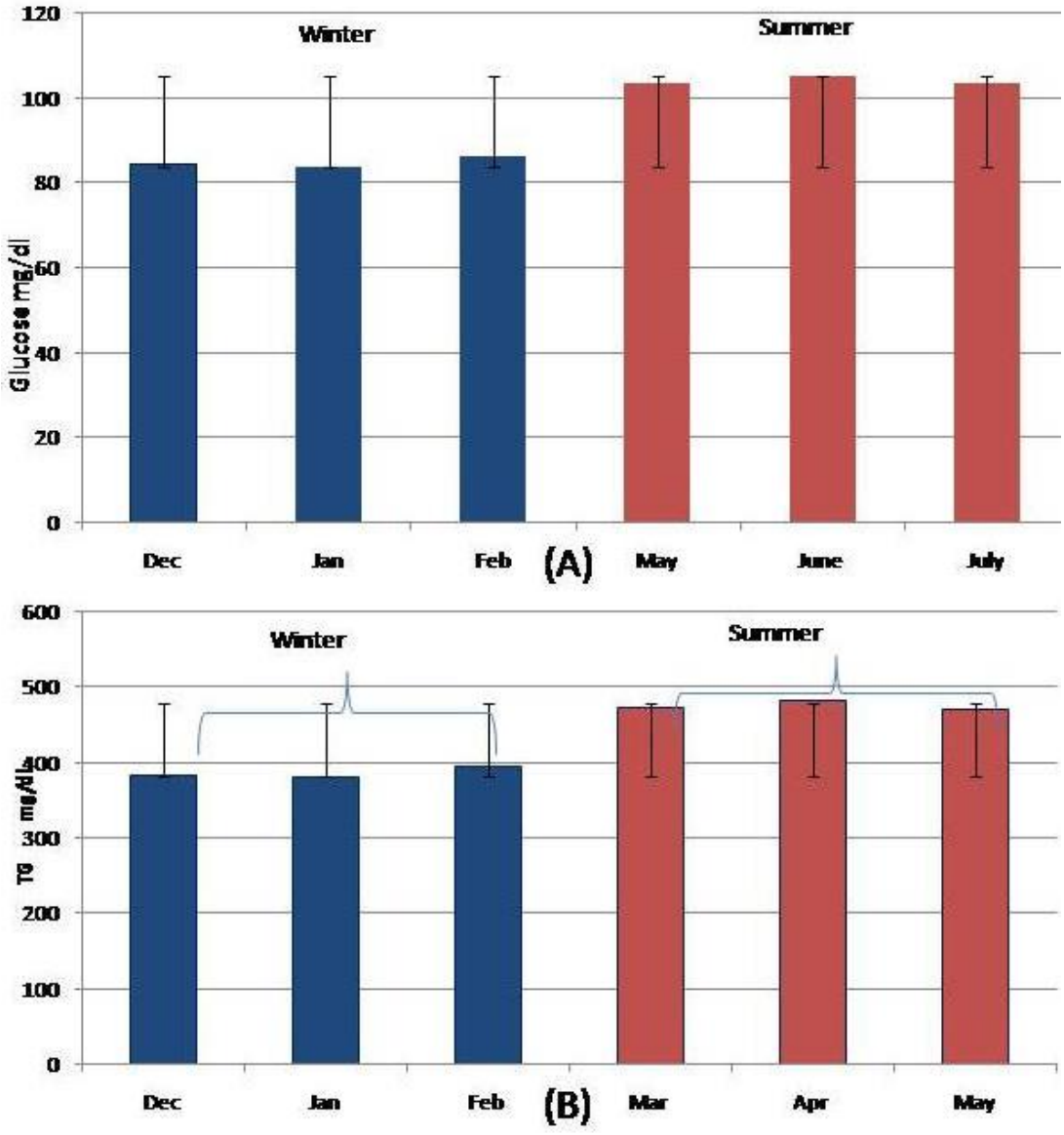


Figure 1. Estimation of Glucose and Triglycerides during Winter and Summer, (A) Glucose estimation during Winter and Summer by standard deviation using error bars. (B) Triglyceride estimation during Winter and Summer by standard deviation using error bas, the results shown are mean values. Each experiment was repeated in triplicates.

The amount of target gene was normalized to an 18s ribosomal gene and was calibrated by formula given as:

2- ΔΔCT

Where, ΔΔCT = ΔCT (sample) - ΔCT (calibrator)

ΔCT (sample) = {CT (target gene) - CT (reference gene)} sample

ΔCT (calibrator) = {CT (target gene) - CT (reference gene)} calibrator

CT represents thresh hold cycle i.e., the cycle number at which fluorescence is detected. Using 2-ΔΔCT method, data was presented as fold change (or fold difference) in target gene expression, normalized to endogenous reference gene relative to calibrator.

Biochemical Investigation

Blood was removed from the caudal vein from each fish separately in an anticoagulant (0.04 g/ml potassium oxalate and 0.02 g/ml sodium fluoride) then centrifuged at 3000 rpm for 5 min. and recovered plasma was immediately frozen in liquid nitrogen. Blood (plasma) glucose was estimated by glucose oxidase followed by peroxidase test whereas glycogen (triglycerides) was estimated by amyloglucosidase method in both the seasons. 0.5ml recovered plasma was incubated for 10 min. at 55°C then 0.1ml of 0.05M acetate buffer (pH 4.5) and 3.5 units of amyloglucosidase was added. The glucose obtained was estimated with purified Glucose oxidase and peroxidase test (Kepler & Decker, 1974)

Results

Investigation of Blood Glucose and Liver Triglycerides

The blood collected from caudal vain of rainbow trout was immediately transferred in to vacutainer tubes containing anticoagulant (0.04g/ml potassium oxalate and 0.02 g/ml sodium fluoride) centrifuged at 3000xg for 5 min. The plasma recovered investigations showed that glucose level was 84.275 mg/dl, 83.9 mg/dl & 86.175 mg/dl in the month of December, January and February(Winter) at recorded mean water temperature 8°C, 4°C and 10°C respectively, where as glucose level in Summer i.e. May>103.5, June>105.32 & July>103.4 was measured at mean water temperature 14.3°C, 17.8°C and 21°C for each respective months (Figure 1A). Glycogen levels (triglyceride) estimation was done by amyloglucosidase method. The results showed significant interaction between triglyceride levels with respect to temperature change in Winter (Dec>384_mg/dl; Jan>382_mg/dl; Feb>396.74_mg/dl) and during Summer (May>471.9_mg/dl; June>481.92_mg/dl; July>471.77_mg/dl) respectively (Figure 1B). The decrease of triglycerides (TG) and glucose level in month of Jan and increase in June justifies role of temperature in the depletion of glycogen reservoirs in Winter and production of glycogen in Summer due to maximum utilization of available diet resources for growth.

Expression of Glucokinase (GK), FBPase and GLUT2 Genes on Temperature Fluctuation

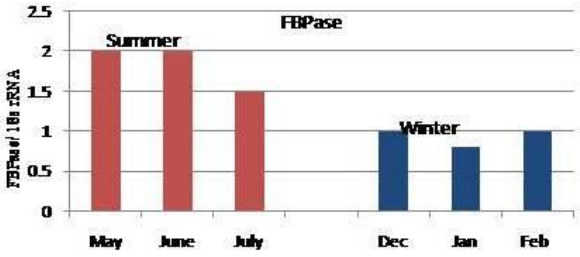
In the experimental procedure to evaluate the expression level of hepatic enzymes fewer than two different climatic seasonal patterns in rainbow trout. The gulcokinase (GK) and FBpase expression was twofold higher in Summer than in Winter (Figure 2B-C), whereas when water temperature in peak Winter decline to subzero and mean water temperature was recorded 4°C, it was observed that mRNA transcript levels of GK and FBpase were extremely low and GLUT2 expression level was same as in Summer and this might be due to the Q10 effect. The amplification of GK>230_bp, FBpase>300_bp and GLUT2>500_bp (Figure 2A) was done for both the seasons.

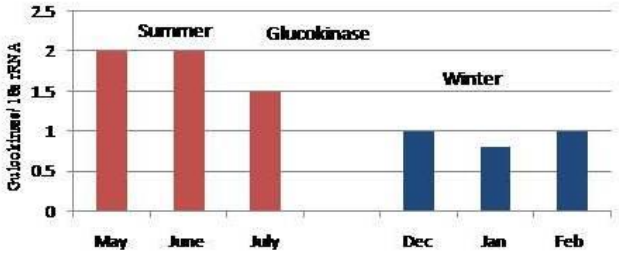
Discussion

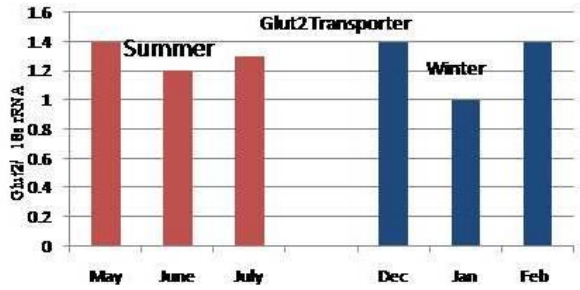
Rainbow trout is a carnivorous fish and due to hyperglycemia have very low affinity for food rich in carbohydrates. The trout glucose is synthesized from non carbohydrate sources via gluconeogenic pathway like from protein (glucogenic amino acids), lipids (Glycerol>triglycerides) and from other metabolic intermediates (pyruvate and lactate). In the present piece of work expressional study of key metabolic intermediates genes such as GK, FBpase and membrane constitute gene GLUT2 was done. Studies have shown their role in maintenance of glucose homeostasis by gluconeogenic pathways. We have observed that in Summer (May, June & July) the expression levels of genes studied are higher due to active feeding abilities of rainbow trout. The glucose concentration in rainbow trout maintained in free flowing raceways showed maximum value in June (106mg/dl) and least difference was observed in the month of May and July, whereas in Winter period, we have observed maximum in February 87mg/dl and least in January 83mg/dl Figure 1A. The reason of glucose values higher in Summer and least in Winter is due to the enhanced feed uptake abilities of rainbow trout in Summer. The glycogen (triglycerides) estimation showed peak value 400mg/dl in February and least 382mg/dl in January, where as in Summer the peak value 481.75 was observed in June and slight decrease was observed in July>467.75 as shown in Figure 1B. Therefore, we can say the gluconeogenic pathway and glucose metabolism is under control of thermal responsiveness. Oku et al. (2014) found temperature dependent differential expression of metabolic enzymes in rainbow trout. The conversion of excess glucose in to lipids by lipogenesis plays important role in the glucose homeostasis of rainbow trout

(Polakof, Panserat, Soengas, & Moon, 2012). The environmental temperature is directly affecting the Growth hormone level in rainbow trout independently of nutritional status (Gabillard et al., 2003).

In rainbow trout and gilthead sea bream carbohydrate rich diet induces expression of glucokinase gene (Panserat et al., 2000b; Caseras, Meton, Vives, Egea, & Fernandez, 2002; Meton, Caseras, Fernandez, & Baanante, 2004). The high glucose level increase potential for glycolysis as was observed in red sea bream and small increase in gluconeogenic enzyme activity (Furuchi & Yoni, 1982). The formation of glucose by gluconeogenic pathway remains highly impaired due to influx of glucose from carbohydrate source as in gilthead sea bream and rainbow trout (Panserat, SkibaCassy, Seiliez, Lansand, & Plagnes Juan, 2009; Kamlam, Medale, Kaushik, Polakof, & SkibaCassy, 2012). Studies have shown starch rich diet up-regulate GK activity in atlantic salmon (Borreback, Waagbo, Christophersen, Trannulis, & Hemre, 1993). Similarly, a high expression of GK was observed in rainbow trout after intake of carbohydrate rich diet (Panserat et al 2000b). The studies are in strong agreement with our findings of high expression of GK gene in Summer due to high abilities of feed uptake and conversion than Winter. With respect to gluconeogensis, high feed uptake has strong association with enhanced expression of FBpase in Summer. Thus is in agreement with the fact that in carnivorous fish glucose does not exert mammalian like inhibitory effect on gluconeogenic pathway (Panserat et al 2000c, Enes, Panserat, Kaushik, & Oliva Teles, 2008; Sundby, Eliassen, Refstie, & Plisetskaya, 1991; Kamlam et al., 2012). The effect of temperature change was also observed in other fishes and in amphibians. In tadpole the low temperature has different effect on membrane lipids with dynamic change in transcript levels for energy, carbohydrate and lipid metabolism (Shunsuki, Koichiro, Akinori, & Kiyoshi, 2016). Salmonids exhibit seasonal variations of growth rate in relation with seasonal temperature and plasma growth hormonal level. Temperature is governing factor for alteration in parameters such as food intake which directly modifies







Temperature In Different Seasons (Month)

Months	Dec.	Jan.	Feb.	May	June	July
Max. (mean)	8±0.5	4±0.5	10±0.5	14.3±1	17.8±1	21±1
Min (mean)	-1.0±0.5	-3.0±0.5	6±0.5	10.3±1	14.6±1	15.3±1

Figure 2. Gene expression of selected genes under study, (A) Agarose gel (2% agarose, 100bp ladder) of FBpase, Glucokinase and GLUT2 genes, the one lane of GLUT2 is represent expression in Winter & Summer both as its expression remains almost static. (B) Expression of FBPase gene during Summer and Winter, (C) Expression of Glucokinase gene during Summer and Winter, (D) Expression of Glut2 gene during Summer and Winter, (E) Temperature of rainbow trout raceway in free flowing system recorded during different months of study. All the expression were measured using real time RT PCR and expression levels were normalized by 18s ribosomal RNA transcript.

level of expression of metabolic genes. We have studied the effect of low temperature induced expression of genes and found that onset of Winter (Nov, Dec and Jan) the expression of these genes are very low in liver and plasma of trout fish comparative to fishes in Summer. The hypothesis was proven because the feed intake capacity is declining as water temperature tends to move down below 10°C. The expression level of GK and FBpase were very low in fishes having water temperature 4°C for almost fifteen days where as GLUT2 was exceptionally maintained. The reason thereof shows the function of GLUT2 as glucose importer while feeding and exporter while off feeding conditions. Studies have proven mRNA level of glucose transporter maintained at steady state due to constitutively presence in the plasma membrane (Olson & Pessin, 1996). Expression of GLUT2 has been maintained high irrespective of nutritional status (Panserat et al., 2001). Studies carried on fat line and skinny line of rainbow trout showed higher abilities to glucose metabolism and excess glucose stored in lipids by lipogenesis in farmer than in latter (Kamal et al., 2012; Kolditz, Borthaire, Richard, Corraze, & Panserat, 2008; Skiba Easy, Lansard, Panserat, & Medale, 2009). The low gain in body weight in Winter is due to the use of non conventional protein source (muscle) for glucose synthesis to carry out metabolic activities through gluconeogenic pathway. This might result in weight loss due to muscle wasting during the Winter and sometimes extended Winter to provide the energy for maintenance of biological activities and need further experimental validations. The interesting findings observed in the present piece of work are astonishing as we have seen 30% of the genetic stock is least affected by lower water temperature during the winter (4°C) which might be due to the synthesis of liver gluconeogenic enzymes at considerably good concentration. The underlining mechanism might be due to adaptive genetics and need further investigations in broader prospective.

Conclusion

The regulation of biochemical pathways is under the control of external environmental stimulus in general and under temperature in particular. The better understanding of the metabolic gene regulation with respect to difference in temperature will help in mitigating the affect of climate change.

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