Journal of
Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

Journal of Fisheries and Aquatic Sciences 1 (1): 1-9, 2006
ISSN 1816-4927

Stress Responses of Matrinxã (*Brycon cephalus*) Subjected to Transportation in Plastic Bags

Luís Antônio Kioshi Aoki Inoue and Gilberto Moraes
Departamento de Genética e Evolução, Universidade Federal de São Carlos,
CP 676; São Carlos, SP, Brasil, 13565 905

Abstract: Hauling and transport are essential procedures in all fish farms. However, these stimuli are harmful to fish that first respond by the plasma catecholamine and cortisol increases. Among the other physiological stress responses, plasma ions balance is disturbed and glucose rises. Marine salt (NaCl) is empirically proposed in fish transport to reduce the stress and ions losses. To evaluate the physiological stress responses of juvenile matrinxã to hauling and transport, two groups of fish (within plastic bags with freshwater and salted water 6 g L^{-1}) were subjected to transport for 4 h. Plasma cortisol increased in both groups. On the other hand, plasma Na^+, Cl^- and protein decreased after the transport. No differences were observed between groups of fish transported in freshwater or salted water. Hepatic glycogen decreased suggesting the high energetic cost to matrinxã cope with hauling and transport. Marine salt added to the water used for transporting fish did not reduce the plasma cortisol increases. Matrinxã is very responsive to the studied stressors recommending special care during these procedures. Moreover, complete recovery occurs between 24 to 96 h.

Key words: Stress, transport, hauling, NaCl, *Brycon cephalus*

Introduction

Fish homeostasis disturbance is observed in consequence of one or more adverse stimulus imposed by several procedures of field and laboratory. One of the most common methods of fish transport is the loading of fish in plastic bags filled with water and oxygen. Although hauling and transport are unavoidable in fish farming, these are relevant sources of stress, which must be considered to prevent fish damages (Sampson and Machintosh, 1986).

In the course of fish transport, plasma catecholamine and cortisol increase (Barton *et al*., 1980; Nikinmaa *et al*., 1983) and many other physiological stress responses can be initiated as a result of the cortisol rise and the activation of several biochemical processes (Iwama, 1998; Perry and Bernier, 1999). Moreover, ions balance is disturbed as a consequence of the catecholamine and cortisol increases. However, these mechanisms are not clear yet (McDonald and Milligan, 1997).

Fish biologists have studied stress in the last few decades. Many practical aspects of the intensive aquaculture are directly related to the physiological and metabolical stress responses. Therefore, fish stress studies are also conducted to improve fish husbandry in order to reduce mortalities and economic losses. Marine salt (NaCl) is usually added to the water of the fish transport containers as a way to stimulate mucus production and also reduce the stress (Nikinmaa *et al*., 1983).

Corresponding Author: Dr. Gilberto Moraes, Departamento de Genética e Evolução,
Universidade Federal de São Carlos, CP 676; São Carlos, SP, Brasil, 13565 905

Although marine salt may reduce the efflux of plasma NaCl and minimize such ions unbalance, sodium chloride use for fishes transport has been controversial, because the positive effects are not always observed. In fact, salt has no effect in some North America fishes transport (Peter and Barton, 1982; Carmichael *et al*., 2001). There is one study evaluating the marine salt use for matrinxã transport in 200 L tanks supplied of water and oxygen diffusion system (Carneiro and Urbinati, 2001). These authors concluded salt in concentration of 6 g L^{-1} may reduce, but not completely avoid transport stress in such conditions. Other study tested the use of clove oil to lightly anesthetize matrinxa during transport and some stress-reducing effects (on cortisol and ions balance) could be observed (Inoue *et al*., 2005). Matrinxã *Brycon cephalus* is a commercially important fish from the Amazon basin. This species is relevant for industry mainly to the market that sells live fish for sport fishing (Cyrino *et al*., 1986; Mendonça *et al*., 1993). Nevertheless, matrinxã is particularly sensitive. Many frustrating consequences of stress during hauling and transport are observed as scales losses, skin damages and mortalities.

In the present research, the energetic metabolism and some physiological stress responses of juvenile matrinxã to cope with transport in plastic bags were evaluated. Salt was tested in concentration of 6 g L^{-1}. The post-stress status was also approached to gauge the ability and the length of time necessary for recovery. This time may indicate the critical period for matrinxã after transportation procedures.

Materials and Methods

Matrinxã from a same strain were reared in 2000 L fiberglass tank (holding tank) for 4 months in the facilities of the Genetics and Evolution Department of the Federal University of São Carlos (Brazil). Fish were fed commercial pellets (30% crude protein) twice a day until reach 60.0±6 g and 16.5±0.5 cm. Water was supplied to the tanks in a re-circulating system of filters that keeps water quality parameters [temperature (25.7±0.9°C), oxygen (5.66±0.07 mg L^{-1}), water conductivity (74.3±4.8 μS cm) and pH (7.0)] appropriate for matrinxã.

Experimental Design

Fish in a holding tank (40 fish m^{-3}) were starved for 24 h before the experimental procedures. A group of 10 fish (control-neither submitted to hauling nor transport) was sampled and killed by a sharp blow to the head. Blood was collected from the caudal vein using heparinized syringes. Liver samples were excised and transferred to liquid nitrogen. Afterwards, 60 fish were equally distributed in 6 plastic bags of 50×85 cm (10 fish/bag), filled with 10 L of water. Marine salt was added to 3 bags to achieve a final concentration of 6 g L^{-1}. The water of all the bags was saturated using pure oxygen and sealed as the usually done in the field. Bags were placed in a car and driven around for 4 h. Thereafter, fish from one bag of each group (salted water and freshwater) were sampled (0 h after stressors). The fish from the other 4 bags were respectively released in 4 tanks (250 L) to recover. Twenty-four hours after, the fish from one tank of each transported fish group were sampled. The fish from the two remaining tanks were sampled 96 h after stressors. Blood and liver were collected as described above. Water quality parameters were monitored in all steps of the experiment.

Laboratorial Techniques

Hematocrit (Goldenfarb *et al*., 1971), total hemoglobin (Collier, 1944) and red blood cells number (Lima *et al*., 1969) were determined. Blood parameters of mean corpuscular volume (MCV), mean

corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were consequently calculated (Lima *et al*., 1969).

Blood aliquots were centrifuged at 14,400 *x* g during 3 min at 4°C for plasma separation. Plasma cortisol was determined through Radio Immune Assay (RIA) in the Laboratory of Endocrinology from the Medical School of Ribeirão Preto, University of São Paulo (FMRP/USP). Sodium (Na^+) and potassium (K^+) were quantified through flame photometry. Chloride (APHA, 1980), total protein (Lowry *et al*., 1951), glucose (Dubois *et al*., 1956) and ammonia (Gentzkow and Masen, 1942) were determined using colorimetric methods.

Liver samples were defrosted and 100 mg were immediately disrupted in 2 N KOH at 100°C in order to determine glycogen as Bidinotto *et al*. (1997).

Statistics

Statistical analyses were performed through the software Graph Pad Instat. When the F-values indicated difference at the significance level of $p<0.05$, the Tukey test was employed for means comparisons.

Results

Fish mortality was not observed during transport and recovery. During the transport, water temperature and pH decreased slightly and the oxygen concentrations were high due to the use of pure oxygen in the bags. Water total ammonia reached values extremely high as always observed in the fish transport (Table 1).

Table 1: Water parameters in the transport of *Brycon cephalus*

Condition	Water temperature (°C)	Water dissolved oxygen (mg L^{-1})	Water total ammonia (mg L^{-1})
Initial	29.2	5.17	0.793
Freshwater			
AT	27.2	9.07	9.987
R_{24}	26.8	4.13	0.845
R_{96}	27.0	4.70	0.799
Salted water			
AT	27.6	9.63	10.685
R_{24}	26.8	4.27	0.851
R_{96}	27.0	4.70	0.782

Fish were transported during 4 h in plastic bags containing 10 L of freshwater or salted water (6 g L^{-1}). After transport (AT) two bags were sampled and the remaining bags were let to recover for 24 h (R_{24}) and 96 h (R_{96})

Table 2: Plasma Na^+, Cl^- and protein of *Brycon cephalus* submitted to transport

Condition	Na^+	Cl^-	Protein
Initial	102.0±11	174±19	0.30±0.03
Freshwater			
AT	42.8±15*	61±3*	0.14±0.01*
R_{24}	98.2±11	171±20	0.24±0.04
R_{96}	111.2±3	169±18	0.36±0.02
Salt-water			
AT	58.3±4*	81±9*	0.15±0.02*
R_{24}	109.0±2	186±6	0.28±0.03
R_{96}	107.7±7	183±17	0.33±0.05

Fish were transported during 4 h in plastic bags containing 10 L of freshwater or salted water (6 g L^{-1}). After Transport (AT) two bags were sampled and the remaining bags were let to recover for 24 h (R_{24}) and 96 h (R_{96}). The values express the mean±SD of plasma concentration of Na^+, Cl^- and protein. The mark (*) means significant difference ($p<0.05$) by Dunnett test between the control and the indicated group

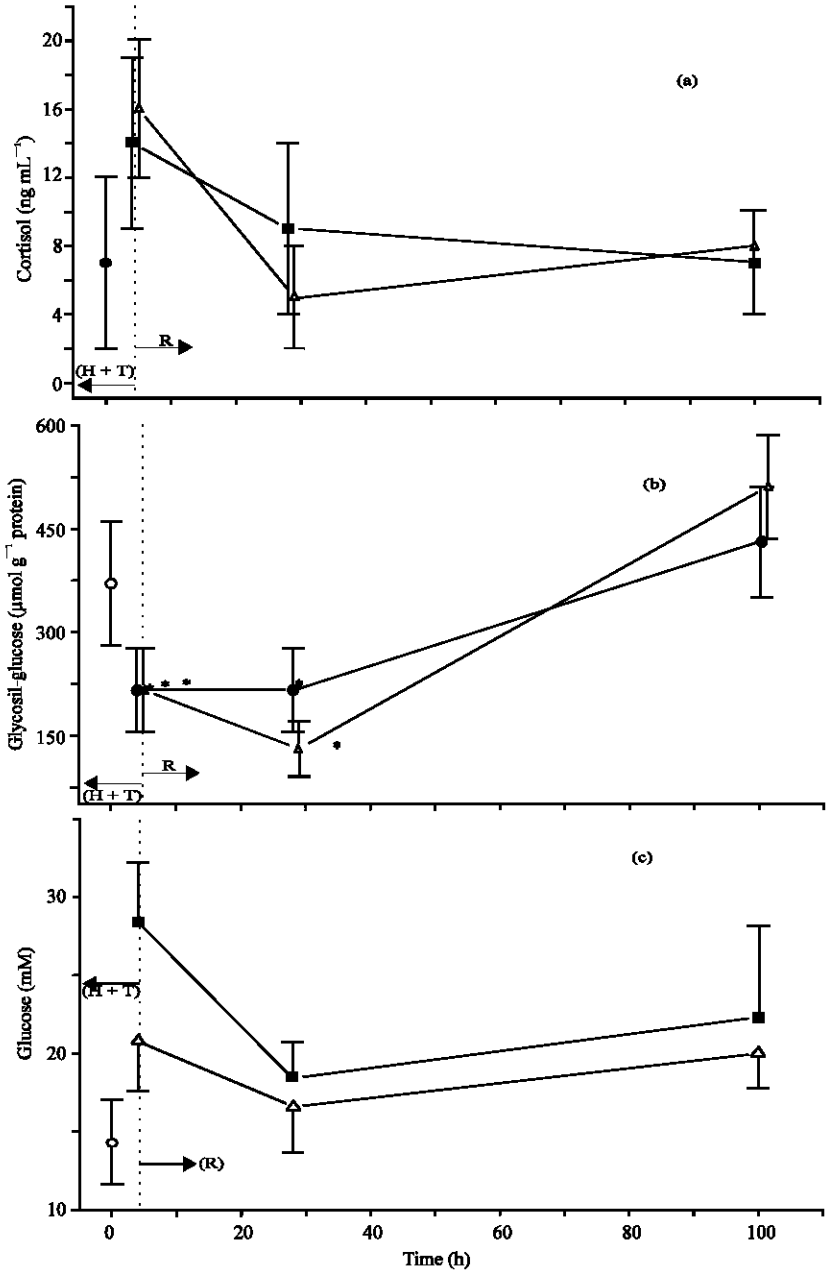

Fig. 1: Plasma cortisol (a), liver glycogen (b) and plasma glucose (c) in juveniles *Brycon cephalus* submitted to hauling (1 min) and transport (4 h) (H+T) and recovery (R) for 96 h. Fish were loaded in plastic bags with fresh water (■) and salted water (Δ) 100 mM NaCl (6 g L^{-1}). Recoveries were held in freshwater. (*) Indicates significant difference between means (±standard error of mean) of control (o) and the indicated groups by the Tukey test at $p<0.05$

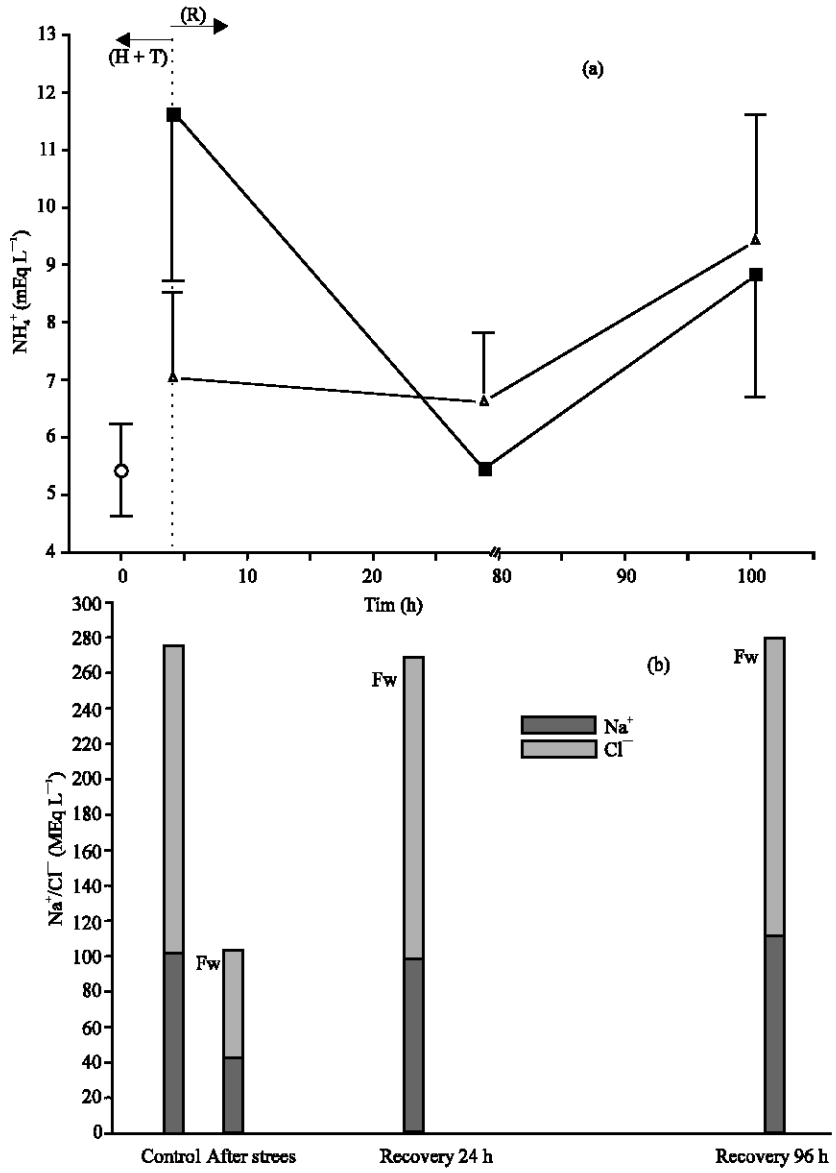

Fig. 2: Plasma ammonia (NH_4^+) in the course of stress and recovery of juveniles *Brycon cephalus* submitted to hauling (1 min) and transport (4 h) (H+T) in Freshwater (Fw) and Salted water (Sw). Recoveries were held in freshwater (R). (*) Indicates significant difference between means (± standard error of mean) of control (o) and the indicated group by the Tukey test at $p<0.05$

The values of hematocrit (30±3%), total hemoglobin (9.8±1.4 g/100 mL), RBC ($2.2\pm0.4\times10^6$ cells mm^{-3}), MCV (137±7 μmm^3), MCH (40±6 pg) and the MCHC (2.5±0.1%) were constant during all the experiment.

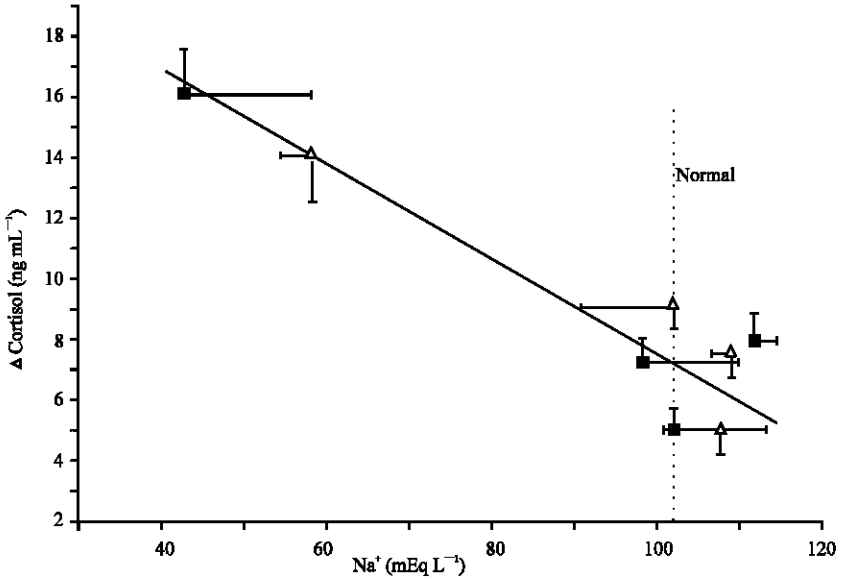

Fig. 3: Plasma concentrations of cortisol relative to plasma Na^+ concentration in juveniles *Brycon cephalus* submitted to hauling (1 min) and transport (4 h) and recovered for 96 h in freshwater (■) and salted water (Δ)

The plasma cortisol doubled ($p<0.05$) after transport in both fresh and salted water groups. Those values returned to the initial levels 24 h after stressors (Fig. 1a). The liver glycogen bulk decreased after hauling and transport (Fig. 1b) remaining low for 24 h. Initial levels of liver glycogen were retrieved after 96 h. Plasma glucose increased in the freshwater group after transport (Fig. 1c).

The plasma sodium, chloride protein concentrations decreased after transport in both freshwater and salted water groups (Table 2). Plasma ammonia concentrations increased in the freshwater group after transport (Fig. 2). The plasma Na^+ decreases were related to the plasma cortisol increases (Fig. 3), but it did not reflect the external salinity.

Discussion

The water quality in the transport is particularly important. In the present work, the water quality was similar to that reported in the field. The use of pure oxygen in the bags provides high concentrations, but it was not apparently deleterious for matrinxã. Fish density within the containers is high which values may reach 300 kg m^{-3} (Carmichael *et al*., 2001; Urbinati *et al*., 2004). Water deteriorated in all bags during transport and salt had no effect for reducing fish ammonia excretion. In both cases, the metabolic activity increased as consequence of stress initiated by crowding and gross movement of the water. Physiological disorders as hypoxia, high ammonia excretion and injuries may take place. The result could also be fish mortality (Staurnes *et al*., 1994), but fish survived during all the experiment.

Blood values of hemocrit, total hemoglobin, RBC, MCV, MCH and MCHC were constant. However, other stressors as exposures of matrinxã to chemicals in concentrations close to lethal have showed to change blood parameters (Avilez *et al*., 2005). By way of comparison, this may indicate our hauling and transport was lighter and shorter stressors than Avilez *et al*. (2005) imposed to matrinxã.

Catecholamine and cortisol increase during hauling and transport (Iwama, 1998; Perry and Bernier, 1999). Adrenaline causes a sequence of physiological responses to acute stress and the increase of blood pressure leads to NaCl losses through the gills (McDonald and Milligan, 1997). Although matrinxã had ion losses after transport as expected, the observed values were unusual (about 50%). In salmon, it is reported fish mortalities after 35% plasma NaCl losses as a consequence of acute handling (McDonald and Milligan, 1997).

It is reported that hard waters may reduce ion losses (McDonald and Rogano, 1986). Matrinxã is a natural species from the Amazon basin (Goulding, 1980), where a large amount of aquatic environments with soft and acidic waters are observed (Esteves, 1988). In view of that, this species should be more resistant to low levels of plasma NaCl than others. The increase of water salinity by the addition of marine salt, in order to reduce fish stress (Carneiro and Urbinati, 2001), is directly related to plasma Na^+ and Cl^- concentration, which is conversely related to cortisol (McDonald and Milligan, 1997). Marine salt is often added to freshwater to reduce fish stress because the water osmolality is near to the physiological one and it may avoid the reduction of the plasma NaCl efflux (McDonald and Milligan, 1997). However, marine salt (6 g L^{-1}) was not effective to avoid ionic losses in matrinxã. The plasma Na^+ decreased in contrast to the cortisol, which increased after the imposition of the stressors. Our values of plasma cortisol were similar in both freshwater and salted water groups. This was also observed in juvenile rainbow trout, in which the plasma cortisol levels were not decreased by the use of salt (Peter and Barton, 1982). Electrolyte disturbances of matrinxã allowed supposing that the effect of catecholamine is drastic in matrinxã and the intensity of the initial stress is certainly crucial for the transport stress responses profiles. Salt could not reduce the matrinxã responses once activated in the initial procedures of the experimental design. Fish handling to load the fish in the plastic bags showed to determine the fish transport physiological stress responses. In fact, Urbinati *et al.* (2004) observed significant cortisol increase immediately after loading of fish in plastic bags. However, Carneiro and Urbinati (2001) reported salt could reduce the cortisol response of matrinxã during transport in 200 L tanks. This condition seems to be less stressful to fishes than the transport in plastic bags. Water movement in the 200 L fish transport containers is lower than in the plastic bags used for fish transport suggesting a slighter stressor.

The water quality and the absence of other sources of stress in the course of recovery contributed to the ionic reestablishment of matrinxã in 24 h. Fish mortality is usually expected as a component of the stress response (McDonald and Milligan, 1997). The normal plasma cortisol level was recovered quickly and was probably the reason for the utter survival of fish.

The increase of plasma glucose concentration is another common response of fish to stressors like hauling and transport (Hattingh, 1976; Carmichael *et al*., 1983). Although marine salt was not effective to prevent stress, lower values of plasma glucose after transport were observed in the salted water group. Glycogen bulks were equally mobilized from the liver in both freshwater and salted water groups. Therefore, the glycogenolysis supplied the energetic metabolism to tolerate the stress in the course of the experiment.

Protein metabolism is usually intense during transport resulting in high ammonia excretion. The plasma ammonia of matrinxã was duplicated in the freshwater group. Ammonia excretion is among the most relevant factors of water deterioration during fish transport, which may result in mortality of many ammonia sensitive species (Arana, 1997). In contrast, tropical warm water fish as matrinxã can tolerate extreme concentrations of ammonia (Carneiro and Urbinati, 2001). Although ammonia diffusion through the gills is the main mechanism of excretion, a system of Na^+/NH_4^+ exchange is also discussed (Carneiro and Urbinati, 2001). Marine salt improved the waste of ammonia in matrinxã. This fact was probably due to the increased external availability of sodium facilitating the ions exchange.

In conclusion, the transport of matrinxã in plastic bags was stressful in both conditions of freshwater and salted water. Marine salt did not present substantial effect on NaCl efflux during transport. Matrinxã is exceptionally resistant to low plasma levels of plasma Na^+ and Cl^-. Hauling and transport of matrinxã requires large amount of energy, which is associated to the catabolism of proteins and carbohydrates. Marine salt reduced the plasma glucose and ammonia concentration recommending the use for transport of matrinxã as prophylactics of these stress responses. Matrinxã responsiveness to hauling and transport in plastic bags is evident indicating high sensitivity to transport. Critical period after these stressors is from 24 to 96 h for complete recovery.

Acknowledgements

The authors are thankful to "Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq" (Processo 150666/2005-5) for the financial support. We are also indebted to Dra. Lucila Elias (FMRP-USP) for suggestions and assistance during this work.

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