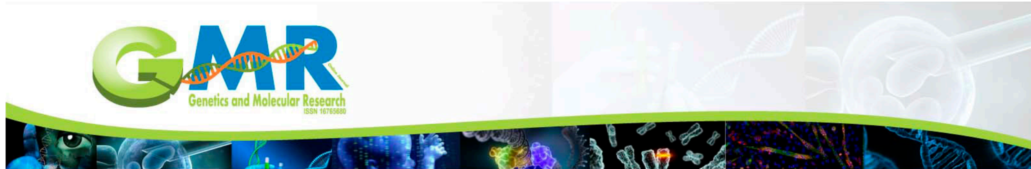

In vivo and *in vitro* inhibitory action of 17β-estradiol and environmental estrogen 4-nonylphenol on gonad-inhibiting hormone (GIH) expression in the eyestalks of *Litopenaeus vannamei*

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ABSTRACT. The gonad-inhibiting hormone (GIH) belongs to a neuropeptide family synthesized and released in an X-organ sinus gland complex of crustacean eyestalks. GIH inhibits crustacean ovarian maturation by suppressing vitellogenin (Vtg) synthesis, whereas estrogen is responsible for the stimulation of vitellogenesis (not established). In this study, the effects of 17β-estradiol (E_2, 10^{-6} M), estrogen receptor antagonist tamoxifen (TAM, 10^{-6}, 10^{-7}, and 10^{-8} M), and the environmental estrogen nonylphenol (NP, 1 μg/L and 100 μg/L) on *LvGIH* expression in the eyestalks of shrimp were determined by quantitative real-time PCR. Results showed that *LvGIH* expression decreased significantly during the *L. vannamei* ovarian maturation cycle. E_2 and NP significantly reduced *LvGIH* transcripts *in*

vivo, but TAM neutralized the inhibitory action of E_2 in a dose-dependent manner (P < 0.05). In addition, the *LvGIH* expression levels decreased significantly in a time-dependent manner (P < 0.05) when ovary fragments were cultured *in vitro* with E_2. The results of this study suggested that estrogen regulates GIH expression in *L. vannamei* eyestalks. E_2 promoted ovarian development not only by directly upregulating vitellogenesis in the hepatopancreas, but it was also capable of downregulating *LvGIH* expression, which indirectly resulted in the stimulation of *L. vannamei* vitellogenesis.

Key words: *Litopenaeus vannamei*; Gonad-inhibiting hormone; 17β-estradiol; Nonylphenol; Estrogen receptor antagonist

Introduction

In decapod crustaceans, a number of neurohormones that regulate vitellogenin (Vtg) synthesis and oocyte maturation are produced in the X-organ sinus gland complex (XO-SG) of eyestalk optic ganglia, the center of the crustacean neuroendocrine system. Gonad-inhibiting hormone (GIH) is an important neuropeptide hormone that has a prominent inhibiting effect on vitellogenesis. In the lobster *Homarus americanus*, GIH levels in hemolymph were high during the immature and previtellogenic stages and low during maturation (De Kleijn et al., 1998). Furthermore, the injection of anti-GIH antibodies can produce ovarian maturation and spawning effects similar to those resulting from unilateral eyestalk ablation in *Penaeus monodon* (Treerattrakool et al., 2014). In *Metanephrops japonicus* and *Litopenaeus vannamei*, recombinant GIH administration can significantly reduce *Vtg* mRNA levels (Ohira et al., 2005; Chen et al., 2014). These results strongly suggest that GIH is a major factor involved in female crustacean reproduction.

In a previous study, Warrier et al. (2001) demonstrated that E_2 can affect vitellogenesis in crayfish, and evidence indicated a correlation between E_2 levels and gonadal development in *Cherax albidus* (Coccia et al., 2010). Furthermore, E_2 has been identified in the hepatopancreas, ovary, and hemolymph of several crustaceans (Couch et al., 1987; Fairs et al., 1990; Warrier et al., 2001). The fluctuation of Vtg levels during ovarian maturation processes was closely correlated with E_2 levels in *P. monodon* (Quinitio et al., 1994), and the amount of Vtg increased when ovarian fragments were incubated with E_2 in *L. vannamei* (Quackenbush, 1992). An environmental endocrine disrupting chemical (EDC) known as 4-nonylphenol (NP) is suggested to have high estrogenic potency (Noppe et al., 2005), and it is known to interfere with normal vitellogenesis (Ghekierea et al., 2006; Hannas et al., 2011; Ara and Damrongphol, 2012). Generally, estrogen exposure in crustaceans can induce Vtg expression (Atienzar et al., 2002; Vandenbergh et al., 2003).

In vertebrates, sex steroid hormones regulate gonadal development, and can feedback to an upstream neuroendocrine factor as a downstream signal factor (Ye et al., 2008). There are reports of the presence of estrogen and androgen receptor (ER and AR) immunoactivities in the brain and thoracic ganglion of the crab, and the results suggest that estrogen and androgen may be involved in the feedback regulation of crustacean neuroendocrine processes (Ye et al., 2008). Further study showed that there could also be a close link between oogenesis, follicle cell proliferation, hepatopancreas cell metabolism, and endocrine regulation. Therefore, it is possible that estrogens might be involved in the regulation of oocytes at early stages in mysid shrimp

(*Neomysis japonica*) (Yang et al., 2012). The brain and eyestalks are well-known major synthesis sites for GIH that is involved in crustacean reproductive endocrine functions (Treerattrakool, 2008; Chen et al., 2014). However, whether estrogens are also involved in the feedback regulation of GIH synthesis and secretion is still unknown.

In the present study, the expression of *GIH* in the eyestalks of *L. vannamei* (*LvGIH*) from oogonium proliferation to maturation stages was examined. In order to demonstrate that estrogens are involved in the regulation of *GIH* expression, we determined the *GIH* expression levels under different treatments with natural estrogen E_2, estrogen receptor antagonist tamoxifen (TAM), and environmental estrogen NP both *in vivo* and *in vitro*. This study offers information to enrich our understanding of the crustacean neuroendocrine system during gonad maturation.

MATERIAL AND METHODS

Animals

Female *L. vannamei* ranging from 8 to 13 cm in total length were purchased from Zhanjiang Hengxing Cultivation Base (Zhangjiang, Guangdong, China). Total RNA of the eyestalks was extracted using the TriZol reagent (Invitrogen, USA). Ovarian development stages were determined based on ovarian histological sections.

Previtellogenic females were selected for E_2, TAM, and NP injection experiments. Animals were kept in seawater at 28ºC under a natural photoperiod, and were fed a commercial prawn diet twice per day.

Gonadal histology

A small piece of ovary was dissected and fixed in Bouin's solution. Subsequently, the fixed ovary was embedded in paraffin and sectioned at 7-μm thickness. The sections were stained with hematoxylin and eosin, and the ovary developmental stages were determined based on the criteria described by Li (2012).

Experimental design and sampling procedures

E_2 (Sigma, USA) stock solution was dissolved in ethanol and diluted to 10^{-6} M with 0.9% saline solution. TAM (Sigma) was dissolved in glacial acetic and diluted from 10^{-6} M to 10^{-8} M. Thirty *L. vannamei* were divided into six groups (five per group): control [0.9% saline), E_2 (10^{-6} M), TAM (10^{-6} M), and E_2 (10^{-6} M with TAM (10^{-6}, 10^{-7}, 10^{-8} M, respectively)]. At 24 hours after injection, the eyestalks were sampled and stored at -80ºC for subsequent RNA extraction and *LvGIH* expression detection.

For *in vitro* incubation, the eyestalks from previtellogenic females (n = 5) were dissected and washed with sterile saline solution. The eyestalks were cut into small fragments, and were then transferred into a well of a 24-well plate (average of 4 eyestalks/well), followed by pre-culture at 25ºC for 2 h. Subsequently, the eyestalk fragments were incubated in RPMI-1640 culture (Hyclone, China) with 10^{-6} M E_2. The controls were cultured in saline solution without E_2. Eyestalk fragments were collected for RNA extraction at 0, 3, 6, 12, and 24 h. Previtellogenic females were immersed in seawater containing NP (final concentrations of either 1 μg/L or 100 μg/L). At 0, 3, and 5 days

after NP treatment, five shrimp from each group were randomly sampled, and the eyestalks were collected for RNA extraction.

Eyestalk *GIH* expression in *L. vannamei*

Total RNA from eyestalks was extracted using the TriZol reagent (Invitrogen) according to manufacturer instructions. DNAse I was used to remove genomic DNA contamination, and 2 μg total RNA was reverse transcribed into first-strand cDNA using M-MLV reverse transcription polymerase (Promega, USA). Specific primers, GIH-R (5'-TCCCAGTCAGTTCCCGTAGA-3') and GIH-F (5'-ACGCCTTGGCTGTATTCCTT–3'), were designed according to the *LvGIH* sequence (Genbank accession No. KF879913). The primers were used to detect *LvGIH* expression using quantitative real-time PCR *SYBR*(R) *Premix Ex Taq*™ (Takara, Japan) according to the manufacturer protocol. An internal control, β-actin transcript, was amplified with the primers Actin-F (5'-TCCTCACCCTGAAATACC-3') and Actin-R (5'-TCAGGATCTTCATCAGGTAGT-3') as a reference for total RNA levels used to calculate relative *LvGIH* expression. All primers were synthesized by Sangon Biotech (Shanghai, China).

Data analysis

All data were subjected to one-way analysis of variance followed by Duncan's multiple range tests. Differences were considered significant when $P < 0.05$. The values are presented as mean values ± S.E.M.

Results

LvGIH expression in eyestalks during ovarian development

Based on the ovarian histology of *L. vannamei*, ovarian developmental can be divided into four different stages (Figure 1). The highest *LvGIH* expression levels were observed at stage I, in which the ovary predominately consisted of oogonia, and the amount decreased gradually during stage II (mainly previtellogenic oocytes), stage III (mainly vitellogenic oocytes), and stage IV (oocyte maturation). As compared to stage I ovaries, the *LvGIH* expression levels doubled at stage II, and the levels increased 12-fold at stage III and sixty-two times at stage IV (Figure 2).

Effect of E2 on *LvGIH* expression *in vitro* and *in vivo*

The *LvGIH* expression levels decreased dramatically in a time-dependent manner during *in vitro* incubation of eyestalks with E_2. The expression levels decreased to a low value after 6 h of incubation, and remained at low transcript levels until the end of the experimental period (Figure 3). Regarding the results of *in vivo* studies, *LvGIH* expression with E_2 injection was significantly lower than the control group after 24 h. However, there was no significant difference between the TAM and the control group. Moreover, when the eyestalks were incubated in 10^{-6} M E_2, *LvGIH* expression levels decreased in a dose-dependent manner with increasing TAM dosage, and there was no significant difference in *LvGIH* expression in eyestalks between the control and the 10^{-6} M TAM group (Figure 4).

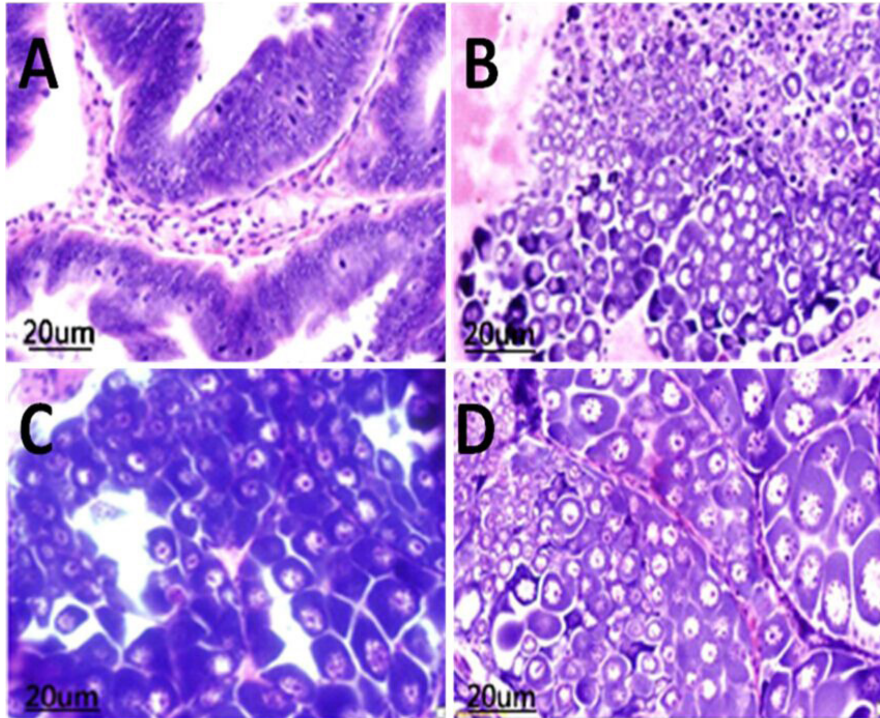

Figure 1. Gonadal histology of female *L. vannamei*. **A.** oogonium proliferation stage (stage I); **B.** previtellogenic stage (stage II); **C.** vitellogenic stage (stage III); **D.** mature stage (stage IV).

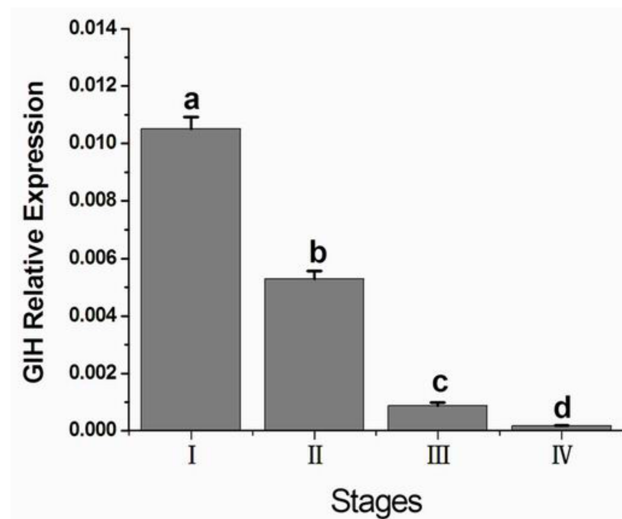

Figure 2. *GIH* expression in different stages of ovarian development in *L. vannamei*. Data are presented as mean ± S.E.M. Values with different letters indicate significant differences ($P < 0.05$) among the various ovarian development stages ($n = 5$).

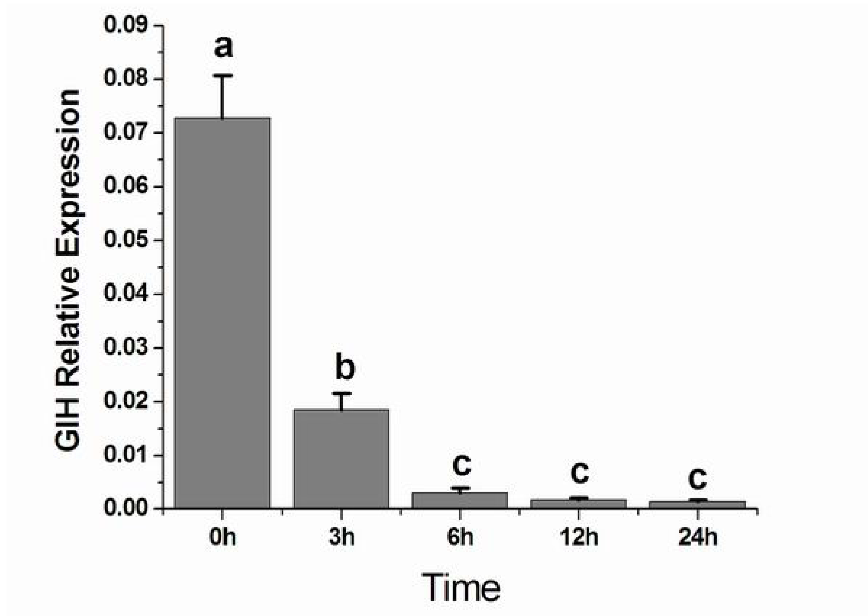

Figure 3. *GIH* expression with E_2 treatment *in vitro* incubation in *L. vannamei.* Data are presented as mean ± S.E.M. Values with different letters indicate significant differences (P < 0.05), n = 5.

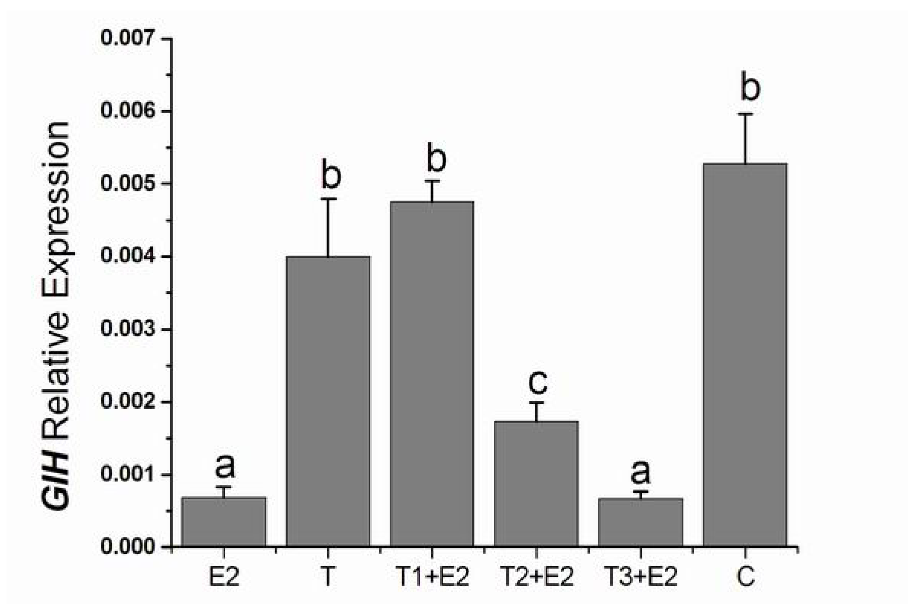

Figure 4. *GIH* expression with E_2 and TAM treatment *in vivo* in *L. vannamei.* E2 = 10^{-6} M 17β-E_2; T = 10^{-6} M TAM; T1+E2 = 10^{-6} M TAM+10^{-6} M 17β-E_2; T2+E2 = 10^{-7} M TAM+10^{-6} M 17β-E_2; T3+E2 = 10^{-8} M TAM+10^{-6} M 17β-E_2; C = control. Data are presented as mean ± S.E.M. Values with different letters indicate significant differences (P < 0.05), n = 5.

LvGIH expression with NP treatment

LvGIH expression decreased with (i) the increasing concentration of environmental estrogen NP and (ii) the duration of immersion. High doses of NP (100 μg/L) can significantly inhibit *LvGIH* expression after three days. However, no significant difference in *LvGIH* expression between the control and the 1 μg/L NP treatment group was observed (Figure 5).

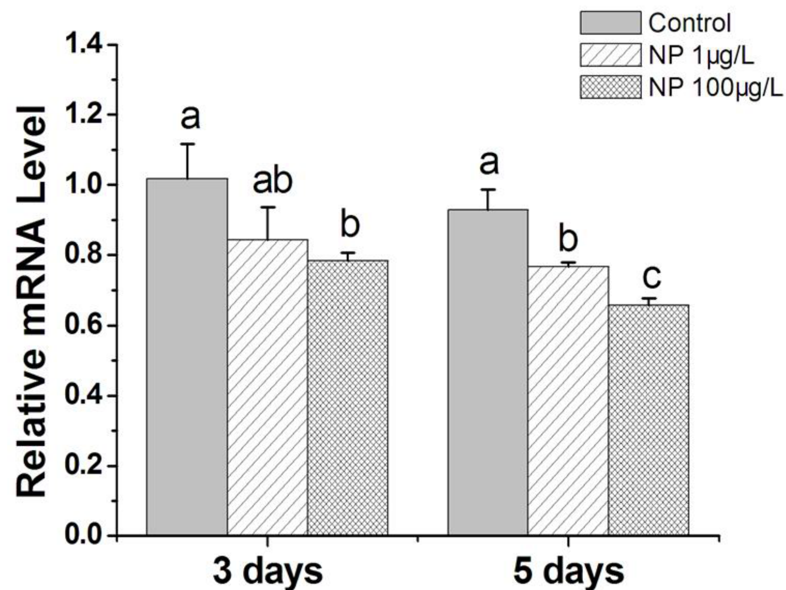

Figure 5. *GIH* expression *in vivo* with NP treatment in *L. vannamei*. Data are presented as mean ± S.E.M. Values with different letters indicate significant differences ($P < 0.05$), n = 5.

DISCUSSION

In crustaceans, female reproduction is controlled by a variety of hormones (Subramoniam, 2000). These hormones include neuropeptides such as gonad-stimulating hormone (GSH) and GIH, which are known to have agonist/antagonist effects on vitellogenesis. Cai et al. (2001) found that ovarian estradiol titers increased rapidly and peaked at the previtellogenesis stage. Results from the same study also showed that high levels were maintained during the vitellogenesis stage, but decreased sharply during the maturation stage in *L. vannamei*. The *Vtg* expression levels increased at the early ovarian developmental stage and peaked at the mature stage, and then decreased sharply to the minimum at the ovarian recovery stage (Li, 2012). In the red mud crab, *Scylla serrata*, the levels of E_2 and progesterone increased sharply at the onset of vitellogenesis (i.e., stage I) and the maximum E_2 levels were detected in the hepatopancreas (Warrier et al., 2001). Moreover, changes in sex steroid hormone levels (estradiol, 17α-OH progesterone, and testosterone) were correlated with the oocyte maturation cycle in crustaceans (Lafont and Mathieu, 2007 for review). In the present study, the highest *GIH* expression levels were observed during oogonium proliferation, followed

by the expressions at previtellogenic and vitellogenic stages, while the lowest levels were observed in mature ovaries. Similar results for high hemolymph GIH levels during the immature and previtellogenic stages were also reported in the American lobster (*Homarus americanus*) (de Kleijn, 1998). Our results were in accordance with that of the American lobster, suggesting that GIH inhibited E_2 levels and resulted in the downregulation of *Vtg* synthesis during ovarian development in *L. vannamei*.

Although E_2 has been demonstrated to play roles in crustacean vitellogenesis in several previous studies (Warrier et al., 2001; Matsumoto, 2008; Shen, 2010), no direct evidence has demonstrated that estrogen exerted feedback regulation on the synthesis and the secretion of GIH. At present, increasing evidence presented in several studies has suggested that vertebrate sex steroids may be involved in the regulation of upstream neuroendocrine factors. For instance, sex steroid hormones have been detected in crustacean ovaries, hepatopancreas, and hemolymph, and the hormone levels were correlated with gonadal development (Couch et al., 1987; Quackenbush, 1992; Cai, 2001; Warrier et al., 2001; Okumura et al., 2004). Moreover, the development of oocytes and follicle cells was regulated by sex steroid hormones (Warrier et al., 2001; Matsumoto, 2008; Shen, 2010). Results from immunocytochemistry studies have demonstrated that estrogen and androgen receptors (ER and AR) exist in the brain and thoracic ganglion of the mud crab (*S. paramamosain)* (Han et al., 2006; Ye et al., 2008) as well as the brain and Hatschek's pit (homologous with the pituitary of fish) of *Amphioxus* (Weng et al., 2001). We postulated that estrogen and androgen may be involved in the feedback regulation of the nervous system, and that it can induce oocyte development and vitellogenesis by interacting with ER subtypes as is seen in vertebrates (Ye et al., 2008). In the present study, *in vitro* incubation and *in vivo* injection with E_2 significantly decreased *LvGIH* expression, and the potent estrogenic chemical NP reduced *LvGIH* mRNA levels. These results suggested that estrogen might be involved in the negative feedback regulation of *LvGIH* expression in eyestalks. Furthermore, the results also demonstrated that the estrogen receptor antagonist TAM is capable of neutralizing the inhibitory action of E_2 on *LvGIH* in a dose-dependent manner. This result further suggests that the above presumption is reasonable. Exogenous E_2 can elevate E_2 levels in hemolymph, resulting in *L. vannamei* ovarian development (Cai et al., 2001), and E_2 levels in hemolymph can remain high during vitellogenesis in *P. monodon* (Fairs et al., 1990). Consequently, it is presumed that estrogen participated in the negative regulation of *GIH* mRNA expression. Estrogen promotes ovarian development by upregulating vitellogenesis directly in the hepatocytes, and it may alternatively inhibit *GIH* expression to indirectly increase vitellogenesis.

In summary, these findings provided evidence that estrogen has negative regulation effects on *GIH* expression in shrimp. Estrogen promoted ovarian development not only by directly upregulating vitellogenesis in the hepatopancreas, but by also downregulating subsequent *LvGIH* expression to indirectly offset inhibition from GIH.

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