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Comparison of the Respiratory Metabolism of Juvenile *Litopenaeus vannamei* Cultured in Seawater and Freshwater

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Abstract *Litopenaeus vannamei*, a euryhaline species, can be cultured at a wide range of salinities. The emergence of freshwater pond-culture of *L. vannamei* is an important prelude to the continued development of shrimp culture in China. In this study, we compared the respiratory metabolism of juvenile *L. vannamei* cultured in freshwater and saltwater by measuring their oxygen consumption rate (OCR), ammonium-type nitrogen excretion rate (AER) and pyruvate kinase (PK) and lactate dehydrogenase (LDH) activities at different molting stages in order to physiecologically characterize juvenile *L. vannamei* under freshwater conditions. The results showed that OCR was significantly higher in saltwater than in freshwater at all stages of molting cycle. However, variation of OCR among molting stages in saltwater was similar with that in freshwater, and the highest OCR was observed at post-molting stage. At all stages of molting cycle, AER was significantly higher in freshwater than in saltwater, and the highest was observed at post-molting stage. The activity of PK was significantly higher in saltwater than in freshwater. Conversely, the activity of LDH was higher in freshwater than in saltwater in general. Significant variation of PK and LDH activities in molting cycle was observed in saltwater and freshwater. The results indicated that aerobic metabolism of juvenile *L. vannamei* was more active in saltwater than in freshwater; while its protein metabolism was more active in freshwater than in saltwater.

Key words *Litopenaeus vannamei*; saltwater; freshwater; respiratory metabolism; molt stage

1 Introduction

The pacific white shrimp, *Litopenaeus vannamei*, is native to the eastern Pacific. Since 1980s, seawater culture of *L. vannamei* developed rapidly in China, especially in eastern coastal areas (BOF, 2004). With the development of breeding techniques, the hypo-salinity culture pattern independent of saltwater appeared and implemented in saline and alkaline soil areas (Zhu *et al*., 2004; Liu *et al*., 2007). Since 2000, numerous freshwater culture were tried in inland regions and met with success (Xie and Gan, 2000; Pan *et al*., 2001; Araneda *et al*., 2008). With the intensification of the freshwater culture, the annual yield of freshwater cultured *L. vannamei* in China soared to 160000 t in 2002.

Respiratory metabolism, an important bio-energetic process in crustaceans (Adcock, 1982), embodies the physiological response to the ambient environment (Dall *et al*., 1992). In addition to environmental factors (Gonzalez *et al*., 2010; Walker *et al*., 2011), the intrinsic conditions also affect the respiratory metabolism (Chae and Paul, 1976; Dong *et al*., 1994). Molting is an essential physiological process for crustaceans. The process has been intensively studied in physiological, histological, endocrine and behavioral aspects (Brown and Cunningham, 1939; Rao, 1965; Waraporn *et al*., 2007; Chantal *et al*., 2008). Researches on the metabolism in molting process have been conducted in many species (Penkoff and Thurberg, 1982; Stern and Cohen, 1982; Cockroft and Wooldridge, 1985; Carvalho and Phan, 1997); however, such researches in *L. vannamei* cultured in freshwater are scarce.

In this study, we investigated the oxygen consumption rate (OCR), ammonium type nitrogen excretion rate (AER) and metabolic enzymes activity of juvenile (in molting process) *L. vannamei* cultured in saltwater and freshwater. We aimed to characterize physiologically of *L. vannamei* cultured in saltwater and freshwater and improve the freshwater culture technique of *L. vannamei* scientifically.

2 Materials and Methods

2.1 Experimental Animals

Juvenile *L. vannamei* was collected from a shrimp farm

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located at Jiaozhou, Qingdao, Shandong, China, transported to Laboratory of Aquaculture Ecology, Ocean University of China in 2 h with continuous aeration, and acclimated for 7 d. Water was controlled at 25.0℃±0.5℃, >7.0 mg L^{-1} dissolved oxygen, salinity 20 during acclimation. The simulated natural photoperiod (14L: 10D) was applied.

Four hundred shrimp individuals were divided into saltwater group and freshwater group. For saltwater group, the salinity was increased to 30 (2–3 per day) by replacing certain percentage of the water with seawater. The shrimp was acclimated continually at 30 for another 15 d ahead of use. For freshwater group, the salinity was reduced 2–3 per day to 5 by replacing the water with tap water aerated for 3 d before use, and then 0.5–1 per day to 0.5. The shrimp was acclimated at 0.5 for 15 d. After acclimation, shrimp individuals with of similar body sizes (4–5 cm) were chosen for experimental use.

2.2 Molting Stages Determination

An ecdysis cycle was divided into different molting stages including post-molting (stages A and B), intermolting (stage C) and pre-molting (stages D_0, D_1, D_2 and D_3). Molting stages were identified and confirmed by comparing the setae morphology under an Olympus microscope (40×) (Drach, 1939; Cesar *et al*., 2006).

2.3 Experimental Apparatus and Sample Collection

The experiment was conducted with two identical sets of flow-through apparatus for saltwater and freshwater, respectively (Fig.1). The respiratory chambers (1 L) were sealed with a rubber plug and installed with both an inlet pipe and an outlet pipe. Flow velocity was adjusted through the gate valve. Water in the system was maintained at 25.0℃±0.5℃ and >7.0 mg L^{-1} dissolved oxygen. The salinity of water used in saltwater and freshwater experiment was equal to that for acclimation.

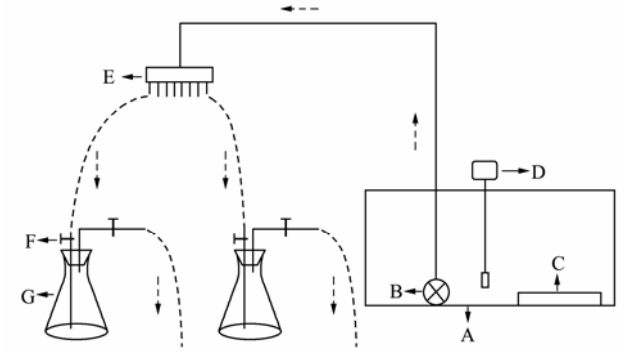

Fig.1 Schematic diagram of breathing apparatus. Dashed arrows represent water flow direction; A, flume tank; B, submersible pump; C, temperature-control system; D, air pump; E, shunting device; F, gate valve; G, respiratory bottle.

At certain molting stages, shrimp was identified and collected everyday from both saltwater and freshwater groups. Collected shrimp was transferred into the respiratory chamber of flow-through apparatus for a 2-h acclimation, during which the gate valves of apparatus were opened and the water was circulated. Three respiratory chambers with no shrimp were set as controls in order to correct the error caused by bacterial respiration. After acclimation, the gate valves were shut down. The experiment lasted 1 h. At the end, water samples were collected for determining OCR and AER. Shrimp testers were dried with paper towels and weighed individually with an MP-120 electronic balancer. The muscle and hepatopancreas of the shrimp were dissected on ice and stored in a freezer for further metabolic enzymes analysis. Ten replicates each molting stage were analyzed. The experiment was conducted in the same time window, 8:00am–9:00am each day, to avoid variation in respiratory metabolism due to circadian rhythm (Carvalho and Phan, 1997).

2.4 Analyzing Methods

Dissolved oxygen of the water samples was measured with Winkler-method (Strickland and Parsons, 1968). Ammonium-type nitrogen concentration of water samples was measured with phenol-hypochlorite method (Koroleff, 1970). OCR (mg g^{-1} h^{-1}) and AER (μg g^{-1} h^{-1}) were calculated with

$$OCR=(C_0-C_t)\ V\ T^{-1}W^{-1},$$

$$AER=(N_t-N_0)\ V\ T^{-1}\ W^{-1},$$

where C_0 (mg mL^{-1}) is the final dissolved oxygen concentration of blank bottle; C_t (mg mL^{-1}) is the final dissolved oxygen concentration of experimental bottle; N_0 (μg mL^{-1}) is the final ammonium-type nitrogen concentration of blank bottle; N_t (μg mL^{-1}) is the final ammonium-type nitrogen concentration of experimental bottle; V (mL) is the respiratory bottle cubage; W (g) is the wet body weight of shrimp testers, and T (h) is experimental time.

O:N ratio was calculated by dividing the atomic number of oxygen consumed by that of nitrogen excreted at each molting stage. Hepatopancreas and muscle were weighed and homogenized in ice-cold Tris/HCl buffer (50 mmol L^{-1}, pH 7.5) using a tissue homogenizer. Temperature was maintained at 4℃ during homogenization. Homogenates were centrifuged at 10000×g and 4℃ for 10 min with supernatants used for enzymes activity assay. The activity of PK in hepatopancreas and LDH in muscle of shrimp testers were analyzed with the method described by Tanaka *et al*. (1962) and Thébault (1984), respectively. The concentration of total protein was assayed with Lowry method (Lowry *et al*., 1951).

2.5 Data Analysis

Data were processed with SPSS 11.5 statistical software. Kolmogorov-Smirnov test and Levene's test were used to check the normality and homogeneity of variance. The data were subjected to two-way ANOVA to check the effects of two factors, molting stage and salinity. If an interaction between molting stage and salinity was detected, the one-way analysis (SNK test) was conducted to

examine how salinity affected OCR, AER, PK and LDH at each molting stage and how molting stages affected OCR, AER, PK and LDH at different salinities. If an interaction between molting stage and salinity was not detected, the one-way analysis was conducted to analyze the difference of average at each molting stage and independent-samples T test was conducted to analyze the difference of average in freshwater and saltwater group. Difference were reported as significant when $P<0.05$.

3 Results

3.1 Oxygen Consumption Rate

OCR of juvenile *L. vannamei* was significantly ($P<0.05$) affected by both salinity and molting stage (Table 1). No interaction between salinity and molting stage was found. In saltwater and freshwater group, OCR changed in the same way in the whole molting cycle. As showed in Fig.2, the average of OCR of saltwater and freshwater group was high from D_3 to B stage, decreased to a low level at C stage, and then increased after D_2 stage, which indicated that molting resulted in high oxygen consumption. The average of OCR of all molting stages was significantly lower in freshwater group than in saltwater group.

Table 1 The two-way analyzing results of OCR and AER of *L. vannamei*

	OCR				AER			
	DF	F	Sig.	Partial eta squared	DF	F	Sig.	Partial eta squared
Molting stage	6	8.505	0.000	0.309	6	10.813	0.000	0.411
Water condition	1	15.201	0.000	0.118	1	168.689	0.000	0.645
Molting stage *vs* Water condition	6	0.450	0.844	0.023	6	4.177	0.001	0.212

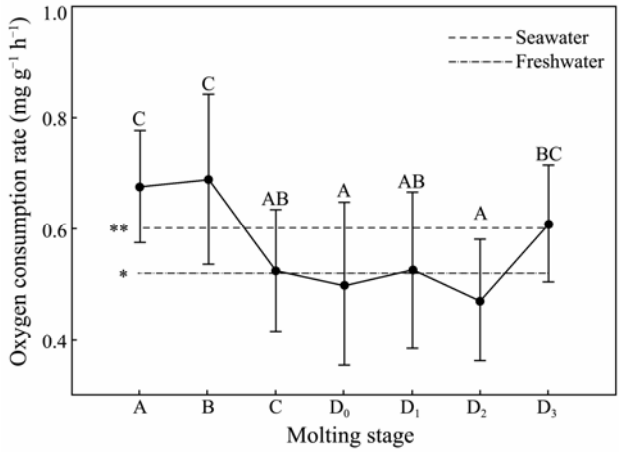

Fig.2 Oxygen consumption rate in juvenile *L. vannamei*. Black balls represent the average of OCR of saltwater and freshwater group at different molt stages. Dash line and dash-dot line represent average of OCR of all molting stages in saltwater and freshwater group, respectively. Different capital letters mean significant difference of average of OCR among molting stages. The different numbers of '*' mean significant difference of average of OCR in saltwater and freshwater group.

3.2 Ammonium-Type Nitrogen Excretion Rate

Salinity and molting significantly influenced AER of juvenile *L. vannamei*, and the interaction between salinity and molting stage was also significant ($P<0.05$) (Table 1). As showed in Fig.3, the whole level of AER in freshwater was significantly higher than that in saltwater ($P<0.05$). The result indicated juvenile *L. vannamei* spent much more nitrogen on physiological activities to maintain the homeostasis in freshwater than in saltwater. In freshwater, AER decreased after molting, touched down at D_2 stage, then increased at D_3 stage and reached the highest at A stage. A different pattern was found in saltwater; AER increased gradually from inter-molting to D_2 stage and decreased at D_3 stage. However, the highest of AER was found at A stage in saltwater and freshwater.

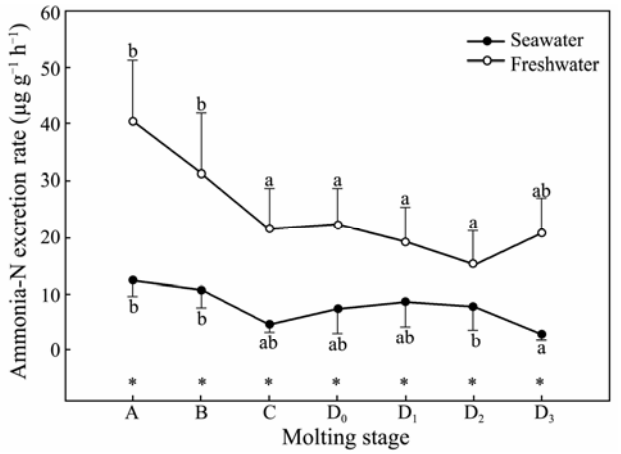

Fig.3 Ammonium-type nitrogen excretion rate in juvenile *L. vannamei.* The '*' means the significant differences between saltwater and freshwater at the same molt stage. Different letters mean significant differences among molt stages in saltwater group, whereas different capital letters mean significant differences among molt stages in freshwater group.

3.3 O/N Ratio

Table 2 shows that mean level of O/N ratio for juvenile *L. vannamei* was higher in saltwater than in the freshwater. The highest O/N ratio was found at D_3 stage in saltwater, whereas the lowest was found at A stage in freshwater.

Table 2 O/N ratio of juvenile *L. vannamei* at different molting stage in freshwater and saltwater

	A stage	B stage	C stage	D_0 stage	D_1 stage	D_2 stage	D_3 stage
Saltwater	40.92	45.05	66.01	38.39	37.84	38.83	126.33
Freshwater	13.15	17.40	19.08	16.48	19.46	23.43	19.97

3.4 Pyruvate Kinase Activity

Two-way ANOVA showed that molting and salinity affected PK in juvenile *L. vannamei* significantly and there was an interaction effect between them ($P<0.05$) (Table 3). Fig.4 illustrates that PK activity was significantly lower in freshwater than that in saltwater at each molting stage except for B stage ($P<0.05$). The highest activity of PK was found at B stage, whereas the lowest was at D_1 and D_2 stages in freshwater. Oppositely, the highest activity of PK was found at D_1 and D_2 stages, and the lowest was found at D_3 stage in saltwater.

Table 3 The two-way analyzing results of PK and LDH activity of juvenile *L. vannamei*

	PK				LDH			
	DF	F	Sig.	Partial eta squared	DF	F	Sig.	Partial eta squared
Molting stage	6	6.841	0.000	0.398	6	6.284	0.000	0.307
Water condition	1	141.709	0.000	0.696	1	4.646	0.034	0.052
Molting stage * Water condition	6	12.160	0.000	0.541	6	2.988	0.011	0.174

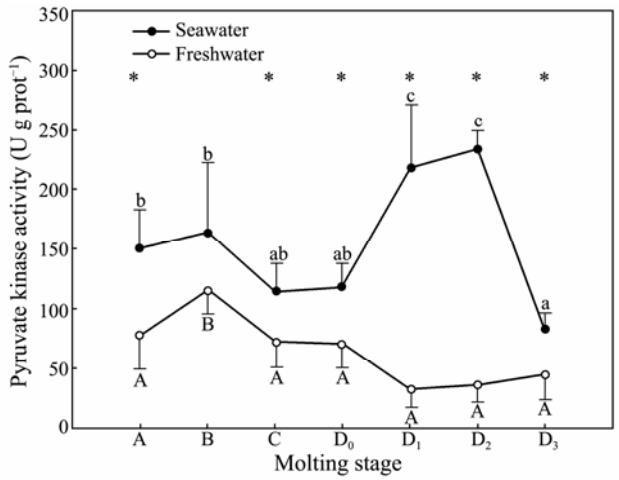

Fig.4 Pyruvate kinase activity in juvenile *L. vannamei*. The '*' means a significant difference between saltwater and freshwater at a molting stage. Different letters mean significant difference among molting stages in saltwater, whereas different capital letters mean significant difference among molting stages in freshwater.

3.5 Lactate Dehydrogenase Activity

Table 3 shows that both salinity and molting significantly affected the LDH activity in juvenile *L. vannamei* and there was an interaction effect between them ($P<0.05$). In freshwater, the fluctuation of LDH activity was wide in a molting cycle (Fig.5). LDH was the highest at D_0 stage, and lower during molting. LDH at A, D_2 and D_3 stages in freshwater was lower than in saltwater, whereas LDH was higher in freshwater at other stages. However, significant difference of LDH was found only at D_0 stage between two water conditions ($P<0.05$).

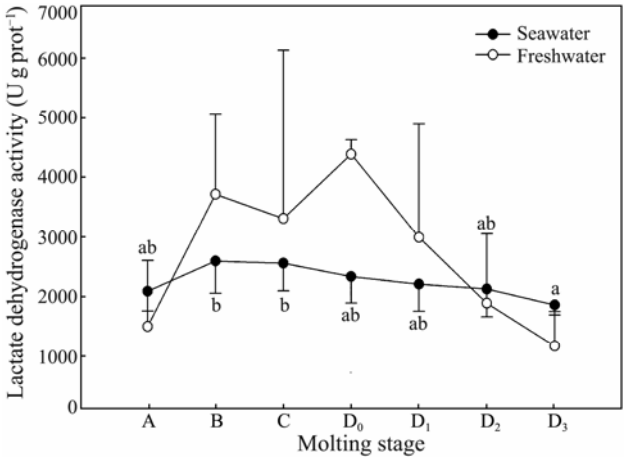

Fig.5 Lactate dehydrogenase activity in juvenile *L. vannamei*. The '*' means the significant difference between saltwater and freshwater at a molting stage. Different letters mean significant difference among molting stages in saltwater, whereas different capital letters mean significant difference among molting stages in freshwater.

4 Discussion

In present study, the first important finding is that the behavior of molting has an obvious effect on the respiratory metabolism of juvenile *L. vannamei*. Carvalho and Phan (1997) found that oxygen consumption reached the highest after molting in *X. kroyeri*, and it was nearly 40% higher than that at stages D_1 and D_2. Emmerson (1985) and Hagerman (1976) also reported that the oxygen consumption after molting was 50% and 100% higher than that at pre-molting stages in *Palaemon pacificus* and *Crangon vulgaris*, respectively. This study demonstrated the increase of OCR during the molting process in saltwater and freshwater. After molting, the soft cuticle makes the shrimp vulnerable to predators. There is a need for the shrimp to reconstitute tissues, synthesize protein and transport elements necessary for cuticle calcification (Dall *et al*., 1992). The calcification process, which was the consequence of natural selection, consumed a significant amount of energy and led to the OCR increase. In addition, the high activity of PK at post-molting stages reflected more energy was produced through anaerobic metabolism process. The energy could be used to feed the biochemical activities during calcification.

High AER after molting was observed in many shrimp species (Regnault, 1979; Stern and Cohen, 1982; Carvalho and Phan, 1997). In present study, increasing of AER after molting in both freshwater and saltwater demonstrated that protein serves as a main source of energy at post-molting stages in juvenile *L. vannamei*. In addition, shrimp needs to adapt to the new osmolality due to a large amount of water absorbance by tissue after molting (Hagerman, 1973). The osmoregulation in shrimp consumes more protein for energy supply (Li *et al*., 2009). This may be one of the reasons for OCR and AER in-

creasing in juvenile *L. vannamei* after molting.

Secondly, the aerobic metabolism and anaerobic metabolism of juvenile *L. vannamei* are obviously different in saltwater and freshwater, respectively. Oxygen requirement and oxygen consumption vary considerably with biotic and abiotic factors, including salinity (Brett, 1987). The response of respiration to changes in salinity generally is more complex in crustaceans (Kinne, 1966). Allan *et al*. (2006) pointed out that the respiratory metabolic rates in crustaceans may be increased or decreased in subnormal salinities and supranormal salinities. The results in this study revealed the decreasing of OCR in juvenile *L. vannamei* under low salinity conditions. Previous studies found that euryhaline shrimp lost salts in response to freshwater due to the osmotic gradient between their body fluids and external medium, which caused the shrimp to actively pump ions, *e.g*. Na^+, Cl^-, across the osmotic gradient in order to counteract the passive efflux of ions. In order to minimize this energy-consuming active ion transport, shrimp lowered their internal osmotic concentration to decrease the osmotic gradient (Schubart and Diesel, 1999). Hurtado *et al*. (2007) also found that osmolarity in *L. vannamei* decreased at low salinity. This further illuminated that *L. vannamei* can actively decrease the osmotic gradient and reduce the consumed energy for respiratory metabolism. According to Potts (1954), the tissue metabolism changed with salinity. However, the total mass of salt-absorbing tissue was so small that the changes in their metabolism were largely masked by the metabolism of the rest tissues. It coincides with the low respiratory metabolism level of juvenile *L. vannamei* in freshwater. However, bacteria respirations in saltwater and freshwater conditions were not compared in this study and the errors will be estimated in the next phase.

Endogenous nitrogen excretion in saltwater species was lower than freshwater species in shrimp with the same body weight (Li *et al*., 2006). The possible reason is that hydrolysis of tissue protein in shrimp is more active in freshwater than in seawater. Deaton (1994) indicated that in the salinity adaption process the aquatic animal released free amino acids (FAA) to extracellular environment through the gill cell, and the FAA decomposition was accelerated. Hurtado *et al*. (2007) found that the total concentration of FAA in muscle decreased in low salinity conditions. Shrimp prefers to use FAA as a metabolic substrate, and ammonium excretion increased significantly at low salinity (Liu, 2008). Therefore, the higher AER under freshwater condition may associate with FAA decomposition as was observed in this study.

As an effective strategy of osmoregulation, *L. vannamei* will increase its Na^+-K^+-ATPase activity at low salinities (Huong *et al*., 2010). This strategy needs more energy consumption to increase Na^+-K^+-ATPase activity. Glycolysis process, for the most part, can produce energy for many physiological activities. PK is an important rate-limiting enzyme in glycolysis process. In this study, we found that PK decreased in freshwater. As reported by Rosas *et al*. (1995), the carbohydrate in hepatopancreas did not vary during later period of a molting cycle in *Penaeus setiferus* and it was probably used up as energy supply after molting. Taking the earlier findings into account, we believe that glycolysis in shrimp hepatopancreas was inhibited and carbohydrate there failed of serving as the main energy source of osmoregulation in freshwater. In addition, Morohashi *et al*. (1991) found there was a close relationship between Na^+-K^+-ATPase activity and free fatty acid concentration. Thus we speculated fatty acid supplied the energy for osmoregulation in juvenile *L. vannamei* in freshwater. LDH is an indicator of anaerobic glycolyzing ability (Liu, 2010). Devi *et al*. (1993) found that the increased LDH activity in muscle may reflect increased dependence on anaerobic carbohydrate metabolism in fiddler crab *Uca pugilator*. In present study, the increased activity of LDH was found in muscle under freshwater conditions. This result demonstrates that glycolyzing ability is enhanced in muscle and carbohydrate is an important energy source for hydrolysis of tissue protein.

Ammonium-type nitrogen can be toxic to crustaceans when accumulated in water (Tomasso, 1994; Barbieri, 2010). Low dissolved oxygen or low salinity could increase ammonium toxicity (Wajsbrot *et al*., 1989; Russo and Thurston, 1991; Chen and Lin, 1992). In this study, the highest OCR and AER were detected after molting in freshwater, suggesting more attention should be paid to ammonium toxicity in intensive freshwater shrimp culture, and low dissolved oxygen should be avoided after centralized ecdysis. Increasing oxygenators is a useful approach to keeping the dissolved oxygen level and reducing ammonium toxicity. In addition, some micro-ecological agents also aid to reduce ammonium toxicity. The high AER average in freshwater indicated that protein metabolism was more rapid and more protein was used to maintain the inner homeostasis, suggesting more protein should be supplied to shrimp by increasing protein content of diet.

Acknowledgements

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