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*Research Article*

# Examination of the Effects of Thoracic Ganglion and Brain Extracts on Vitellogenin Gene Expression in the Ovary of Whiteleg Shrimp, *Litopenaeus vannamei*, and Possible Vitellogenesis-Stimulating Activity

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In order to examine the role of the central nervous system in regulating ovarian maturation in the whiteleg shrimp, *Litopenaeus vannamei*, we incubated ovarian explants taken from adult shrimp with either brain or thoracic ganglion extracts at the level of five tissue equivalents for 6 h. For assessment of the effects of each tissue extract, transcriptional expression of vitellogenin mRNA in incubated ovarian tissue fragments was analyzed by quantitative real-time PCR. Results showed that crude brain extracts and a 90% aqueous acetonitrile eluate upregulated expression levels in a significant manner. These findings suggest that factors originating from brain tissue are involved in ovarian maturation, but that the thoracic ganglia do not likely harbor significant quantities of stimulatory substances. In the future, this knowledge may be useful in developing technology for inducing maturation and spawning in captive female penaeid.

## 1. Introduction

The shrimp aquaculture industry has grown dramatically over several decades to become a major global industry such that total world shrimp production has reached around 5 million tons, of which over 80% is comprised of the Pacific whiteleg shrimp, *Litopenaeus vannamei* [1]. To support this large industry, it is essential for shrimp hatcheries to produce large quantities of postlarvae [2]. However, it remains difficult to obtain sufficient numbers of matured female broodstock in a simultaneous manner under captive conditions. In order to induce ovarian maturation in shrimp more efficiently in captivity, it is essential to understand the endocrine mechanisms that regulate shrimp reproduction.

Reproduction in crustaceans is thought to be regulated by the antagonistic action of two endocrine factors: vitellogenesis-inhibiting hormone secreted from the X-organ sinus gland complex of the eyestalk and vitellogenesis-stimulating hormone (VSH) assumed to be secreted by central nervous tissues (the brain, thoracic ganglia, and abdominal ganglia) of mature females [3]. Thus far, the neuroendocrine factors identified in crustaceans are predominantly inhibitory in nature and are synthesized in the sinus glands of the eyestalks [4]. Hence, eyestalk ablation is a commonly practiced method of promoting ovarian maturation in domestic hatcheries. However, this procedure has many adverse effects on the physiology of the broodstock and is also considered to be undesirable from the point of view of animal welfare [2]. Therefore, in order to develop more appropriate maturation-inducing procedures, it is necessary to develop new technology based on the functioning of natural hormones.

The stimulatory effects of various tissues including the brain, thoracic ganglia, and mandibular organs on ovarian development have been examined by both *in vitro* methods and *in vivo* implantation or injection of their extracts over the past several decades [5–11]. A potential vitellogenesis-stimulating factor from the thoracic ganglia was

characterized as a 10 kDa peptide hormone in the kuruma prawn, *Penaeus japonicus*; this factor could be inactivated by trypsin, suggesting that it was a peptide [12]. It is possible that there may be a suite of factors that fulfill the role of VSH; nevertheless, the full identity of such factors/hormones remains elusive. Therefore, in this study, we evaluated the biological functioning of brain and thoracic ganglion extracts on vitellogenin (*Vg*) mRNA expression in the ovary of *L. vannamei* as a first attempt to better understand the effects of the intraspecific brain and thoracic ganglia on ovarian maturation with the aim of ultimately identifying putative VSH.

## 2. Materials and Methods

The brains and thoracic ganglia were dissected out from subadult and adult *L. vannamei* females (132 individuals in total) purchased from International Mariculture Technology Engineering Inc. (Tokyo, Japan) and stored at −80 ˚C until use. The frozen pooled brains and thoracic ganglia were homogenized in a cold aqueous acetonitrile solution (30% acetonitrile and 0.9% NaCl; 1 mL per g tissue) using a sterilized mechanical homogenizer (Omni TH Tissue Homogenizer with a generator probe, TH-115, OMNI International Inc., Gainesville, VA, USA) for 30 s on ice. The homogenates were then centrifuged at 15,000 g for 15 min. The supernatant was collected, and an aliquot equivalent to 30 brains/thoracic ganglia was separated as a crude extract; the remaining portion was applied to a Sep-Pak C18 cartridge for further purification. Fractions were eluted manually with increasing percentages (10%, 60%, and 90%) of aqueous acetonitrile. Each eluate was concentrated under reduced pressure by using a centrifugal concentrator (VE-96W, Titech, Koshigaya, Japan) to remove acetonitrile, aliquoted to obtain a dose of five brain/thoracic ganglion equivalents for one well of *in vitro* culture, and stored at −80˚C until use. A portion of each extract (1.5 brain/thoracic ganglion equivalent) was also subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10–20% gradient gel (e-PAGEL, 331840/E-T1020L, ATTO Corporation, Tokyo, Japan) as a preliminary means of characterizing the putative substances contained in the eluate. Eluted proteins/peptides were stained with Quick-CBB Plus (178-00551, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

The culture medium for *in vitro* incubation was prepared based on 1 × Medium 199 containing Earle's Salts (Invitrogen), with 1 × antibiotic-antimycotic (Life Technologies, Japan), 25 mM HEPES, 2 mM glutamine, 0.4 mM taurine, NaCl (41.8 mM), KCl (1.04 mM), $MgSO_4$•$7H_2O$ (0.4 mM), $MgCl_2$•$6H_2O$ (1.7 mM), $NaHCO_3$ (0.1 mM), $CaCl_2$•$2H_2O$ (1.4 mM), and 0.1 mg/mL of AlbuMAX I (11020021, Gibco, Auckland, New Zealand). The pH and osmolality of the medium were adjusted to 7.4 and 790–800 mOsm, respectively. Ovarian explants were obtained by dissecting out tissues from a total of six previtellogenic shrimps that had been anaesthetized in chilled sea water in compliance with Japanese policy on animal use [13]; body weight was 49.3 ± 3.7 g (mean ± SEM), and the gonadosomatic index was 0.9 ± 0.1% (mean ± SEM). To maintain consistency in lobe-specific expression during incubation, bilaterally-paired ovarian lobes (2, 3, 4, 5, and 10) were used for *in vitro* incubation. A paired-lobe (left and right sides) was divided into two groups: control and experimental [14] (Additional file 1). Experimental treatment groups were incubated with either brain or thoracic ganglion extracts at a volume of five tissue equivalents into a total of 1 mL of the incubation medium, while the control treatment was incubated in the same manner but with equal volumes of elution buffer. As an experimental control (buffer-treated vehicle and a negative control (eyestalk extracts)), the lobes were incubated, respectively, in the medium/elution buffer or medium/extract of two eyestalk equivalents (Additional file 2). The designated lobes were cut into approximately 5 mg pieces and transferred into 24-well culture plates (BD Biosciences, Franklin Lakes, NJ, USA) to have one lobe per well. The plates were incubated for 6 h under an oxygen-enriched atmosphere with gentle shaking (30 rpm) at 28˚C in constant darkness. Following incubation, the lobes were snap-frozen and stored at −80˚C until use in total RNA extraction for the quantification of *Vg* mRNA expression levels.

Transcriptional expression of *L. vannamei Vg* mRNA in the incubated ovarian explants was determined using QuantiFast Probe RT-PCR + ROX Vial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the design of primers and probes, we utilized a cDNA sequence by encoding the *Vg* gene that was first reported by Raviv et al. [15] (GenBank ID: AY321153), which also corresponds to *LvVg1b* and *LvVg1e* of the *LvVg1* gene group, as reported by [16]. Total RNAs extracted from the incubated ovarian explants with RNeasy Mini Kit (250) (Qiagen, Hilden, Germany) were used as templates. *Vg* and *β-actin* were amplified by quantitative real-time PCR using the ABI PRISM 7500 sequence detection system v2.3 (Applied Biosystem, Foster City, California, USA), as given in a previous report [17]. Sequences of primers and probes used in amplification are given in Table 1. The amplification conditions for both genes were initial 50˚C for 10 min, 1 cycle of 95˚C for 5 min, and 40 cycles of 95˚C for 10 s and 61˚C for 30 s. All samples were analyzed in duplicate, and the expression of *Vg* mRNA was normalized to that of *β-actin* as described previously [18]. Values are shown as a mean ± SEM. The differences between the values for the vehicle control and those for the experimental group were tested for significance using Student's *t*-test. Differences were considered significant at $p < 0.05$.

## 3. Results

The elution patterns of proteins/peptides in each tissue extract as obtained by SDS-PAGE are shown in Figure 1. Band patterns varied among the extracts, but most of the protein/peptide bands appeared in the 10% aqueous acetonitrile Sep-Pak fraction. However, in the 60% acetonitrile Sep-Pak fraction, a prominent band between 20 and 15 kDa was detectable, and a faint low molecular weight band could be detected at a slightly lower position than 10 kDa in the

Table 1: Primers and probes used for quantitative real-time PCR of *Vg* mRNA and the *β*-actin housekeeping gene in *Litopenaeus vannamei* (designed based on cDNA sequence encoding vitellogenin (GenBank ID: AY321153) and *β*-actin (GenBank ID: AF300705)).

| Target gene | Primer/probe name | Sequences (5′–3′) | Length of amplification (bp) |
|---|---|---|---|
| Liv-*Vg* | Lv-*Vg*-qF01 | 5′-CAGAGAGTC-ATC-GACTAC-ACC-ATGT-3′ | 103 |
| | Lv-*Vg*-qR01 | 5′-GAATGAGGCGAACGACCTTT-3′ | |
| | Lv-*Vg*-Prb (TaqMan) | 5′-TTC-CAT-TCG-GAA-CGAGCATTTGCT-3′ | |
| Liv-*β*-actin | Lvact-F01 | 5′-CGACCT-CAC-AGACTA-CCT-GATGAAGAT-3′ | 138 |
| | Lvact-R02 | 5′-GTGGTCATCTCCTGC-TCG-AAGT-3′ | |
| | Lv-act-Prb (TaqMan) | 5′-CGA-CCACCGCCG-AGC-GAGAAATCGTTCGT-3′ | |

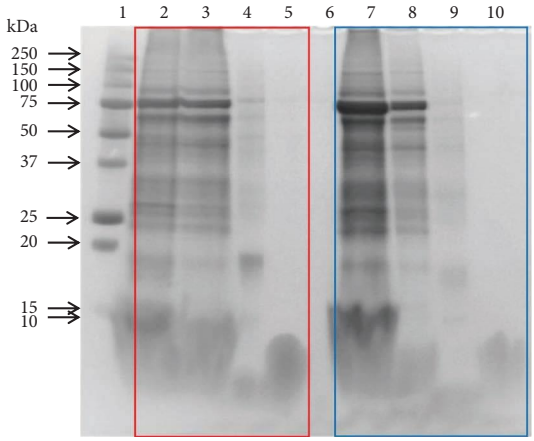


Figure 1: SDS-PAGE patterns of tissue extracts for the thoracic ganglia (TG) and brain; patterns for TG extracts are shown in red and the brain in blue. Lane 1: markers (Bio-Rad #1610373); lanes 2–5 and 7–10: crude extract, 10%, 60%, and 90% aqueous acetonitrile fractions for the TG and brain, respectively. Lane 6: blank. The volume of each sample applied to a 10–20% gradient gel was 1.5 tissue equivalents. Most peptides/proteins were eluted in the 10% acetonitrile Sep-Pak fraction.

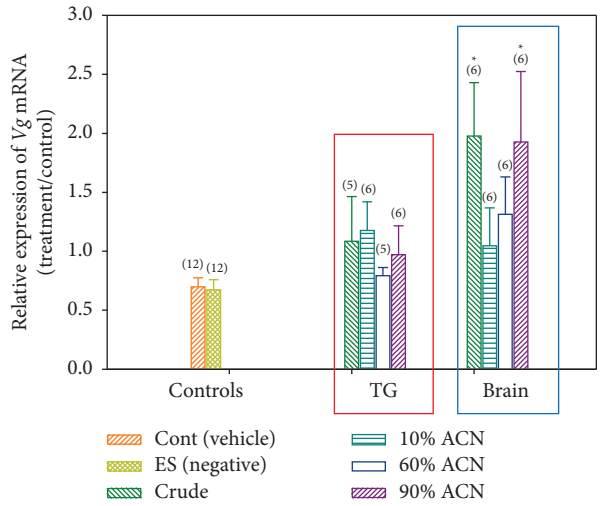


Figure 2: Results of the ovarian tissue culture. Control (vehicle); experimental control (buffer-treated and ES: eyestalk (negative control)). Results for TG extracts are shown in red and those of the brain in blue. Numbers in parentheses above each bar indicate the number of individuals analyzed in each group. *Significant difference ($p < 0.05$). The expression of *Vg* mRNA in ovarian explants was significantly increased by incubation with crude and 90% aqueous acetonitrile (ACN) brain extracts.

90% acetonitrile Sep-Pak fraction. The results of the ovarian culture revealed that *Vg* mRNA expression levels were increased by approximately two-fold in ovarian explants incubated with crude brain extracts ($t(5) = -2.79$; $p = 0.01$) and a 90% acetonitrile eluate ($t(5) = -2.04$; $p = 0.04$) (Figure 2) ($p < 0.05$). Based on these results, it may be considered that substances present in the brain are possibly capable of stimulating *Vg* mRNA expression in the ovary of *L. vannamei*.

## 4. Discussion

The results of the present study corroborate earlier studies in which ovarian maturation was induced by implantation of the lobster thoracic ganglia [9] and injection of the lobster brain extract to *L. vannamei* [11]. In addition, other earlier studies have also demonstrated a stimulating effect of brain and thoracic ganglion implants or extracts on oocyte development in various penaeid species [6, 7]. However, Alfaro and Vega [19] did not find any vitellogenesis-stimulatory effects for either interspecific thoracic ganglion implantation or extract injection in both male and female *L. vannamei in vivo*. On the other hand, Chen et al. [20] reported that subadult female *L. vannamei* brain tissue exhibits vitellogenesis-inhibiting activity when cocultured with hepatopancreatic cells. As these results show a discrepancy with those of the present study, it may be possible that the brain functions in a temporal manner as a function of the developmental stage.

To our knowledge, the present study is one of the few examples of experimentation employing the functional analysis of the intraspecific brain and thoracic ganglion extracts in *L. vannamei*. The application of tissue extracts at a dosage level of five tissue equivalents suggested that stimulatory factors specifically reside in crude and 90% acetonitrile-eluted brain extracts. Based on examination of SDS-PAGE patterns for the 90% acetonitrile-eluted brain extracts (Figure 1), it may be considered that the putative vitellogenesis-stimulating factor could be either a 10 kDa proteinaceous component as previously reported by Yano [10] or possibly a lipophilic or terpenoid-related substance such as is found in insects. In the present study, due to limited availability of tissues, we were able to test the effects of only one dosage level, i.e., five tissue equivalents. In subsequent work, it will be necessary to examine the effects of brain extracts in a dose-dependent manner. In addition, based on the results of this study and examination of the scientific literature, we hypothesize that the putative stimulating factor is found primarily in maturing female individuals, but in subsequent experimentation, it may be valuable to examine tissues from subadult females and males for purposes of reference.

In conclusion, examination of the chemical properties of this putative factor, and more ideally, its specific identification, is an essential prerequisite for exploring strategies towards the development of practical applications for penaeid shrimps. Along these lines, the results obtained here are expected to be useful for advancing the current understanding of the reproductive endocrinology of *L. vannamei* and for developing a new methodology for inducing maturation of female broodstock in captivity.

## Data Availability

All raw data that support the results of this study are available upon reasonable request to the corresponding author.

## Ethical Approval

All experiments complied with institutional regulations and the Japanese policy on animal use [13].

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Marcy N. Wilder, Bong Jung Kang, and Zakea Sultana designed the experiments. Zakea Sultana and Marcy N. Wilder performed the experiments and collected raw data. Zakea Sultana and Bong Jung Kang analyzed the data. All authors contributed to the article writing and approved the submitted version.

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## Supplementary Materials

*Supplementary Figure 1.* Schematic diagram of *L. vannamei* ovarian lobes used in *in vitro* culture (adopted from [13] (General and Comparative Endocrinology, 246: 301–308)). Lobes divided into left and right sides were used as control and experimental groups, respectively. *Supplementary Figure 2.* Ovarian culture scheme. Lobes for the experimental control (vehicle) and the negative control (eyestalk extracts) were incubated in the medium/elution buffer or medium/extract of two eyestalk equivalents. Experimental treatment groups were incubated with either brain or thoracic ganglion extracts at a volume of five tissue equivalents in a total of 1 mL of the incubation medium, while the control treatment was incubated in the same manner but with equal volumes of elution buffer. (*Supplementary Materials*)

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