Studies on multiple vitellogenin genes in the kuruma prawn *Marsupenaeus japonicus*: identification of novel vitellogenin and hormonal regulation of gene expression

（クルマエビのビテロジェニン遺伝子に関する研究：
新規ビテロジェニンの同定とホルモンによる発現制御）

A thesis submitted in fulfillment of the requirements for the degree of
Doctor of Philosophy
at
Graduate School of Bioresources,
Mie University, Japan

by

Marwa Said abdEl-Halem El-Desoky

September 2023

Table of contents

List of Tables

List of Figures

Abstract

The reproductive biology of crustaceans is of great importance for the fisheries and aquaculture industries. Vitellogenesis, the process of yolk formation, is a critical factor in reproduction for both oviparous vertebrates and invertebrates. Vitellogenin (Vg) is the precursor of the major egg-yolk protein, hence is used as a marker of vitellogenesis. The kuruma prawn *Marsupenaeus japonicus* is an economically important fisheries species in Japan and several Southeast Asian countries. A single Vg gene, known as *Maj-Vg1*, was identified in this species, and the hepatopancreas, and ovary were recognized as Vg synthesis sites based on its gene expression pattern. The *Maj-Vg1* expression in the ovary was inhibited by the vitellogenesis-inhibiting hormone produced by the eyestalk ganglia. However, the presence of multiple Vg genes, recently reported in some decapods, and the hormonal regulation of Vg gene expression in the hepatopancreas is still ambiguous. Therefore, in this thesis, the search for a novel Vg gene from the transcriptome data of the hepatopancreas was conducted, and the relationship between its gene expression and the progression of vitellogenesis was also examined. Furthermore, the effects of various hormones on Vg gene expression in hepatopancreas were studied using *ex-vivo* assays to gain a deeper understanding of vitellogenesis in this species.

The full-length cDNA sequence for the novel vitellogenin, Maj-Vg2 was successfully

identified. The deduced Maj-Vg2 is composed of 2,554 amino acid residues. Amino acid sequence similarity between Maj-Vg1 and Maj-Vg2 was 54%. The phylogenetic analysis demonstrated that Maj-Vg2 forms a distinct clade with Vg2 of several *Penaeus* species, such as *Metapenaeus ensis, Fenneropenaeus merguiensis*, and *Litopenaeus vannamei*, separate from the clade containing Vg1 and Vg3 of these species.

The expression of *Maj-Vg2* was restricted to the hepatopancreas. *Maj-Vg2* showed low expression during the previtellogenic stage, then increased during the early and late yolk globule stages of wild prawns. Moreover, eyestalk ablation significantly induced *Maj-Vg2* expression in immature prawns. This result was similar to the increase in *Maj-Vg1* expression observed in the ovary, indicating that Maj-Vg2 is involved in vitellogenesis and may play a complementary role in yolk protein synthesis during ovarian development.

A vertebrate-type sex steroid hormone estradiol and the crustacean homolog of insect juvenile hormone methyl farnesoate show no significant effect on gene expression of both *Maj-Vg1* and *Maj-Vg2* in the hepatopancreas. However, the synthetic insulin-like peptide (ILP1) significantly increased the expression levels of the two *Vg*s in the hepatopancreas of adolescent prawns. Specifically, *Maj-Vg1* expression was significantly upregulated with 1 μM ILP1, 1.3-fold higher in average relative value to control. *Maj-Vg2* expression was induced with 0.1 and 1 μM, 1.2- and 1.3-fold higher to control, respectively. In the ovaries of adolescent and immature adult prawns, only 0.1 μM ILP1 upregulates *Maj-Vg1* expression, 1.5- and 2.2-fold

higher than the control, respectively. These results suggest that the ovarian-specific ILP1 is likely involved in regulating vitellogenesis in *M. japonicus*.

Overall, this is the first study focusing on the identification of novel vitellogenin Maj-Vg2 in *M. japonicus* and hormonal regulation of the gene expression of *Vg*s by ILP1.

1. General Introduction

Fisheries and aquaculture

Aquaculture is the practice of farming aquatic organisms such as fish, mollusks, crustaceans, and aquatic plants. The fisheries and aquaculture sectors play a critical role in ensuring food security and nutrition worldwide. As the global population continues to grow, the demand for seafood increases, and the contribution of these sectors needs to be increased accordingly. From 1961 to 2019, global consumption of aquatic food increased at an annual rate of 3%, almost double the rate of annual world population growth (1.6%) during the same period. In 2020, aquaculture accounted for 56% of the amount of aquatic animal food products available for human consumption (FAO 2022).

Crustacean fisheries, such as shrimp, lobsters, and crayfish, play a vital role in marine fisheries worldwide. These fisheries are growing faster than any other seafood group (Boenish et al. 2022). To enhance efficiency and promote sustainability, various aquaculture techniques have been developed, including adjusting feed composition, optimizing culture systems, and manipulating hormonal regulation in these species. The production of crustaceans has been increasing rapidly in recent years. In 2020, approximately 17 million tons of crustaceans were produced, which is more than double the production recorded in 2000 (FAO 2022). The kuruma prawn *Marsupenaeus japonicus* (Fig. 1) is a significant penaeid species found throughout the Indo-West Pacific (Fig. 2), with particular importance in Japanese fisheries and aquaculture

(Hayashi 1992). Understanding the reproductive mechanisms of decapod crustaceans is critical for promoting the sustainability of these economic species.

Reproductive biology of crustaceans

Reproduction in female crustaceans is a complex biological process that involves a multi-linked endocrine system. Underlying the intricate processes underlying their reproductive biology is crucial for conservation efforts and aquaculture management. One significant aspect of crustacean reproduction is vitellogenesis, which is the production and accumulation of Vt in the developing oocytes, is crucial for ovarian maturation (Huberman 2000; Jimenez-Gutierrez et al. 2019). The precursor of Vt is called vitellogenin (Vg), which is synthesized in specific tissues such as ovary and/or hepatopancreas. It is then transported to the ovary for uptake and accumulation in the oocytes. Vg serves as a significant source of amino acids, carbohydrates, and lipids, providing essential nutrients to the eggs. These vital nutrients are subsequently used by the developing embryo and early larvae for their growth and development (Meusy 1980). Thus, Vg is considered as an index of the onset of ovarian maturation.

Vitellogenesis in crustaceans is influenced by a range of factors, including endocrine regulation, environmental cues, and nutritional status. Environmental factors, such as temperature, photoperiod, and water quality also exerts influences on the success of vitellogenesis (Jayasankar et al. 2020). In vertebrates, hormones coordinate the actions along the hypothalamic-pituitary-gonadal axis (Kanda 2019) while in crustaceans, several

neuropeptides are released by the neurosecretory tissue X-organ/sinus gland complex (XOSG) and the central nervous system (Christie 2011; Hopkins 2012; Alfaro-Montoya et al. 2019). For instance, the eyestalk of crustaceans secretes a group of four major neuro-endocrine hormones known as the crustacean hyperglycemic hormone (CHH)-family. These hormones, including CHH, molt inhibiting hormone (MIH), mandibular organ-inhibiting hormone (MOIH) and vitellogenesis/gonad inhibiting hormone (VIH/GIH) play a significant role in the regulation of ovarian maturation in crustaceans (Böcking et al. 2002; Nagaraju 2011; Webster et al. 2012). In addition to these peptides, there are some peripheral factors such as gonadal hormones (Tsutsui et al. 2018; Tsutsui et al. 2020), ecdysteroids which are produced by the Y-organ, and methyl farnesoate which is sesquiterpenoid hormone produced by the mandibular organs also regulates reproductive physiology in crustaceans (Fingerman and Nagabhushanam 1992; Subramoniam 2000; Paran et al. 2010). VIH, a member of CHH family, is of particular interest due to its inhibitory effect on vitellogenesis. VIH suppresses vitellogenin production in the target tissues or inhibits protein uptake by the oocytes (Chen et al. 2014; Qiao et al. 2015). For example, six CHH-family peptides from seven peptides produced by the XOSG, inhibited the expression of *Vg* (currently *Maj-Vg1*) in the ovary of *M. japonicus* (Tsutsui et al. 2005a; Tsutsui et al. 2013a). Consequently, eyestalk ablation (ESA) is a commonly employed technique in crustacean research, particularly in studies investigating reproduction, growth, and molting as a way of the hormonal manipulation. This procedure involves the surgical removal or partial

removal of one or both eyestalks (Fig. 3), which contain neuroendocrine centers responsible for synthesizing and releasing vital hormones. ESA is often used in hatcheries to improve gonadal maturation, enhance broodstock production and optimize the timing of spawning in crustaceans by removing the source of VIH (Okumura et al. 2006; Uawisetwathana et al. 2011). However, this technique has negative impacts on the quality and quantity of the offspring, as well as performance reduction and death of broodstock (Palacios et al. 1999). Specifically, the surgical procedure itself carries the risk of injury or infection, and the recovery process following ablation may vary among species. Additionally, the hormonal changes induced by ESA are not always specific, as other neuroendocrine centers may compensate for the loss of eyestalk control. As a result of ESA, not only VIH but also other hormones produced by eyestalks may be lost or their production and release may be altered. Therefore, alternative techniques have been explored, such as administering exogenous compounds to stimulate vitellogenesis (Guan et al. 2014; Merlin et al. 2015; Chen et al. 2018) or using VIH antibodies (Treerattrakool et al. 2014) and RNA interference (RNAi) (Feijó et al. 2016) to reduce levels of the circulating inhibitory hormone by silencing VIH gene (Jayasankar et al. 2020). Nonetheless, developing effective alternatives to ESA remains a challenge especially after the limited success of RNAi technology in inducing ovarian maturation.

The existence of multiple *Vg* genes have been reported recently within a single decapod species, such as the sand shrimp *Metapenaeus ensis* (Tsang et al. 2003; Kung et al. 2004), the

morotoge shrimp *Pandalus japonica* (Jeon et al. 2011), the whiteleg shrimp *Litopenaeus vannamei* (Wang et al. 2020), and the banana shrimp *Fenneropenaeus merguiensis* (Zhao et al. 2021) . To date, it is unclear if additional *Vg* genes are present in *M. japonicus*, warranting further analysis to fully understand the regulatory mechanisms of vitellogenesis. In oviparous vertebrates and insects, the sites of Vg synthesis have been well-established. Specifically, the fat body is recognized as the primary site for Vg synthesis in insects (Nose et al. 1997) and the liver fulfils this role in vertebrates (Wang et al. 2000). On the other hand, in crustaceans, the site of Vg production was still debated until the 1990s. Recent advances in molecular biological studies have revealed that the ovary and/or hepatopancreas serve as the sites of Vg synthesis in crustaceans. Briefly, these studies suggest that both the hepatopancreas and ovary are involved in Vg synthesis in shrimps and crabs, while prawns such as *Macrobrachium rosenbergii* and crayfish such as *Cherax quadricarinatus* primarily rely on the hepatopancreas for Vg synthesis (Subramoniam 2011).

In *M. japonicus*, up to now, only a single *Vg* gene (*Maj-Vg1*) has been identified previously. This gene is expressed in the follicle cells of the ovary and the parenchymal cells of the hepatopancreas (Tsutsui et al. 2000). Furthermore, it has been reported that the expression of *Maj-Vg1* was inhibited by six VIHs in the ovary when subjected to *ex-vivo* assay (Tsutsui et al. 2013a). However, the precise molecular mechanisms underlying the function of the neuropeptides produced by XOSG complex in relation to vitellogenesis remains inadequately

understood. Furthermore, the regulatory mechanism controlling the expression of *Maj-Vg1* in the hepatopancreas is still ambiguous. It is unknown whether there are additional *Vg* genes present in this species. Therefore, further characterization of potential additional *Vg* genes, including their sites of synthesis, expression dynamics, and regulatory mechanisms would greatly contribute to a better comprehension of vitellogenesis in *M. japonicus*. Focusing on these aspects could pave the way for innovative approaches in crustacean reproductive managements.

Therefore, this study aims to gain a comprehensive understanding of the *Vg* genes in *M. japonicus* through firstly, searching for a novel *Vg* gene from the transcriptomic data of the hepatopancreas of *M. japonicus.* Secondly, studying the relationship between *Vg* expression levels and the progression of vitellogenesis in this species. Thirdly, clarifying the effect of various hormones on Vg gene expression levels in hepatopancreas using *ex-vivo* assays.

Through these objectives, I hope to contribute to a better understanding of the reproductive biology of the *M. japonicus*, and potentially inform efforts to improve aquaculture practices for this economically important species.

Fig. 1. The kuruma prawn *Marsupenaeus japonicus* (Bate,1888)

Fig. 2. Map for distribution of *M. japonicus* in the Indian and Southwestern Pacific Oceans

https://www.fao.org/fishery/en/aqspecies/2584/en

Fig. 3. Eyestalk ablation (ESA) of prawns. Female *M. japonicus* underwent unilateral (A) or bilateral (B) ESA to artificially induce ovarian maturation.

Chapter 1. Identification of an additional hepatopancreas-specific vitellogenin gene in *M. japonicus*

1.1 Background

Vitellogenesis, a critical reproductive process in decapod crustaceans, is responsible for the accumulation of yolk proteins and other nutrients in the ovary, leading to the growth of oocytes (Huberman 2000; Jimenez-Gutierrez et al. 2019). This process involves the synthesis of vitellogenin (Vg), the precursor of Vt, in specific tissues, its transportation to the ovary for uptake, and accumulation in the oocytes as several Vt subunits (Wilder et al. 2010). Vt plays nutritive role in embryogenesis and is involved in the importation of essential minerals, lipids and other materials into the developing oocytes (Wilder et al. 2010). Significant progress in understanding vitellogenesis has been achieved throughout the cloning of Vg genes/cDNAs in crustaceans. Initially, by using the partial amino acid sequences information of Vg and Vt, researchers successfully cloned Vg genes/cDNA in certain species. Based on this knowledge, subsequent studies were able to clone Vg genes in some other crustacean species, with the ovary and/or hepatopancreas being the Vg synthesis sites (Tsutsui et al. 2000; Okuno et al. 2002; Abdu et al. 2002; Mak et al. 2005a; Zmora et al. 2007; Tiu et al. 2009; Wang et al. 2020; Zhao et al. 2021).

Although significant progress has been made in understanding vitellogenesis, the regulating mechanisms remain poorly understood. One of the reasons hindering this progress is the lack

of sufficient knowledge regarding the number of vitellogenin genes and their functional expression levels in decapods. Previous studies have reported the presence of multiple Vg genes in a single decapod species, such as *P. japonica*, *M. ensis*, *L. vannamei*, and *F. merguiensis* (Tsang et al. 2003; Kung et al. 2004; Jeon et al. 2011; Wang et al. 2020; Zhao et al. 2021). However, it remains unclear whether multiple *Vg* genes are present in *M. japonicus* and how their expression in the hepatopancreas is regulated.

In this chapter, I focused on the hepatopancreas and identified an additional Vg, designated as Maj-Vg2, using hepatopancreas transcriptomic data and subsequent cDNA cloning. The complete primary structure of Maj-Vg2 was determined, and its phylogenetic relationship with Vgs from other decapod crustaceans was assessed. The identification of Maj-Vg2 contributes to a better understanding of regulatory mechanisms of vitellogenesis in crustaceans and sheds light on the possible presence of multiple *Vg* genes and their expression in the hepatopancreas.

1.2. Materials and Methods

1.2.1. Transcriptomic analysis of the hepatopancreas

In this study, adult *M. japonicus* were procured from local prawn farms in Okinawa Prefecture, Japan. The hepatopancreas of a female prawn in the previtellogenic stage weighting 26.4 g with a 1.0% GSI was selected for dissection. The hepatopancreas of the prawn was excised and stored in RNA*later* solution (Thermo Fisher Scientific, Waltham, MA, USA) at −20°C until RNA extraction. The previtellogenic stage of the prawn's ovaries was confirmed

by histological analysis (Okumura et al. 2007), while its molting stage was identified as intermolt (C0) based on the previous report (Chan et al. 1988).

Total RNA was extracted from the above tissues using a combination of Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and the NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The integrity of the extracted RNA was estimated by the visualization of the clear bands. The concentration of RNA was determined by using the NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA). A cDNA library was generated from 1 μg of total RNA obtained from the hepatopancreas using the NEBNext ultra directional RNA library prep kit for Illumina, NEBNext Poly(A) mRNA Magnetic Isolation Module, and NEBNext multiplex oligos for Illumina (index primers set 1; New England BioLabs, Ipswich, MA, USA). The protocols were followed according to the manufacturer's instructions with slight modifications (Tsutsui et al. 2020). The resulting library fragment size ranged between 223 and 1496, with an average of 565 bp as estimated using the Agilent high-sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA). The sequencing was conducted with MiSeq using Reagent Kit v3 (Illumina, San Diego, CA, USA) in the paired-end mode with a read length of 300 bases. The raw data for paired-end sequencing was deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive with the accession number DRA012835. Data was processed using the DDBJ Read Annotation Pipeline and the supercomputer at the Research Organization of Information and Systems (ROIS), National

Institute of Genetics (NIG), Japan. Reads with a quality score (QV < 20) were trimmed from the 5′ and 3′ ends, and reads containing ≥ 30% of low-quality bases (QV < 14) with less than 25 bp were excluded before assembly. The preprocessed reads were *de novo* assembled using the Trinity platform (Haas et al. 2013).

The assembled contigs described above were subjected to the Basic Local Alignment Search Tool+ (BLAST+; version 2.3.0) analysis to perform a tblastn homology search and find contigs that exhibited significant similarity with the amino acid sequence of known *M. japonicus* Vg (Maj-Vg1, GenBank accession no. BAB01568). All Primers, excluding dT-adaptor and N_adapt01, were designed based on the nucleotide sequences of the contigs that encode the putative Vg (Table 1.1) and utilized for cDNA cloning, as described below.

1.2.2. cDNA cloning of *Maj-Vg2*

Adult *M. japonicus* weighting 27 g body weight and a GSI of 2.9% were procured from a local fish market in Aichi Prefecture, Japan. The hepatopancreas was then dissected, and total RNA was isolated as described above. The integrity of the extracted total RNA was evaluated and the concentration was measured by the spectrophotometer. To synthesize first-strand cDNA, 71 ng of total RNA, 66.7 pmol of dT-adapter primer, ProtoScript II Reaction Mix, and ProtoScript II Enzyme Mix (New England Biolabs) were used following the manufacturer's instructions. Target DNA fragments were then amplified using different denaturation temperatures based on the Tm of the primers, and different extension times according to the

expected length of the amplicons, using the primer sets listed previously. The final PCR mix included 0.2 μM of each primer, 200 μM of dNTPs, 1.25 U of DNA polymerase (New England Biolabs), 1× buffer, and 1 μL of cDNA. PCR products were analyzed by using gel electrophoresis (1% agarose gels). Gel-purification using FavorPrep TM PCR Clean-Up Mini Kit (Favorgen Biotech, Japan) was performed. The resulting PCR products were ligated into the pGEM-T easy vector (Promega, Madison, WI, USA) by overnight incubation at 4ºC, and transformed into the competent *Escherichia coli* cells. Plasmids containing the desired size of inserts (Positive white colonies) were selected and sequenced by Macrogen Japan Corp. (Tokyo, Japan). The nucleotide sequences obtained were used to reconstruct a full-length cDNA encoding Maj-Vg2.

1.2.3. Bioinformatic analyses

The prediction of the open reading frame (ORF) of *Maj-Vg2* was performed using the ExPASy translate tool (https://web.expasy.org/translate/). To identify potential signal peptides, the SignalP 5.0 server was utilized (http://www.cbs.dtu.dk/services/SignalP/). In addition, a homology comparison of the deduced amino acid sequence of Maj-Vg2 with other crustacean Vgs deposited in the GenBank database was conducted using a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The PROSITE database (https://prosite.expasy.org/) was used to analyze potential functional motifs of Maj-Vg2.

1.2.4. Phylogenetic analysis of *Maj-Vg2*

Several decapod Vgs with related report(s) were selected from the GenBank database (Table 1.2) and used to construct a molecular phylogenetic tree. The amino acid sequences, including Maj-Vg2, were aligned using the ClustalW algorithm (Thompson et al. 1994) integrated into Mega 7 software (Kumar et al. 2016). The neighbor-joining method (Saitou and Nei 1987) was employed to construct a molecular phylogenetic tree with 1,000 bootstrap replicates.

1.3. Results

1.3.1. Characterization of full-length cDNA and deduced amino acid sequence of Maj-Vg2

The use of BLAST analysis on the transcriptome data from *M. japonicus* hepatopancreas led to the identification of three candidate contigs for *Maj-Vg2*, primarily derived from a single transcript as confirmed by subsequent cDNA cloning (Fig. 1.1). The full-length cDNA was 7830 bp with 22 bp of a 5' untranslated region (UTR), an ORF of 7662 bp encoding a protein of 2554 amino acids, and a 3' UTR of 146 bp, excluding the poly (A) tail (GenBank accession number LC726372). Notably, the canonical polyadenylation signal AATAAA was not present, but its variant TATAAA, reported in several animal species (Hajarnavis et al. 2004; Tian et al. 2005; Sheppard et al. 2013), was located 28 and 19 nucleotides upstream of the poly (A) tail. The 18 amino acid residues of the N terminal were predicted to be a signal peptide. Two potential cleavage sites (R-X-K/R-R) recognized by the endoprotease of the subtilisin family were found (Barr, 1991) (Fig 1.2).

Functional annotation of Maj-Vg2 revealed the presence of the vitellogenin-N domain, a domain of unknown function 1943 (DUF1943), and the von Willebrand factor D (vWFD) domain. The overall domain structure and arrangement were the same for Maj-Vg2 and Maj-Vg1. Protein BLAST analysis demonstrated high homology between other decapod Vgs and Maj-Vg2 (Table 1.3). In particular, the Vg-like protein (XP_042857980) predicted based on the genome project of *M. japonicus* (Kawato et al. 2021) exhibited the highest overall homology to Maj-Vg2 (98.8%), thus providing evidence for the existence of Maj-Vg2. The homology between Maj-Vg2 and Maj-Vg1 was 54% for the full-length amino acid and 71% for the region corresponding to the small subunit (Figs. 1.3 and 1.4). Similarly, comparison with other Vgs revealed that the small subunit region containing the vitellogenin-N domain had relatively higher homology than the full-length (Table 1.3).

1.3.2. Phylogenetic analysis of Vgs of decapods

A molecular phylogenetic analysis was conducted using deduced amino acid sequences to examine the evolutionary relationship of Vg among various crustacean species. The resulting phylogenetic tree indicated that Vg sequences from *Penaeus* species formed a large clade, distinct from Caridea, Portunoidea, and Astacidea groups (Fig. 1.5). Notably, a separate cluster (group 2) was formed by Vg2 of *M. ensis*, *F. merguiensis* (MeVg2), *L. vannamei* (LvVg2), and Maj-Vg2 within the Penaeus species clade, which was distinct from the group consisting of Vg1 and Vg3 (group 1).

1.4. Discussion

Research has demonstrated that, akin to various oviparous vertebrates and invertebrates, multiple Vg genes are present within a single species among decapod crustaceans. For example, the morotoge shrimp *P. japonica* possesses *Pj-Vg1* and *Pj-Vg2* (Jeon et al. 2011), while the banana shrimp *F. merguiensis* has *FmVg1*, *FmVg2*, and *FmVg3* of (Zhao et al. 2021). The sand shrimp *M. ensis* also possesses two *Vg* genes, *MeVg1* and *MeVg2* (Kung et al. 2004; Tsang et al. 2003). Furthermore, *LvVg2* and *LvVg3* have been identified in the whiteleg shrimp *L. vannamei*, in addition to the previously reported *LvVg1* (Raviv et al. 2006; Wang et al. 2020). In *M. japonicus*, a commercially significant species in the global market, I identified a new molecular species Maj-Vg2 based on the transcriptomic data from the hepatopancreas, which is distinct from known Maj-Vg1 (Tsutsui et al. 2000). These findings suggest that multiple *Vg* genes are present in many decapods.

A molecular phylogenetic analysis based on the amino acid sequences has also indicated the existence of multiple Vgs in decapods. In penaeid shrimps, the analysis revealed two major groups (groups 1 and 2 in Fig. 1.5), wherein two or three Vgs were observed and *Vg* gene duplication was likely to have occurred. *Vg* duplication has also been reported in vertebrates (Finn and Kristoffersen 2007; Babin 2008), and more recently, in decapod crustaceans (Wang et al. 2020). Thus, the presence of multiple Vgs is strongly expected to be common in penaeids, but it is also expected to be found in other decapods, such as brachyurans, astacideans in the

future, for which only a single *Vg* has been reported until now. For instance in portunoidea, the green mud crab *Scylla paramamosain* has two reported *Vg* sequences; however, due to the demonstrated over 95% amino acid identity between the deduced amino acid sequences of the two, with slight differences in length, they are believed to be two isoforms produced by alternative splicing (Jia et al. 2013; Yang et al. 2016). Therefore, their relationship is distinct from that between groups 1 and 2 in penaeid shrimps.

The advancement in the analysis of *M. japonicus* genomic DNA (Kawato et al. 2021) has influenced the current study. Specifically, the high degree of similarity; 99.0%, observed in the nucleotide and deduced amino acid sequences of the Vg-like protein (XP_042857980) predicted by the genomic database and Maj-Vg2 provides strong evidence for the presence of Maj-Vg2 on the *M. japonicus* genome. It is worth nothing that slight variations in nucleotide sequences observed between the two proteins may be attributed to the populational differences and/or geographical distance. A similar case was previously reported, wherein two *Maj-Vg1* cDNAs obtained from cDNA libraries of the ovary (AB033719) and hepatopancreas (AB176641) of separate prawns displayed 99.4% identity in their nucleotide sequences (Tsutsui et al. 2005b). Notably, an updated genome annotation also suggests the possible existence of a “third” Vg (XP_042867234), which shares 49% homology with Maj-Vg1 and 45% with Maj-Vg2. However, most of the nucleotide sequence encoding the third Vg is not found in the ovary (DRA010103) and hepatopancreas (DRA012835) transcriptome data used

in the current study (data not shown). Thus, further analysis of this gene product will be a subject for future research.

The comparison of amino acid sequences between Maj-Vg1 and Maj-Vg2 revealed a similarity of 54%. A functional domain search revealed the presence of the vitellogenin-N domain, DUF1943, and vWFD domain in both Vgs as shown in Fig. 1.3, but the absence of the DUF1080 domain found in some Vgs reported in other decapods (Kung et al. 2004; Wang et al. 2020; Zhao et al. 2021). Therefore, it is reasonable to assume that Maj-Vg2 shares a similar molecular function with Maj-Vg1. Amino acid composition analysis showed that the differences between the two Vgs were minimal, with variations of 1.0% at most. Essential amino acids (Teshima et al. 2002) showed even smaller differences (Table 1.4). The results suggest that the two Vgs complement each other as a source of nutritional supplementation during early development. A similar comparison between Pj-Vg1 and Pj-Vg2 of *P. japonica*, and between MeVg1 and MeVg2 of *M. ensis*, revealed differences in amino acid compositions of less than 1.8%, further supporting the complementary nature of Vgs in these species as a nutritional source.

Table 1.1 Sequences for primers and adaptors used for cDNA synthesis and cloning.

Primer	Nucleotide sequence
HPVg_F01	GTGGTGACCGTGTTCT
HPVg_R01	GGTGTTGGGAAACGGTTA
HPVg_F02	GTCACGGAACTCGAATGGTCCAAG
HPVg_R02	CTCAGTAGCCATGGATGGGTGAAC
HPVg_F03	ACAGATTCTGGCCACCAAAGCTC
HPVg_R03	GACAATGGGTTCTCCAAGAGGCATAG
HPVg_F04	GGACGTCAGGATTCACCATGAGAAC
HPVg_R04	CACTCTTGGGCAAGACTGCTG
HPVg_F05	GACAATGAACTCTGGAGAAGGAC
HPVg_R05	GCTCTCAAACTCTACAGTGCAAG
HPVg_F06	TTGGTTTCGATGTGGAAATCAAG
HPVg_R06	TCAATCAGCCACGTAAAGTCT
HPVg_F07	ATCGAGATTCCATTGCATAAACCT
dT-adaptor	TATCTAGAGGCCGAGGCGGACGACATG-d$(T)_{27}$VN
N_adapt01	TATCTAGAGGCCGAGGCGGAC

Table 1.2 Species names, GenBank accession numbers, and references for the Vgs used in the molecular phylogenetic analysis.

Species	Accession no.	References
Callinectes sapidus	ABC41925	Zmora et al. 2007
Cherax quadricarinatus	AAG17936	Abdu et al. 2002
Fenneropenaeus chinensis	ABC86571	Xie et al. 2009
Fenneropenaeus merguiensis 1	QXJ08926	Zhao et al. 2021
2	QXJ08927	Zhao et al. 2021
3	QXJ08928	Zhao et al. 2021
Homarus americanus	ABO09863	Tiu et al. 2009
Litopenaeus vannamei 1	AAP76571	Raviv et al. 2006
2	ROT77686	Wang et al. 2020
Macrobrachium rosenbergii	BAB69831	Okuno et al. 2002
Marsupenaeus japonicus	BAB01568	Tsutsui et al. 2000
Metapenaeus ensis 1	AAN40701	Tsang et al. 2003
2	AAT01139	Kung et al. 2004
Pandalus hypsinotus	BAD11098	Tsutsui et al. 2004
Pandalus japonica 1	ACU51164	Jeon et al. 2011
2	AHD26978	Jeon et al. 2011
Penaeus monodon	ABB89953	Tiu et al. 2006b
Penaeus semisulcatus	AAL12620	Avarre et al. 2003
Portunus trituberculatus	AAX94762	Yang et al. 2005
Scylla paramamosain	ACO36035	Jia et al. 2013
Upogebia major	BAF91417	Kang et al. 2008

Table 1.3 The percentage of amino acid identities with Maj-Vg2 and other decapod Vgs

Species	Accession no	Amino acid identity%	
		Full-length	Small subunit[1]
M. japonicus	XP_042857980	99	98
F. merguiensis Vg2	QXJ08927	80	89
M. ensis Vg2	AAT01139	64	75
P. indicus	QIA62006	60	79
P. monodon	ABB89953	55	80
M. japonicus Vg1	BAB01568	54	71
F. merguiensis Vg3	QXJ08928	53	80
F. merguiensis Vg1	QXJ08926	52	68
H. americanus	XP_042241566	44	60
C. quadricarinatus	AAG17936	41	55
S. paramamosain	ACO36035	35	50
P. trituberculatus	AAX94762	35	49
C. sapidus	ABC41925	34	51
P. japonica Vg2	AHD26978	39	55

[1] Amino acid sequence corresponding to the small subunit: from N-terminus to the first cleavage site.

Table 1.4 The percentage of variation in the essential amino acids (EAAs) of Maj-Vg1 and Maj-Vg2

EAAs	Maj-Vg1 (%)	Maj-Vg2 (%)
Arginine	4.68	5.32
Histidine	2.40	2.35
Isoleucine	7.38	7.17
Leucine	7.31	7.32
Lysine	5.99	6.38
Methionine	2.94	2.94
Phenylalanine	3.44	3.84
Thereonine	6.53	6.42
Tryptophan	0.66	0.70
Valine	8.54	7.87

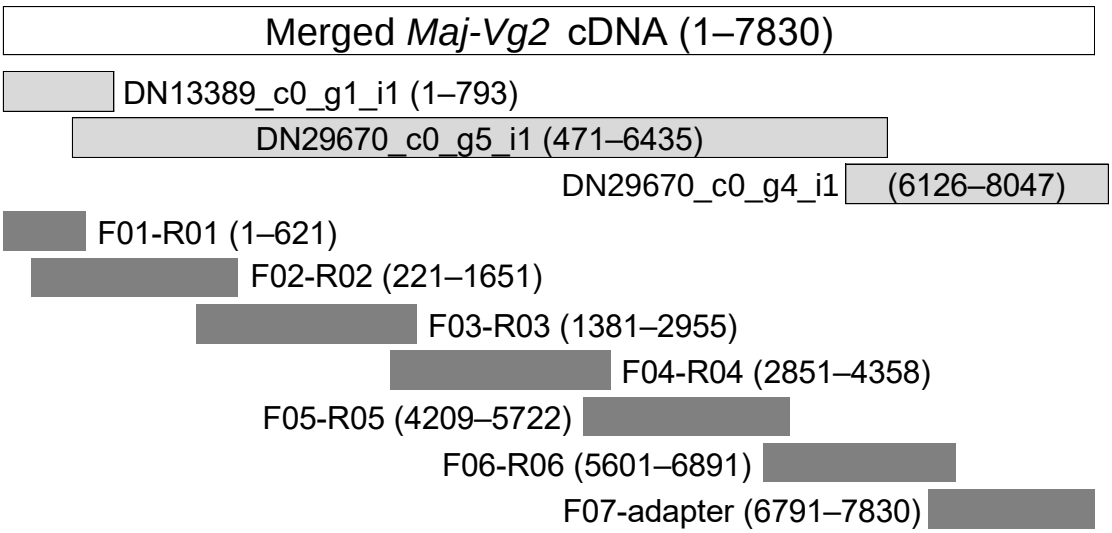

Fig. 1.1. Characterization of *Maj-Vg2* full-length cDNA. Contigs of DN13389_c0_g1, DN29670_c0_g5, and DN29670_c0_g4, shown in light gray, were obtained from the hepatopancreas transcriptome analysis. Nucleotide sequences of seven overlapping DNA fragments shown in dark gray were obtained by PCR, their nucleotide sequences were confirmed from several clones, and they were used for the reconstruction of full-length cDNA. Nucleotide numbers are shown in parentheses for respective contigs and DNA fragments.

1 GTGGTGACCGTGTTCTGCCACCATGACGACCTCAAGCCTCGTCTTCGTGCTCGCTCTCGTGGCAGGTGGACTTGCAGCGC
M T T S S L V F V L A L V A G G L A A

81 CCTGGGGAACTGAGGCGCCGAGATGCTCCACCGAATGCCCCATCACCGGATCCCCGAAGCTGGCCTACCAACCCGAGAAG
P W G T E A P R C S T E C P I T G S P K L A Y Q P E K

161 ACCTACACCTACGCCTACTCCGGCAAGTCTCGAGTCCAGCTCAAGGGCGTTGAGGACGGCGTCACGGAACTCGAATGGTC
T Y T Y A Y S G K S R V Q L K G V E D G V T E L E W S

241 CAAGCAGGTGAAGCTGACATGGATCACTCCCTGCGACATGGCCATCACGATCAAACACGCCAAGGTCGATGGTGCTGCAG
K Q V K L T W I T P C D M A I T I K H A K V D G A A

321 GAGCCGAGGTGCAGTTCCTGGAGCGCTATCCTTTGGTGGTGGCCGTGACAGATGGGCGCATCCAGCATGTGTGTACCCAC
G A E V Q F L E R Y P L V V A V T D G R I Q H V C T H

401 CCTGGCGACGCCCCTTGGTCCATCAACACGAAGAAGGGAATCGCCACGGCTCTGCAGAACTCACTCCCGTCACTCTCCCC
P G D A P W S I N T K K G I A T A L Q N S L P S L S P

481 TCTCAGCTCAGGACTCACTCTTACAGAGACGGATGCTGTAGGAGAGTGTCCAACAAAGTATGAGATTAAGACTGAAGGAG
L S S G L T L T E T D A V G E C P T K Y E I K T E G

561 AAAAGGTCATTGTTGTTAAGGAAAAGAACCACCGCCACTGCCATAACCGTTTCCCAACACCTGCTGAAACACCTGCACCA
E K V I V V K E K N H R H C H N R F P T P A E T P A P

641 TGGCTGAAGGCTCCCCTGCCAATCGAAGAGTCCAGGTCAGAGTGCAAGCAGGAAATCACCAATGGCATCTACACCGCCGT
W L K A P L P I E E S R S E C K Q E I T N G I Y T A V

721 CACCTGTGAGGACAAGAACATCGTTCGACCTGCCTTTGGAGTCTACAAGTACGTGGAGGCCAATCAAGAGTCAACACTTC
T C E D K N I V R P A F G V Y K Y V E A N Q E S T L

801 GCTTCATCTCTGAGTCCAGGGACACCTCAGCCATCAGTGCCATCCCTCGGGGAGAATTCGAAATTGAAAGCCTCTTGTTC
R F I S E S R D T S A I S A I P R G E F E I E S L L F

881 AACCATGAAACAGGGAAGGAGCCTGAGCTGGCACCTGAGGTGGATGCGGTCATGAAGGAGATCTGCCAGAAGACCATGGA
N H E T G K E P E L A P E V D A V M K E I C Q K T M E

961 GACTGTTGAGGCTGATGCTGCTGAACTGGTTGACAAGGCCCTTCACTTATTGCGTCGTGTTCCTGAGACAGTTGTTGGAG
T V E A D A A E L V D K A L H L L R R V P E T V V G

1041 CAACTGCAGAGAAGGTTCGAGGAGGACGTTATTGTGCAAACTCCGCCAGGTTGGAGAGCATCTTCCTGGATGCCATTGCC
A T A E K V R G G R Y C A N S A R L E S I F L D A I A

1121 TTCCTGTATGAGTCTGGTGCTGTCAAGATCATGGTCCAGGAAATAGAAAGTGGACGAGCAACAGGAGGACGTCTTGCTCT
F L Y E S G A V K I M V Q E I E S G R A T G G R L A L

1201 TTACACAGCTGCTCTCTACCTCACCCCACGACCCGACATCGAAGCTGTGAAGGCGCTCACACCCTTGTTCGAGAGCCCTC
Y T A A L Y L T P R P D I E A V K A L T P L F E S P

1281 GGCCTGTGCCTTCTGTGGCTCTGGCTGCTGCCACTATGGTCAACAACTACTGCCGTCACACTCCTCACTGCAGCGAGAAA
R P V P S V A L A A A T M V N N Y C R H T P H C S E K

1361 GCTCCAGTCAAGAGAATTGCACAGATTCTGGCCACCAAAGCTCAACGTCAGTGCTCTCCTTCTGCCGGTGAACAAGTTGA
A P V K R I A Q I L A T K A Q R Q C S P S A G E Q V E

1441 GAAAGAAGCTCTTGCAACATTCAAAGCACTAGGCAACATGGGTGTAGTTACACCTGCAGTAACAAGAGCAGCAGTCGGCT
K E A L A T F K A L G N M G V V T P A V T R A A V G

1521 GTATTGAACAAGAGGGAGTAGAAACTAGCATCCGAGTAGCAGCTGCACATGTCTTCAGGCATACTCAGTGTGCTCGCTAT
C I E Q E G V E T S I R V A A A H V F R H T Q C A R Y

1601 GTTACTGAAAAACTTAGCGACATCGCAGTTCACCCATCCATGGCTACTGAGGTTCGCATCGCAGCTTATTTGGGAGCAAT
V T E K L S D I A V H P S M A T E V R I A A Y L G A I

1681 CAGATGTGCTGAAGAGGAACATCTCCAGAAGATCATTTCAAAGGTTTCCGAAGAAAGGAATACTCAAGTACGAGGTTTCA
R C A E E E H L Q K I I S K V S E E R N T Q V R G F

1761 TTCTGAGCCACTTGTTGAACATCCAAGAATCCGCCTCACCTGACAGAGAACGCCTCCGTTACCTCCTCACCAACTTTGTT
I L S H L L N I Q E S A S P D R E R L R Y L L T N F V

1841 ATTCCCAGAGACTTTGATGGAGACATCAGGAAATATTCTCGCAATATCGAGATGTCTTACTTTGCTCCTTCATTCGGTAT
I P R D F D G D I R K Y S R N I E M S Y F A P S F G M

1921 GGGTGCTGGTGTGGAATCCAACATCATCTACACCCCTGAATCCTTCCTTCCTCGTTCCGTTGATCTGAACCTCCGAACAA
G A G V E S N I I Y T P E S F L P R S V D L N L R T

2001 CTATTGAGGACTTGAACATCAATTTAGGAGAGGCTGGCATCCGTCTTGAAGGTTTAGACCCAATCATTAAGGAACTGGTT

T I E D L N I N L G E A G I R L E G L D P I I K E L V

2081 GGTCCAGAAGGATATCTTCGCAAGGCCTCTTTTGGACGCATTTTGAAAGATGTGCTTGCTTTTGCTGAAGAGAAGGGACA
G P E G Y L R K A S F G R I L K D V L A F A E E K G H

2161 TCGCATTGCCGAGCATTTGGAAGACACACTTCGAGAAAAGAGGGCTATCAGCATGTCTACCATTTCCAGATTTTTCAAAA
R I A E H L E D T L R E K R A I S M S T I S R F F K

2241 AGCTCTATGGAGAAAGGAAAGAGGGCGAAGTCCGAGCTGATGTATTTGCAAGAATCTTTGGACATGAAGTAACCTATGCT
K L Y G E R K E G E V R A D V F A R I F G H E V T Y A

2321 TCCATTGCTGAAGACCTGAAGGAATTGGATGCTGACAGAATAATTGAGTCACTTTTCTCTTACTTCGATGAAATTCTTCC
S I A E D L K E L D A D R I I E S L F S Y F D E I L P

2401 CAATATTCACAATCTAGACATCGATTCTGCACGCACAGGCCAAATATTCCTTGACTACTCTTTGCCCACCATTCAAGGTA
N I H N L D I D S A R T G Q I F L D Y S L P T I Q G

2481 CTCCACTCAAGATTAAGTTGGAAGGAACTGCTGTTGTAGGAATCAAACTGGCTGGTGACATTAATATCATTGAACTCTTC
T P L K I K L E G T A V V G I K L A G D I N I I E L F

2561 ACCAACCCAGCACATGTTGAGAGGAGTCTCAAACTGATCCCAAGTGCACCAGTTGCAGTCCATGGTTTTGTTGGCTATGA
T N P A H V E R S L K L I P S A P V A V H G F V G Y D

2641 CTGTCACATTGCTAAGGCAGGGACTGAGCTGAAGAGCACCATTGCAACGGCCAATGGAGCCACCATTAACATCAGAAAGA
C H I A K A G T E L K S T I A T A N G A T I N I R K

2721 CTGAAGACAATGCATTTGAGTTTGCACTGGATCTTCCAGAGAGGATGGAACTACTCAGTGTCAAGGCAGAGACCAATCTT
T E D N A F E F A L D L P E R M E L L S V K A E T N L

2801 GTCAAGGCTGTTGGTAAGAGAGTGATGAAGGTTAGTCCACCCTCCATGAGGGACGTCAGGATTCACCATGAGAACTGCAT
V K A V G K R V M K V S P P S M R D V R I H H E N C M

2881 GGAAGCTCTTGAACCAGTGTTTGGTCTGAAGATGTGCCATGAGATGAGCTTCCCTGATATCTTCCGTAGTACTGCTATGC
E A L E P V F G L K M C H E M S F P D I F R S T A M

2961 CTCTTGGAGAACCCATTGTCGCCAAGTTGTACATTGAGAAAACCGACCCTTCCATGAGAGGTTACCGAATGACTACTGCC
P L G E P I V A K L Y I E K T D P S M R G Y R M T T A

3041 ATCAAGAACAAGAAGAGTAATAAGGTCATCAAGGTGAACATGGAGACCCAAGGAGCTGCGACACCACGACAGGCTGAAAT
I K N K K S N K V I K V N M E T Q G A A T P R Q A E M

3121 GACTATGTCCTATACCAAGGAAGAGAGGTCCCACGCTGTTTATGCCAAATTTGAGTCTTCTAGCATCTCAGCAGGACTGT
T M S Y T K E E R S H A V Y A K F E S S S I S A G L

3201 GGACTACCTTCACCAATGAGGAAGAGCACAAGGCCATTGAGACCTTTGTAAAGCTCAGGTCCAATGAATTTGATATTTCA
W T T F T N E E E H K A I E T F V K L R S N E F D I S

3281 CGAGGATTTAAGGTCGACATCATTGGAAAGGAAGTTGCTAATGAAGCGCAATATGAAGTAAACGTGTTCACAAGTCGGAA
R G F K V D I I G K E V A N E A Q Y E V N V F T S R N

3361 CAGGAGGTTTGCTACCTCATCTAAGATTGTGGAAGCCAAGTTCATCAAGAAGATTAATGACCCGGCGGTGGAAATAATTT
R R F A T S S K I V E A K F I K K I N D P A V E I I

3441 GCAGGACAATGAATGAACTGAAGGACTATGTTGATTTTAACTTCGAAGTTGATGCAGACTTTAGATACAGCCCATATACC
C R T M N E L K D Y V D F N F E V D A D F R Y S P Y T

3521 TGTATGTTTATTCCAACTGAAGTACGGAAGATAGAATTCCACACTGGTATTAGAGGTTGGAAAATAGCATCTGCTATCCA
C M F I P T E V R K I E F H T G I R G W K I A S A I H

3601 CAAAATGACTGGGTCTACTGAAGCTAGTGAGCACGTTGCTACCTTTAGTGTGGAAGAGGGAAACAGTGAGATTATGTCAG
K M T G S T E A S E H V A T F S V E E G N S E I M S

3681 TGAAGGCTGTCATGAATACCAAAGGAAGAATGTTCAGGAACATGATTATTCATAACGAAGTTGCAGTTAATTTTGGACAA
V K A V M N T K G R M F R N M I I H N E V A V N F G Q

3761 CATTCATATAGAGCTTGCTATGACCTATTCCTTGGCGCATCGAAGATGGGAACAAGTGTAGAAGTGACTAAGCCCAAAGA
H S Y R A C Y D L F L G A S K M G T S V E V T K P K E

3841 GAACTTGAAGATGTTTGAATTTGGAGCTTTGTATGAGCGATCCAGCAATGCACACACTGTCCAAGTGTTGGTTGATGCTC
N L K M F E F G A L Y E R S S N A H T V Q V L V D A

3921 CGGAATACATGCGAGCTATCAAGTTTGAAAGCAAGTTCAATGAGGAAGAAAACGGCAAATATGCCGTTGAAGTTGCCGTC
P E Y M R A I K F E S K F N E E E N G K Y A V E V A V

4001 AAACAAGGTCAGCGTATTCTGCTGGAACTTGATGGTCCGGTGACCCTCATTTTCTCACCGAGAAAGCTGAAGATCGAAGC
K Q G Q R I L L E L D G P V T L I F S P R K L K I E A

4081 TGAACTTAAAGTTGCTGTCCTTGATATGGAACCTCACATCATTTCTACAACTATACTTGGAAGTAACAGCAAACAAATTC
E L K V A V L D M E P H I I S T T I L G S N S K Q I

4161 TGGCTTTTGAAATGAAGAACAGACAAGAAAGCCTGTTTGCATTTAAATGGACAATGAACTCTGGAGAAGGACCCGAACAG
L A F E M K N R Q E S L F A F K W T M N S G E G P E Q

4241 AAAACAACAAGCAGCACCAAGCTCGTAGTCCCTGCCCTCATGGAGTTCATGCTTGATACCACAGTAATGCATGAGAACGT
K T T S S T K L V V P A L M E F M L D T T V M H E N V

4321 CCATGTGAGCCTCAACACAGCAGTCTTGCCCAAGAGTGCGTCTGCACATCGCGTTAAGGCTTTTGTAGATATTGACGGTG
H V S L N T A V L P K S A S A H R V K A F V D I D G

4401 GAAATAAGAAGATGAATGCTGAATTCGCCTGGGATGCAGATCGTAACCCTAACAACAAGATTGTTGTTGACGCTAATGTA
G N K K M N A E F A W D A D R N P N N K I V V D A N V

4481 ATCAGCAGTTCTTCTGACCTGGGTCATGCTTCTGTTCACGGAAACGTCATAATAGCTGGAGAACCATACCATATGAAGCT
I S S S S D L G H A S V H G N V I I A G E P Y H M K L

4561 GAACCTGAATGCTGAAGATATCATGGCATCTGGTTTTGAGCTGGAAGTAACCATACCTTCTCAGAGGACTTTTGCCGTAG
N L N A E D I M A S G F E L E V T I P S Q R T F A V

4641 AAGCCAGCTATAAGATCGAGGACCAACAACCTACCACCAAAGTTATTACCGTCTTCAGATATAAGAACACTGAGGGTGAA
E A S Y K I E D Q Q P T T K V I T V F R Y K N T E G E

4721 GAGCATAAATTCACAGGTTCTGTTGCAGCTGAGAGGCTCGATGGGCCATACTGCTATGCTCTTGAGACCAAGGTGGTGTA
E H K F T G S V A A E R L D G P Y C Y A L E T K V V Y

4801 TGTAGCTCCTGAAGGAAAGGAAACAAGGTTGGAGACGATTCTGAAACATCATAAAAAGCCAGAGGCACATGTGATATTAT
V A P E G K E T R L E T I L K H H K K P E A H V I L

4881 TTAAGGTTGATGCTGAAGGCCTTATCCTAAGGAAGCCTCTCATGTTCGAATTCGCCGTTGAAAACAAAGAAGGTTCTTAT
F K V D A E G L I L R K P L M F E F A V E N K E G S Y

4961 GAAGGCAAGTGCATGATGACAAGAAATGCTCCCAAAACTGTCTTTGACTGGAATGTGAGGATCCATCCCCATGGTGAGAT
E G K C M M T R N A P K T V F D W N V R I H P H G E I

5041 TGAGGCCATTGAAGCAGGACTGGACACTAAGGCTGCAGTTCAGCTACTGAAGATTATCCGTGCTGTGGTTACTTTTGAGA

```
      E  A  I  E  A  G  L  D  T  K  A  A  V  Q  L  L  K  I  I  R  A  V  V  T  F  E

5121 AGGAGGAGAGAATCGAAAGGTCCGAGCCAGCCAACTATCGCTATCGCTTCAGCAAGCCAACACCAACATCTTACAATATT
     K  E  E  R  I  E  R  S  E  P  A  N  Y  R  Y  R  F  S  K  P  T  P  T  S  Y  N  I

5201 CTTGTAAAGACTCCATCACGTACAATGGAGGGTGAAGCTCACATTTCTCCTTCACAGTCAGGAATCAAGTTCTATTCTAA
      L  V  K  T  P  S  R  T  M  E  G  E  A  H  I  S  P  S  Q  S  G  I  K  F  Y  S  N

5281 CAAGCATAGTTCTCAGTCCCCATATGAGATTGGTTACAAGGCTACACACGAGGGCAACCAAGGCAAGTGGGAGAGCCAAA
      K  H  S  S  Q  S  P  Y  E  I  G  Y  K  A  T  H  E  G  N  Q  G  K  W  E  S  Q

5361 TAAACCATCCAGTTCTTCCCAAAGCCATGCAGTTTGCACTTCAGTACACAGCTGGTCAAGGAACAGTGGAAGGAACTGTA
     I  N  H  P  V  L  P  K  A  M  Q  F  A  L  Q  Y  T  A  G  Q  G  T  V  E  G  T  V

5441 GAGCTGGATATTTTCCCATCCAGTGAGGACAAGATTACTGGCCACCTTGCATCAACACGAGTTTCAGAAAGCACCATTAG
      E  L  D  I  F  P  S  S  E  D  K  I  T  G  H  L  A  S  T  R  V  S  E  S  T  I  R

5521 AACTGAAGCTTCCATTGTCAGCAGAATTCTTAGAGTGAGCCCCCAAATCATCCTAACCACAGCCGTCGCTCCAGACACTG
      T  E  A  S  I  V  S  R  I  L  R  V  S  P  Q  I  I  L  T  T  A  V  A  P  D  T

5601 TTGGTTTCGATGTGGAAATCAAGAAATCTGCATCTGCCCCGCCATCCCTCAAGGTTATTGCCAAATACGACAAAACTACT
     V  G  F  D  V  E  I  K  K  S  A  S  A  P  P  S  L  K  V  I  A  K  Y  D  K  T  T

5681 CCAAGGAATGCAGTCCTGGCTTGCACTGTAGAGTTTGAGAGCACCCCAGTATTTGAAGTGTCTGGGGTAGTGAAACCTGA
      P  R  N  A  V  L  A  C  T  V  E  F  E  S  T  P  V  F  E  V  S  G  V  V  K  P  D

5761 TGAAACAGCCACATGTAACGGCCTGGCAATGTCTGCTGTCGTACAGGCACCTATTTTAGGTACACATCACATCTACTCCA
      E  T  A  T  C  N  G  L  A  M  S  A  V  V  Q  A  P  I  L  G  T  H  H  I  Y  S

5841 CCATGTGTAAACCAGCCTTTGTGGAGGTGACCACCATCAGACAGGGCGCTGATAGGAAATACATTGCTAGGATTGGCGTA
     T  M  C  K  P  A  F  V  E  V  T  T  I  R  Q  G  A  D  R  K  Y  I  A  R  I  G  V

5921 CAGGCACCAGACAATTTAGAATTTAGTCTGAGTCAAGGAACAACACAGTCACAGGAGGAGAGTAATATCATCCTTGCTCG
      Q  A  P  D  N  L  E  F  S  L  S  Q  G  T  T  Q  S  Q  E  E  S  N  I  I  L  A  R

6001 CCTGGAGATGGTTGACCCTGCAGTAATCAATATTGGCTTTGCTTTTGAGCGTGAAGAAATCTACCGTGTGAAGGAACTTA
      L  E  M  V  D  P  A  V  I  N  I  G  F  A  F  E  R  E  E  I  Y  R  V  K  E  L
```

```
6081 TCTGGGAAGAGCTCTCAAATGTCTTGAGCTCCATGGAATCTGGATTTGAGGATATCATAAGAGAAGTCGCAGGGAGTGCA
     I  W  E  E  L  S  N  V  L  S  S  M  E  S  G  F  E  D  I  I  R  E  V  A  G  S  A

6161 GCAAACGTTCCAGCACCAGAGTTCTACACACTGATAGGTGAAGCCAAAAGGGAACTGATGATGATCTACCAGGATCTGGT
      A  N  V  P  A  P  E  F  Y  T  L  I  G  E  A  K  R  E  L  M  M  I  Y  Q  D  L  V

6241 AGAAGATAGCGGGCCAATCTTTGAAGAAATGCCTTCCTTTGGTCGCATCAGGAACAGCTTTAGGAAAATCGTTCGACTTT
       E  D  S  G  P  I  F  E  E  M  P  S  F  G  R  I  R  N  S  F  R  K  I  V  R  L

6321 GGGCTCAGCTAGAGAAAAGCATCCTTGAACAACGTGAGAGGATGTTCAGTGTTTGTCTAAATATGGTCAAGGATATTACA
     W  A  Q  L  E  K  S  I  L  E  Q  R  E  R  M  F  S  V  C  L  N  M  V  K  D  I  T

6401 GCTCGGATGTATACAGTCATGAAGGAAACGATGGAAGTTCTAGAGACAGGAGAGCTGCCTGAACCTGTACGCCGTATGGT
      A  R  M  Y  T  V  M  K  E  T  M  E  V  L  E  T  G  E  L  P  E  P  V  R  R  M  V

6481 AGAAGGACTGAAAAGGACTGAAGTTTTTGAGATCGTAAAGAGGTTATCGGATGCTGTGTTGGATAAGTACCCAGAGGAAT
       E  G  L  K  R  T  E  V  F  E  I  V  K  R  L  S  D  A  V  L  D  K  Y  P  E  E

6561 ATGAGGCCATTAAGTATGTTATAACTAACATGATGAGTACTCTTGAGAGAGACTATAATCTGATGTTCGAGAGGATCATG
     Y  E  A  I  K  Y  V  I  T  N  M  M  S  T  L  E  R  D  Y  N  L  M  F  E  R  I  M

6641 GAAGTTCCTGCTTTCCAAAGGATCATCAACTGGATCATGAAAAACCTGAGCCCTGGTCGTCTGGCAGCAGTAGAGGCAAA
      E  V  P  A  F  Q  R  I  I  N  W  I  M  K  N  L  S  P  G  R  L  A  A  V  E  A  N

6721 TGCACTGGCAGAAATACTTCTTGAGGATTTCCACGTCCTCGTCATGAAGGCTGAAGGGAACCAACTTAAGATCGAGATTC
       A  L  A  E  I  L  L  E  D  F  H  V  L  V  M  K  A  E  G  N  Q  L  K  I  E  I

6801 CATTGCATAAACCTCTGTATTCAGTGGTGCAGTTTATCAAGGATTTGATAAACCCATACGAAATCCGCAAAGACTTTACG
     P  L  H  K  P  L  Y  S  V  V  Q  F  I  K  D  L  I  N  P  Y  E  I  R  K  D  F  T

6881 TGGCTGATTGAGGGCACTCTGCCTTATACCCTGGAAGACCTAATTTGGATGTATTACTCCTTGATTCCACATCGTATCAC
     W  L  I  E  G  T  L  P  Y  T  L  E  D  L  I  W  M  Y  Y  S  L  I  P  H  R  I  T

6961 AGATTTGTTGCCCCCCTACCCTCGAACAGCCATGGTAGTCGGAGGTACCGAGGTCCTCACCTTCGACGGCCTTGTGGTGC
       D  L  L  P  P  Y  P  R  T  A  M  V  V  G  G  T  E  V  L  T  F  D  G  L  V  V

7041 GAGCGCCTCGAAGCCCCTGCAAGGTTCTGTTGGCCACCCACGGTTCCCATAGCATTATGATGTCCCACCCGGAGCCCTCA
     R  A  P  R  S  P  C  K  V  L  L  A  T  H  G  S  H  S  I  M  M  S  H  P  E  P  S
```

7121 GCTCCTGCACAACTTGAGCTCAAAACACCCGATGCCACTGTTGTTATCAAGCCTGACACTGAAGTCCTTGTTAATGGTCA
A P A Q L E L K T P D A T V V I K P D T E V L V N G Q

7201 ACCAATCAGGGGATCGGAGGAGACCGTTGGAAAGATTAGGATCGAGAAGAAGGCTGGAGAGATAGTGGTAGGATGTCCTT
P I R G S E E T V G K I R I E K K A G E I V V G C P

7281 TAATGAAGGTCATTGTAGCTAAGAAGGGCCAAGTTGTGGCCATAGAGGCTTCAGGATGGACTTTCGGCCGCGTAGCAGGA
L M K V I V A K K G Q V V A I E A S G W T F G R V A G

7361 CTTCTGGGTCCCAACAACGGGGAAGTCGGAGATGATCGCCTCATGCCCAACGGCGCAGAAGCCTCTAGCCCTCGCGAGTT
L L G P N N G E V G D D R L M P N G A E A S S P R E L

7441 GGTAGCTGCTTGGCAGGAGAGGAAGCAGTGCTCCATGCCTGAGATTCCTCCTGCCATAGCCACAGTAGCTCGTGTGATCA
V A A W Q E R K Q C S M P E I P P A I A T V A R V I

7521 AGTGTGAAGCACTCCTCAGCATCCGATCACAGTGCATTGCGGTAGTTGAGCCGGAGCCCTTCATTAGGATGTGCCATGCA
K C E A L L S I R S Q C I A V V E P E P F I R M C H A

7601 GCCCAAGATGCTTGCGACGCCATTGCAGCTTACAAAACCTTCTGTGCTTTAAAAGGAGTTGAAGAAGCATCCCCAATGCC
A Q D A C D A I A A Y K T F C A L K G V E E A S P M P

7681 TTGCTAGTAGATGGACAGCCCTCGATCAGTCCTTAATGAAACTTGATGATGACGACCTGCCCACGTATGAATAACGAGTA
C * * M D S P R S V L N E T * * * R P A H V * I T S

7761 TCTTTTTGCTTTTGTAATATTTCACTAAAAAATGTAAAAAAA<u>TATAAA</u>AAT<u>TATAAA</u>CCCTTGGCCTTGTAAAAAAAAAA
I F L L L * Y F T K K C K K I * K L * T L G L V K K K

Fig. 1.2. Nucleotide sequence of *Maj-Vg2* and its deduced amino acid sequence. Nucleotide numbers are shown in left. Predicted signal peptide was italicized, and putative cleavage sites are shown in bold letters with underline. Polyadenylation signal variants are underlined.

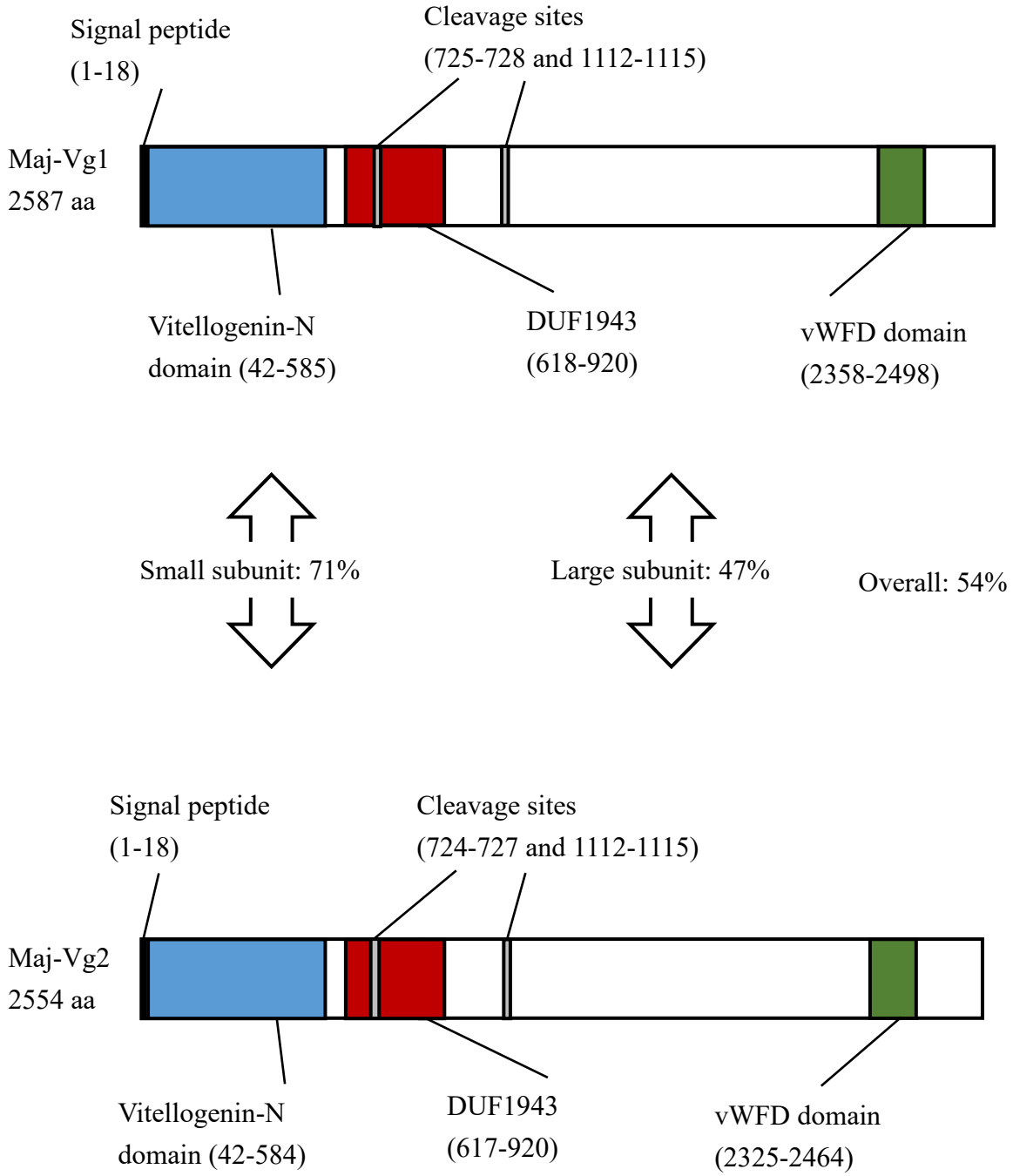

Fig. 1.3. Schematic view of Maj-Vg1 and Maj-Vg2. Positions of the three domains, signal peptide, and two putative cleavage sites are shown in parentheses. Amino acid identities between small subunits, large subunits, and overall are also shown.

```
Maj-Vg1  MTTSSLLFVLALVAGGLAAPWGADLPRCSTECPISGSPKLAYQPEKTYTYQYSGKSRVQLKGVDDGVSETEWAARVDLTW 80
Maj-Vg2  MTTSSLVFVLALVAGGLAAPWGTEAPRCSTECPITGSPKLAYQPEKTYTYAYSGKSRVQLKGVEDGVTELEWSKQVKLTW 80
         ******:***************:: ********:*************** ************:***:* **: :*.*** 
Maj-Vg1  ISPCDVAISFNNMKMDGARGPIAARTLERHPLVVAVVDGRVQHVCAHPDDEPWAINLKKGVASAFQNSIPSLSTVSSGMT 160
Maj-Vg2  ITPCDMAITIKHAKVDGAAG-AEVQFLERYPLVVAVTDGRIQHVCTHPGDAPWSINTKKGIATALQNSLPSLSPLSSGLT 159
         *:***:**:::: *:*** *   .: ***:******.***:****:**.* **:** ***:*:*:***:****.:***:* 
Maj-Vg1  VTETDVVGKCPTTYQIETEGEKVIVVKEKNHRHCQQRYPTPAQTPAPWLKAPLPIEESKSECKQEITNGIYTSIMCHDKN 240
Maj-Vg2  LTETDAVGECPTKYEIKTEGEKVIVVKEKNHRHCHNRFPTPAETPAPWLKAPLPIEESRSECKQEITNGIYTAVTCEDKN 239
         :****.**:***.*:*:**********************::*:****:***************:************:: *.*** 
Maj-Vg1  IVRPAIGIYQYVEAHQESTLHFISETTDTSAISAIPRGEMHIESLLYNHETMKDPQLAPELDQLMKEICEKTKDTVEAET 320
Maj-Vg2  IVRPAFGVYKYVEANQESTLRFISESRDTSAISAIPRGEFEIESLLFNHETGKEPELAPEVDAVMKEICQKTMETVEADA 319
         *****:*:*:****:*****:****: ***********:.*****:**** *:*:****:* :*****:** :****:: 
Maj-Vg1  AALVAKALHLLRRVPETVVVEIAQKVRQGHYCSDSAKLESIFLDAVAFLHESGAVKVMVQEILHGRATGGRLALYTAALY 400
Maj-Vg2  AELVDKALHLLRRVPETVVGATAEKVRGGRYCANSARLESIFLDAIAFLYESGAVKIMVQEIESGRATGGRLALYTAALY 399
         * ** **************   *:*** *:**::**:********:***:******:*****  **************** 
Maj-Vg1  LTPRPTIEALKALAPLFESPLPMPSLLLATASMVNHYCRHTPHCHQEAPVERIAEILAAKVEGHCSPSIGVEEKEEALAI 480
Maj-Vg2  LTPRPDIEAVKALTPLFESPRPVPSVALAAATMVNNYCRHTPHCSEKAPVKRIAQILATKAQRQCSPSAGEQVEKEALAT 479
         ***** ***:***:****** *:**: **:*:***:******** ::***:***:***:*.: :**** * : ::**** 
Maj-Vg1  FKALGNMGVVTPAVTRAAAQCIEKEGLETSIRVAAAQAFRQANCDRPAVQKLVDIATRPTFETEVRIASYLAAIRCAEKE 560
Maj-Vg2  FKALGNMGVVTPAVTRAAVGCIEQEGVETSIRVAAAHVFRHTQCARYVTEKLSDIAVHPSMATEVRIAAYLGAIRCAEEE 559
         ******************. ***:**:*********:.**:::* * ..:** ***.:*:: ******:**.******:* 
Maj-Vg1  HLEQIIEKISEEENTQVRGFVLGHLINIQESTCPTKENLKYLLTNVVIPTDFEKDFRKFSRNVEMSYHAPAFGMGADLES 640
Maj-Vg2  HLQKIISKVSEERNTQVRGFILSHLLNIQESASPDRERLRYLLTNFVIPRDFDGDIRKYSRNIEMSYFAPSFGMGAGVES 639
         **::**.*:***.*******:*.**:*****:.* :*.*:*****.*** **: *:**:***:****.**:*****.:** 
Maj-Vg1  NIIYAPGSFIPRAVNLNMKAAVDETHMDLAEIGARFEGIDSIIEELFGPEGYLRKATFGKIMQDITGFAEEKGLKVMEHI 720
Maj-Vg2  NIIYTPESFLPRSVDLNLRTTIEDLNINLGEAGIRLEGLDPIIKELVGPEGYLRKASFGRILKDVLAFAEEKGHRIAEHL 719
         ****:* **:**:*:**::::::: :::*.* * *:**:*.**:**.********:**:*::*: .****** :: **: 
Maj-Vg1  KQTLRTKRSIDSSVISDFFGKLYGEGRS-HTHAEVFARIMGHEITYADVAESLKGVTADTLIETFFSFFEESLEQMKGLN 799
Maj-Vg2  EDTLREKRAISMSTISRFFKKLYGERKEGEVRADVFARIFGHEVTYASIAEDLKELDADRIIESLFSYFDEILPNIHNLD 799
         ::*** **:*. *.** ** ***** :. ..:*:*****:***:***.:**.** : ** :**::**:*:* * :::.*: 
Maj-Vg1  LNTARTAQLYMDYSLPTIQGTPLKLKLAGTAVAGLKMEGDFNIAQILSDPGNLQTGIKLFPALSVQATGFVGFECRLTRV 879
Maj-Vg2  IDSARTGQIFLDYSLPTIQGTPLKIKLEGTAVVGIKLAGDINIIELFTNPAHVERSLKLIPSAPVAVHGFVGYDCHIAKA 879
         :::***.*:::**************:** ****.*:*: **:** :::::*.::: .:**:*: .* . ****::*::::. 
Maj-Vg1  GIEMENTISSATGASINIRTTENKKIQMELEIPEKMELLNIQAETYLVKAVGKKLTKITPPTVRDVRVTHAACLNAVEPV 959
Maj-Vg2  GTELKSTIATANGATINIRKTEDNAFEFALDLPERMELLSVKAETNLVKAVGKRVMKVSPPSMRDVRIHHENCMEALEPV 959
         * *::.**::*.**:****.**:: ::: *::**:****.::*** *******:: *::**::****: *  *::*:*** 
```

```
Maj-Vg1  LGIKVCYNINMPDVFRANGLPLGEPAIAKLYIEKADPSMRGYLMTAAIKNKKGNKFIKLNVEAAGATTPRRAEMTLSYTK 1039
Maj-Vg2  FGLKMCHEMSFPDIFRSTAMPLGEPIVAKLYIEKTDPSMRGYRMTTAIKNKKSNKVIKVNMETQGAATPRQAEMTMSYTK 1039
           :*:*:*:::.:**:**:..:***** :*******:******* **:******.**.**:*:*: **:***:****:****
Maj-Vg1    EEGSHIVSAKLDSSSIAAGVWATLTNEEGHKAMETYVKFDYGQIAISRGIKLDMIVKEESAGKEFEVNVFSGRSRRFTPE 1119
Maj-Vg2    EERSHAVYAKFESSSISAGLWTTFTNEEEHKAIETFVKLRSNEFDISRGFKVDIIGKEVANEAQYEVNVFTSRNRRFATS 1119
           ** ** * **::****:**:*:*:**** ***:**:**:  .:: ****:*:*:* ** :   ::*****:.*.***:..
Maj-Vg1    SHIVEAKFIKKTNGPEVNVDVICRTRNALAQYFDLNIEVGADFMEFSPEGVYPARYIPKVSILLPVALRKMEVHANTVAW 1199
Maj-Vg2    SKIVEAKFIKKINDP--AVEIICRTMNELKDYVDFNFEVDADFRYS-----------PYTCMFIPTEVRKIEFHTGIRGW 1186
           *:********* *.*   *::**** * * :*.*:*:**.***             * ..:::*. :**:*.*:.  .*
Maj-Vg1    KLASYIREGSQSGESRELISAFKLSKGRNDIIYVQATHKIEGTLPQNIVIENEATVEVGRSSYRAMYDIFYHPEKIGASV 1279
Maj-Vg2    KIASAIHKMTGSTEASEHVATFSVEEGNSEIMSVKAVMNTKGRMFRNMIIHNEVAVNFGQHSYRACYDLFLGASKMGTSV 1266
           *:** *:: : * *: * :::*.:.:*..:*: *:*. : :* : :*::*.**.:*:.*: **** **:*  ..*:*:**
Maj-Vg1    EVFRTAGNEKVAEMEAIYENTGEKYYTKFLVEAPGYIRPVRIEATAEEETGGRYALESAIKYGERTVFEVTGPVMARFTS 1359
Maj-Vg2    EVTKPKENLKMFEFGALYERSSNAHTVQVLVDAPEYMRAIKFESKFNEEENGKYAVEVAVKQGQRILLELDGPVTLIFSP 1346
           ** :.  * *: *: *:**.:.: : .:.**:** *:*.:::*:. :** .*:**:* *:* *:* ::*: ***   *:.
Maj-Vg1    KTAKLQANIKLSAMASEPYIIGANFVFGNKKQMIAMEIKEREEPVFGVEWKMVQES--AEKTTLSIAFVLPALIENKVDA 1437
Maj-Vg2    RKLKIEAELKVAVLDMEPHIISTTILGSNSKQILAFEMKNRQESLFAFKWTMNSGEGPEQKTTSSTKLVVPALMEFMLDT 1426
           :. *::*::*::.:  **:**.:.:: .*.**::*:*:*:*:*.:*..:*.* . .   :*** *  :*:***:*  :*:
Maj-Vg1    VITEDLVHVSFNNLVLPKTSYRRRVKGFADVNIGEKRANVEFSWDADKSPEKKLVVDASLISSPSNPGHAEIHGNIVIAG 1517
Maj-Vg2    TVMHENVHVSLNTAVLPKSASAHRVKAFVDIDGGNKKMNAEFAWDADRNPNNKIVVDANVISSSSDLGHASVHGNVIIAG 1506
           .: .: ****:*. ****::  :***.*.*:: *:*: *.**:****:.*::*:****.:***.*: ***.:***::***
Maj-Vg1    EPYHMKLILTATNLLEYMEGENGFKLLLTTPSQKTIVLGASCDVQLEGTTTKVVSVIEYKNMENKEYKYTSVIALEKLGG 1597
Maj-Vg2    EPYHMKLNLNAEDIMASG-----FELEVTIPSQRTFAVEASYKIEDQQPTTKVITVFRYKNTEGEEHKFTGSVAAERLDG 1581
           ******* *.* :::        *:* :* ***:*:.: ** .:: : .****::*:.*** *.:*:*:*. :* *:*.*
Maj-Vg1    PYDYVVKAKVIYKQPETQEIMLETEVKHQWTPEEHLVAFKVGAKAPVLKMPLMIAFSIHNTRGSFVGFCKIERNTPSNVF 1677
Maj-Vg2    PYCYALETKVVYVAPEGKETRLETILKHHKKPEAHVILFKVDAEGLILRKPLMFEFAVENKEGSYEGKCMMTRNAPKTVF 1661
           ** *.:::**:*  ** :*  *** :**: .** *:: ***.*:. :*: ***: *::.*..**: * * : **:*..**
Maj-Vg1    EWKIQMTPEGGIEVVEAGLDMKAINEVLKIVHAVVTFEEEGYQAYGQETAKYQYRFTRPSPTTYIMQMRTPTRTIEGRAK 1757
Maj-Vg2    DWNVRIHPHGEIEAIEAGLDTKAAVQLLKIIRAVVTFEKEERIER-SEPANYRYRFSKPTPTSYNILVKTPSRTMEGEAH 1740
           :*:::: *.* **.:***** **  ::***::******:*      .*.*:*:***::*:**:* : ::**:**:**.*:
Maj-Vg1    LSPRESGIKFYPNKGKAEAKYEVGYKANHQGSWGQHASNIEVRMNHPTLPKPIMVAAHYTAIGETIKGTIELDIFPEEEN 1837
Maj-Vg2    ISPSQSGIKFYSNKHSSQSPYEIGYKATHEGNQGKWES----QINHPVLPKAMQFALQYTAGQGTVEGTVELDIFPSSED 1816
           :** :******.** .::: **:****.*:*. *:  *    ::***.***.: .* :***   *:**:******..*:
Maj-Vg1     KITGTLETQRISENAIRVEVFLTGKILQVNPKAIVTVAYAPETFALDVVFHKTPSTAPVFALAAKYDKTSAHNAAATFTV 1917
Maj-Vg2     KITGHLASTRVSESTIRTEASIVSRILRVSPQIILTTAVAPDTVGFDVEIKKSASAPPSLKVIAKYDKTTPRNAVLACTV 1896
            **** * : *:**.:**.*. :..:**:*.*: *:*.* **:*..:** ::*:.*:.* : : ******:.:**. : **
```

Fig. 1.4. Deduced amino acid sequence alignment of Maj-Vg1 and Maj-Vg2. The numbers indicate amino acid positions. Asterisk represents conserved amino acids. Colon and dots represent amino acid with strong and with weak similarity, respectively. Signal peptides are denoted by underline. Cleavage sites are shown by dark grey background. Vitellogenin-N domain, DUF 1943, and vWFD domains are shown by blue, red, and green letters, respectively.

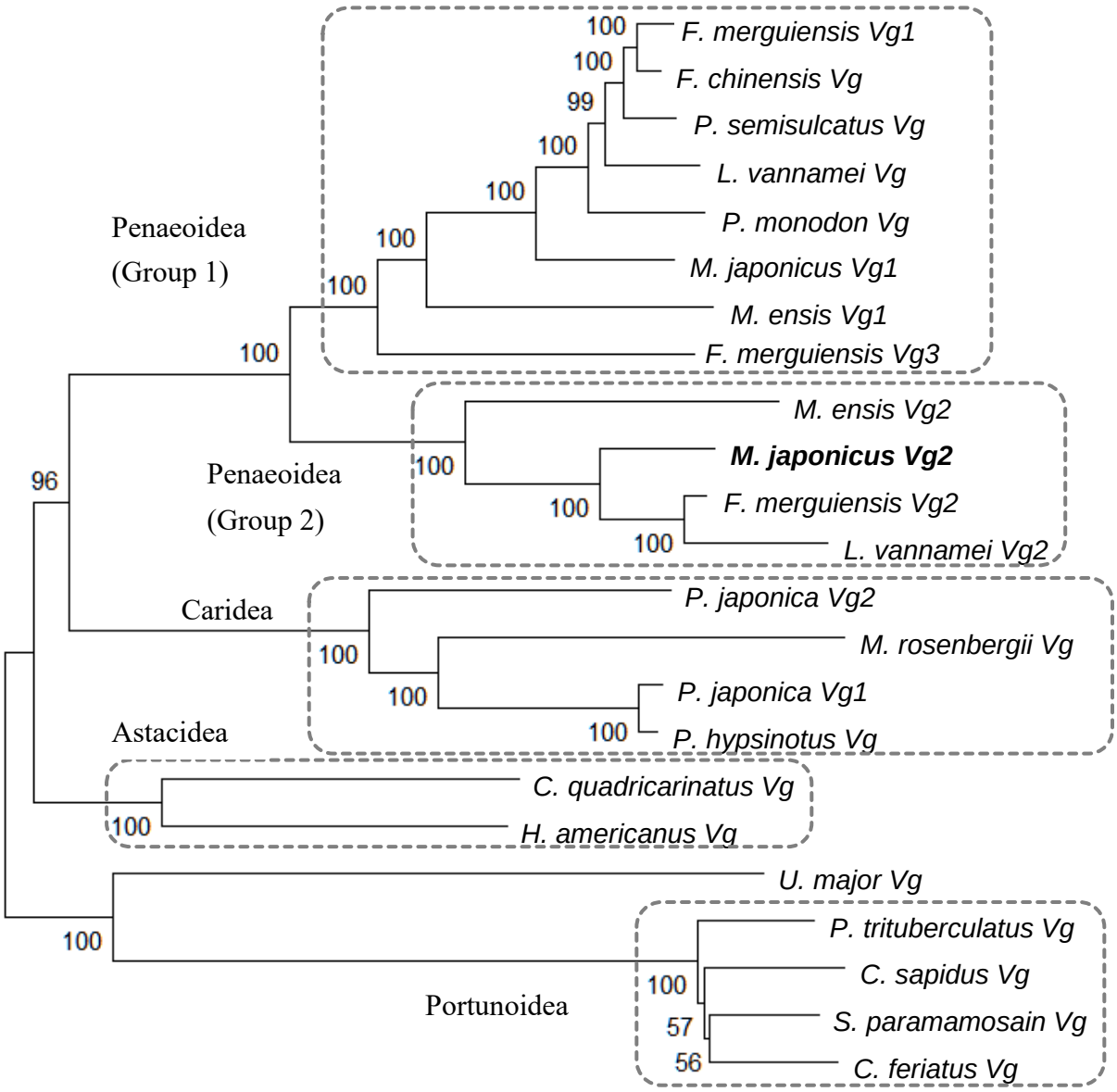

Fig. 1.5. Molecular phylogenetic tree of decapod Vgs. Only sequences for which relevant publications have been published were selected for analysis (see Table 1.2). The phylogenetic tree was constructed by the neighbor-joining method. Values at the nodes represent the percentage of 1,000 bootstrap replicates. The scale bar shows the number of substitutions per site.

Chapter 2. Expression dynamics of vitellogenins during natural maturation and after eyestalk ablation

2.1. Background

The synthesis of Vg in decapod crustaceans is known to occur primarily in the ovary and/or hepatopancreas, i.e., endogenous, exogenous or combination of both. Exogenous Vg is synthesized in tissues outside the ovary then transported through the haemolymph to be accumulated in the developing oocytes as yolk (Meusy 1980). Molecular approaches using Vg genes and cDNAs have helped in identifying the sites of Vg synthesis in different species. In Penaeidea, Vg synthesis is likely to occur in both the ovary and hepatopancreas, while in Caridea, Astacidea and Portunoidea it principally happens in the hepatopancreas (Wilder et al. 2010). In *M. japonicus*, cDNA cloning has allowed for the determination of *Vg* expression levels at different stages of vitellogenesis, as well as the dynamics of expression levels during both natural and artificial maturation (Tsutsui et al. 2000; Tsutsui et al. 2005b). Previous molecular analyses, such as northern blot and quantitative RT-PCR, have revealed the dynamics of *Maj-Vg1* expression levels in both the ovary and hepatopancreas of *M. japonicus* during vitellogenesis. Specifically, the expression levels were observed to increase during endogenous and exogenous vitellogenic stage in the ovary and hepatopancreas, while levels decreasing during late exogenous stage in the ovary. The dynamics of the expression for the additional Vg (*Maj-Vg2)* in the hepatopancreas have not yet been studied and will be the focus of this chapter.

Vg expression has been also used as a molecular indicator for the characterization of hormones involving in the vitellogenesis. The VIH/GIH, a regulator of *Vg* expression belonging to CHH superfamily and produced by the XOSG system located in the eyestalk, has been found to inhibit *Vg* expression in the ovary of some species such as *L. vannamei* and *M. japonicus*. This is consistent with the well-known phenomenon of ESA promoting ovarian development (Brown and Jones 1949; Tsutsui et al. 2005a; Tsutsui et al. 2007; Tsutsui et al. 2013b).

Despite the potential benefits of hormonal manipulation for seedling production, the development of alternative techniques remains a challenge. VIH gene knockdown using RNA interference (RNAi) has shown less efficacy in promoting ovarian maturation compared to ESA (Treerattrakool et al. 2008; Treerattrakool et al. 2011; Feijó et al. 2016; Duangprom et al. 2022). Therefore, further understanding of vitellogenesis with a focus on multiple *Vg* genes is necessary to establish an alternative to ESA. Investigating the expression dynamics of these multiple genes during natural maturation and after ESA, as well as their regulatory mechanisms, may help comprehensively understand vitellogenesis in *M. japonicus* and other decapod crustaceans.

2.2. Materials and Methods

2.2.1. Expression levels of vitellogenins in eyestalk-ablated prawns

Immature adult *M. japonicus* (weighing from 17.7 to 30.5 g; average: 24.2 ± 0.6 g; n = 28) were sourced from local prawn farms in Tokushima Prefecture, Japan. The prawns were then transported to the Mie Prefectural Fish Farming Center and acclimatized for approximately two weeks in a 2500-L tank supplied with filtered seawater at 20°C. The prawns were fed a commercial diet (Vitalprawn, Higashimaru Co., Kagoshima, Japan) during the acclimatization period. On the day when acclimatization was complete (day 0), 14 prawns of the experimental group were subjected to bilateral ESA and kept individually in a small cage placed in the 2500-L tank. The remaining 14 prawns of the control group were housed in another cage within the same tank. On day 0, the hepatopancreas and ovary were dissected from 4 prawns of each group. On day 3 and day 10, the tissues were collected from 5 prawns of each group. The dissected tissues were partly stored in RNA*later* solution at −20°C for RNA extraction and partly stored in Davidson's fixative solution for histological analyses.

Total RNA was extracted from the hepatopancreas and ovary using a previously described method (refer to Chapter 1). The quantity of the isolated RNA was assessed by observing clear bands. The concentration of total RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher). Subsequently, 1 μg of total RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) using a random primer included in the kit as per the manufacturer’s instructions. The resulting cDNA was then used for quantitative real-time PCR, as described in the following section.

2.2.2. Expression levels of vitellogenins in wild prawns

Wild *M. japonicus* (weighing from 35.1 to 70.2 g; average: 54.5 ± 2.2 g; n = 19) caught in Enshu-nada, Japan, were purchased from a local fish market in Aichi Prefecture in October 2018 and February 2019. On the day of procurement, the prawns were immediately dissected, and their hepatopancreas and ovary were stored in RNA*later* solution and Davidson's fixative solution, as described earlier. The ovarian developmental stage of each prawn was determined by histological analysis, which revealed that 9, 4, and 6 prawns were in previtellogenic, early yolk globule, and late yolk globule stages, respectively (refer to Results section). Total RNA extraction and subsequent cDNA synthesis were performed as described previously.

2.2.3. Quantitative real-time PCR (qRT-PCR)

The cDNA solutions were diluted to a concentration of 10 ng of total RNA per μL. The primers and TaqMan probe sequences used in this study are listed in (Table 2.1). Those used for *Maj-Vg1* were designed in a previous report (Tsutsui et al. 2018). The primers employed for the preparation of standard DNA fragments are also listed in (Table 2.1). The standard DNA for each target gene, along with the qPCR amplicon sequences, were amplified by PCR using the primer sets and cDNAs from the hepatopancreas or ovary. Standard DNA fragments having the desired length were purified using AMPure XP (Beckman Coulter Life Sciences Japan, Tokyo, Japan), and 2 ng/μL standard solutions were prepared. The qPCR reactions were conducted using 2 μL of cDNA sample, 400 nM of each primer, 200 nM of probe, and 1× Luna

Universal Probe qPCR Master Mix (New England BioLabs). The 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was utilized for real-time monitoring of the fluorescence signal generated during PCR. Standard curves were generated using each standard DNA ranging from 4000 to 0.04 pg prepared by serial 10-fold dilutions, and arbitrary values ranging from 4000 to 0.04 were assigned correspondingly. Then, the threshold cycles were utilized to determine the relative expression levels based on the standard curves. As each standard had almost the same length, similar amplification efficiencies were achieved in qPCR (> 95%, data not shown). The utilization of the standard curves facilitated a relative comparison of the expression levels between *Maj-Vg1* and *Maj-Vg2*, as well as the expression levels between the hepatopancreas and ovary.

2.2.4. Statistical analysis

Gene expression levels were represented as the mean ± standard error of the mean (SEM). To determine the statistical differences in gene expression levels, the Mann-Whitney U test was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

2.3. Results

2.3.1. Expression levels of vitellogenins in eyestalk-ablated prawns

In this study, the expression profiles of the two *Vg* genes were assessed in prawns that underwent bilateral ESA. Consistent with prior findings (Tsutsui et al. 2005b), individuals

subjected to ESA exhibited a remarkable increase in GSI, suggesting that vitellogenesis was promoted (Fig. 2.1a). Notably, *Maj-Vg1* expression in the ovary was significantly upregulated following ESA, with discernible differences between control and ablated groups becoming apparent as early as day 3 (Fig. 2.1b). Additionally, *Maj-Vg2* expression levels in the hepatopancreas were significantly elevated in ablated prawns compared to control prawns on days 3 and 10 (Fig. 2.1c). Conversely, *Maj-Vg1* expression in the hepatopancreas remained relatively stable throughout the experiment, with significant differences being observed only on day 3 (Fig. 2.1d).

2.3.2. Expression levels of vitellogenins in wild prawns

The expression patterns of the two *Vg* genes were investigated in wild prawns with previtellogenic and vitellogenic ovaries. As previously reported, *Maj-Vg1* was expressed in both the hepatopancreas and ovary (Tsutsui et al. 2000; Tsutsui et al. 2005b). *Maj-Vg1* expression in the ovary was observed to be low during the previtellogenic stage, gradually increasing during the early yolk globule stage, and subsequently decreasing during the late yolk globule stage (Fig. 2.2a). Similarly, *Maj-Vg1* expression in the hepatopancreas displayed a similar trend, although high expression levels persisted during the late yolk globule stage before declining with increasing GSI (Fig. 2.2b). In contrast to *Maj-Vg1*, *Maj-Vg2* expression was not detected in the ovary (Fig. 2.2c), only in the hepatopancreas, where its expression remained low in the previtellogenic stage, but increased rapidly during the early yolk globule

stage, and showed a tendency to decrease during the late yolk globule stage (Fig. 2.2d).

2.4. Discussion

In this chapter, the expression of *Maj-Vg2* was restricted to the hepatopancreas, whereas *Maj-Vg1* was detected in both the hepatopancreas and ovary, indicating that the hepatopancreas, a major extraovarian source, along with the ovary are Vg synthesis sites in *M. japonicus*. This finding is consistent with prior reports conducted on other decapods such as the black tiger shrimp *Penaeus monodon* (Tiu et al. 2006b), *M. ensis* (Tsang et al. 2003), *L. vannamei* (Wang et al. 2020), and *F. merguiensis* (Zhao et al. 2021). Additionally, the expression of multiple *Vg* genes in a tissue-specific manner has been described. For example, in *F. merguiensis*, *FmVg2* was mainly expressed in the hepatopancreas, while *FmVg1* and *FmVg3* were predominantly expressed in the ovary (Zhao et al. 2021). Both *MeVg1* and *MeVg2* were primarily expressed in the hepatopancreas of *M. ensis*, but *MeVg1* was also expressed in the ovary (Kung et al. 2004; Tsang et al. 2003). Although the biological significance of such multiple Vg production by different tissues remains unclear, elucidating the dynamics of Vg production provides important insights into the regulatory mechanisms of vitellogenesis.

To investigate the regulatory mechanisms of vitellogenesis in *M. japonicus*, I conducted an evaluation of the two *Vg* gene expression levels in wild-caught prawns, including previtellogenic and vitellogenic individuals. I also used prawns that had undergone ESA to induce vitellogenesis. In the wild-caught prawns, it was observed that the overall expression

patterns of *Maj-Vg1* in the hepatopancreas and ovary were consistent with those reported in the previous study (Tsutsui et al. 2000), with high gene expression levels observed from early to late yolk globule stages in the hepatopancreas and during the early yolk globule stage in the ovary. In contrast, *Maj-Vg2* expression in the hepatopancreas showed an increase during the early yolk globule stage and a decline in the late yolk globule stage. These results suggest a tissue-specific and dynamic contribution to Vg synthesis with *Maj-Vg2* playing a complementary role in the production of yolk protein. Temporal and locational expression changes of multiple *Vg* genes during vitellogenesis have been reported in other species, such as *F. merguiensis* and *L. vannamei* (Zhao et al. 2021; Wang et al. 2020). Therefore, it is likely that the presence of multiple *Vg* genes and multiple sites of Vg production contributes to the rapid ovarian development of penaeid shrimps. In case eyestalk-ablated prawns, *Maj-Vg1* expression in the ovary and *Maj-Vg2* expression in the hepatopancreas were significantly increased, while *Maj-Vg1* expression in the hepatopancreas remained low. Previous research solely attributed the increase in GSI after the ESA to an increase in *Maj-Vg1* expression in the ovary (Tsutsui et al. 2005b). However, the present study shows that *Maj-Vg2* expression also contributes to the increase in GSI. This finding suggests that *Maj-Vg2* complements *Maj-Vg1*, but it also indicates that vitellogenesis process induced by the ESA differs from the natural process at the molecular level. Overall, the expression of *Maj-Vg2* is relatively low compared to *Maj-Vg1* in the ovary and hepatopancreas, suggesting that the contribution of Maj-Vg2 to

vitellogenesis is auxiliary or complementary. To determine its actual role, it is necessary to demonstrate that the protein is present in the hemolymph and oocytes. Detection with specific antibodies against recombinant Maj-Vg1 and Maj-Vg2 proteins or with mass spectrometry can be useful for this purpose.

Interestingly, *Maj-Vg2* expression in the hepatopancreas was induced by ESA, displaying a pattern of expression similar to that of *Maj-Vg1* in the ovary. It is widely acknowledged that the XOSG complex produces VIH, which negatively regulates Vg synthesis. In *M. japonicus,* six peptides of the CHH-family from the XOSG down-regulate *Maj-Vg1* expression in the ovary, suggesting their potential role as VIH (Tsutsui et al. 2005a; Tsutsui et al. 2013a). However, the hormonal regulation of *Maj-Vg2* expression in the hepatopancreas is still uncertain, as the regulatory mechanisms of *Maj-Vg1* expression in the hepatopancreas also remain unknown. The findings from the ESA experiment of this study suggest that the regulation of *Maj-Vg1* expression in the hepatopancreas is distinct from that of *Maj-Vg1* in the ovary. To gain further insight into the mechanism of vitellogenesis in *M. japonicus,* further research should investigate the effects of various hormones, particularly the six CHH-family peptides from XOSG and other hormone candidates identified in the ovary (Tsutsui et al., 2020), on the expression of *Vg1* and *Vg2* in the hepatopancreas. In the following chapters, the effect of some hormones such as the ovarian specific Maj-ILP1 (Chapter 3), MF and 17β-estradiol

(Chapter 4) on yolk protein genes (*Maj-Vg1* and -*Vg2*) in the hepatopancreas and ovary of *M. japonicus* will be examined by *ex-vivo* assay and discussed in details.

Table 2.1 Sequences of standards, primers and probes used for qPCR.

Primer	Nucleotide sequence
For qPCR standards	
StVg1_F01	GCCCTACCACATGAAACTGAT
StVg1_R01	TGTATTGTGAATGGAGAACGCAA
StVg2_F01	GGAGGGTGAAGCTCACAT
StVg2_R01	AGATGCAGATTTCTTGATTTCCACAT
For qPCR[1]	
Mj-Vg2 qF	TGGTTACAAGGCTACACACGA
Mj-Vg2 qR	TGACCAGCTGTGTACTGAAGT
Mj-Vg2 qPr	ACCATCCAGTTCTTCCCAAAGCCATGCAG

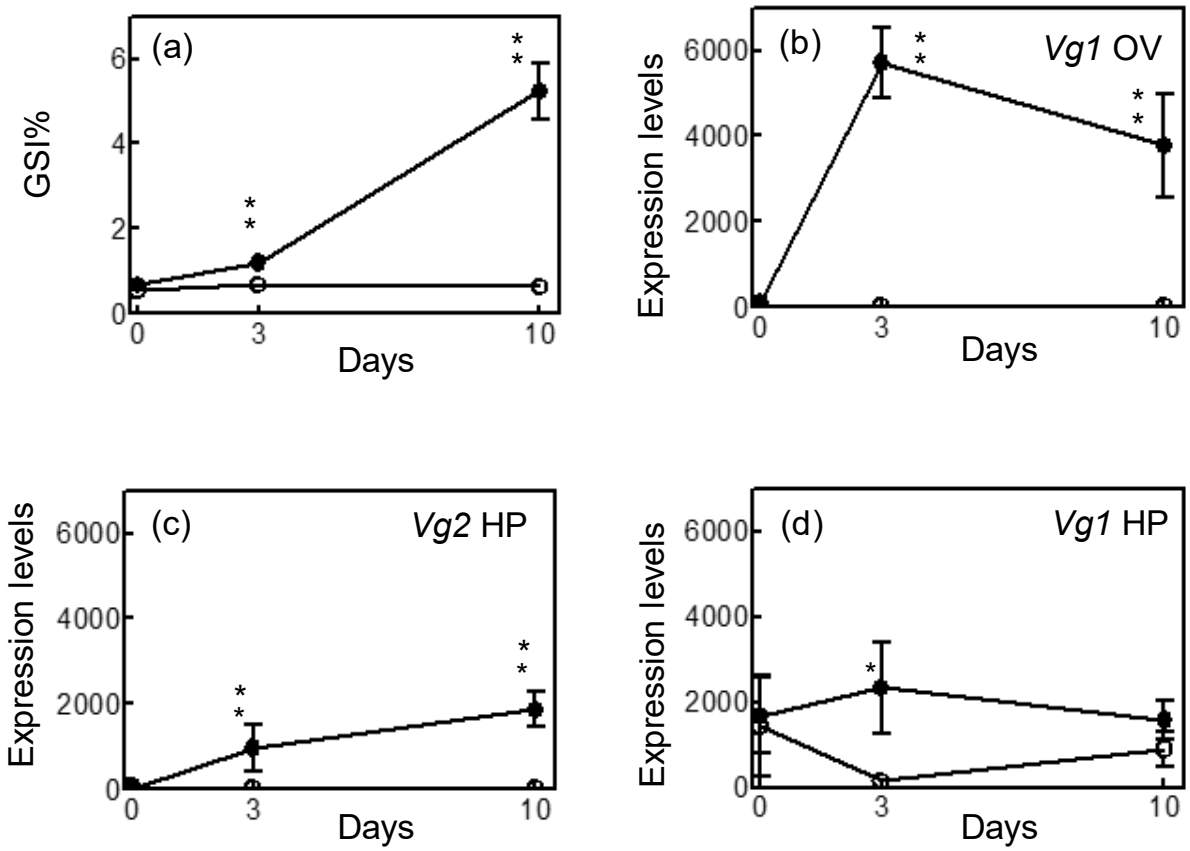

Fig. 2.1. Expression levels of *Maj-Vg1* and *Maj-Vg2* in immature prawns with and without ESA. Changes of GSI (a), and changes in expression of *Maj-Vg1* in the ovary (b), *Maj-Vg2* in the hepatopancreas (c), and *Maj-Vg1* in the hepatopancreas (d), are shown. Open circles indicate intact prawns, and closed circles indicate eyestalk-ablated prawns (mean ± SEM, n = 5 prawns). Asterisks indicate significant differences in mean values in the comparisons of respective days by the Mann-Whitney U test (*, $p < 0.05$; **, $p < 0.01$).

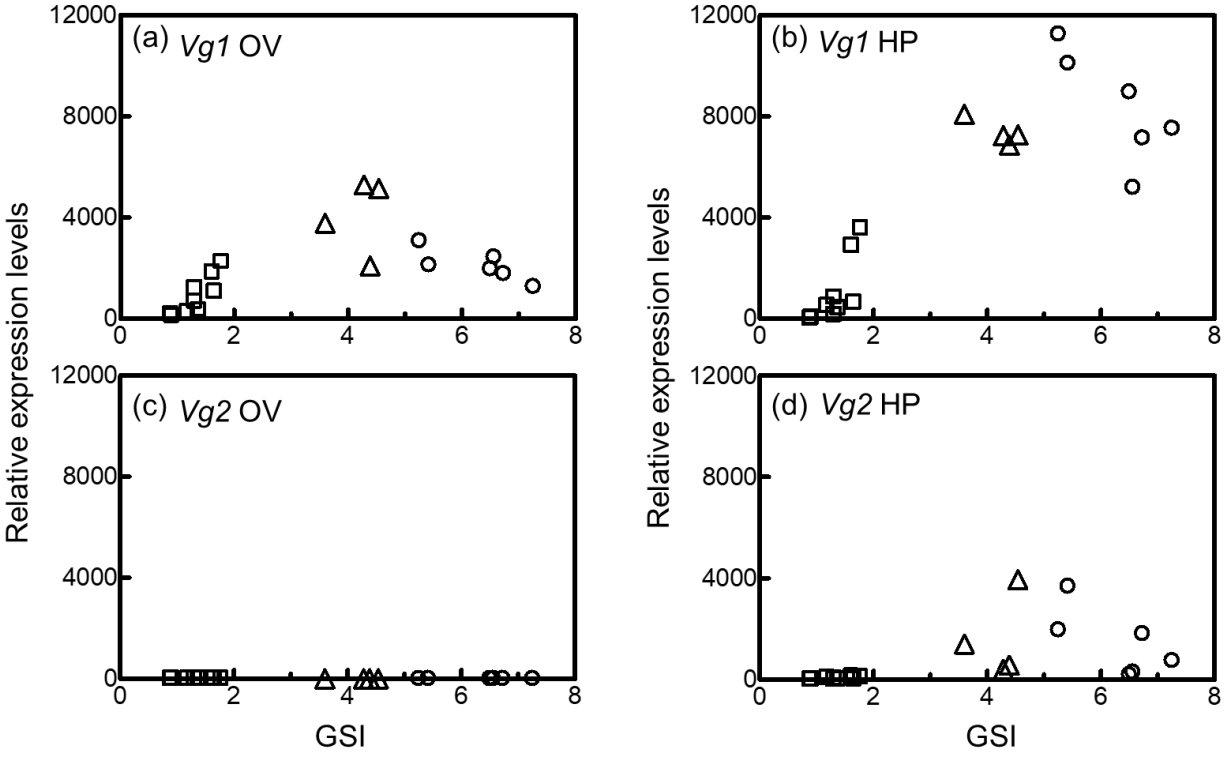

Fig. 2.2. Expression levels of *Maj-Vg1* and *Maj-Vg2* in wild prawns. Expression of *Maj-Vg1* in the ovary (a), *Maj-Vg1* in the hepatopancreas (b), *Maj-Vg2* in the ovary (c), and *Maj-Vg2* in the hepatopancreas (d) are shown with the comparison with GSI. Squares, triangles, and circles indicate previtellogenic ($n = 9$), early yolk globule ($n = 4$), and late yolk globule stages ($n = 6$), respectively.

Chapter 3. Insulin-like peptides as potential regulators of vitellogenesis in *M. japonicus*

3.1. Background

The investigation into the involvement of a hormone-like substance in the processes of yolk accumulation or final maturation of oocytes can be accomplished by evaluating its impact on either yolk protein synthesis or the progression of germinal vesicle breakdown (GVBD). For instance, the bioassay focusing on the GVBD has been utilized to characterize the gonad-stimulating substance belonging to the ILP family in starfish (Mita et al. 2009), although its establishment in *M. japonicus* remains incomplete. On the other hand, a bioassay using Vg as an index of vitellogenesis is available to investigate the former aspect (Tsutsui et al. 2005a). Vg is the precursor to the major yolk protein, which is synthesized and subsequently accumulated within the oocytes during vitellogenesis, thereby contributing to ovarian development (Wilder et al. 2010). In *M. japonicus*, the expression of *Maj-Vg1* is significantly upregulated in the ovary and hepatopancreas during vitellogenesis (Tsutsui et al. 2005b). Building upon this knowledge, an *ex-vivo* bioassay has been established, employing the ovarian *Maj-Vg1* gene expression as an indicator of vitellogenesis, and has proven useful in characterizing hormones involved in this process (Tsutsui et al. 2013a; Tsutsui et al. 2020). Briefly, the inhibitory effect of six CHH-family peptides, known as Pej-SGP-I, -II, -III, -V, -VI and -VII, produced by XOSG on the expression of *Vg* gene (*Maj-Vg1*) in the ovary was assessed by using the ovarian culture system (Tsutsui et al. 2013a). The stimulatory effect of

recombinant neuroparasin-like peptide (Maj-NPLP) on Vg synthesis in the ovary was evaluated by using the same ovarian culture system (Tsutsui et al. 2020). However, the effects of these hormones on *Maj-Vg1* in the hepatopancreas is unknown until now. The novelty of this thesis is the identification of new *Maj-Vg2* exclusively expressed in the hepatopancreas (Chapter 1). Based on this information, there is a need to establish a bioassay system to examine the effect of these hormones in the hepatopancreas. Therefore, an *ex-vivo* assay for the hepatopancreas of *M. japonicus* was constructed for the first time within the context of this chapter. Studying the effects of gonadal hormones on *Maj-Vg* mRNA expression in the hepatopancreas as well as the ovary will help to outline the endocrine regulation of vitellogenesis in *M. japonicus*.

Insulin-like peptides (ILPs) are a diverse family of signaling molecules found in various animal species (Okamoto et al. 2016). Mammalian ILPs, such as insulin and insulin-like growth factors (IGFs), play crucial roles in regulating glucose metabolism and growth through the insulin/IGF signaling (IIS) pathway (Bathgate et al. 2002). Other ILPs, such as relaxins and INSLs, bind to G protein-coupled receptors and regulate various physiological functions, particularly reproduction (Speck et al. 2022).

ILPs are conserved in invertebrates as well, with putative genes encoding ILPs, insulin receptors (IRs), and downstream components found in many species. For example, the nematode *Caenorhabditis elegans* contains 40 ILPs in its genome regulating aging, stress resistance, larval development, and reproduction (Pierce et al. 2001; Fernandes de Abreu et al.

2014; Matsunaga and Kawano 2018). The fruit fly *Drosophila melanogaster* has 8 ILPs known as Drosophila insulin-like peptides Dilps (Grönke et al. 2010; Garelli et al. 2012). They are regulating lifespan, body growth, development and reproduction (Semaniuk et al. 2021). In this model organisms, IIS is believed to affect physiological functions such as reproduction as a nutrient-sensing pathway. ILPs have also been shown to play roles in the regulation of reproductive processes in mollusks, the cuttlefish *Sepiella japonica* (Lü et al. 2022) and in echinoderms, the starfish *Asterina pectinifera* (Mita et al. 2009).

In decapod crustaceans, four types of ILP have been identified, including insulin, relaxin, gonadulin, and insulin-like androgenic gland factor (IAG) (Veenstra 2020). Previous studies have focused mainly on IAG (Nagamine et al. 1980), and its former-found ortholog androgenic gland hormone (AGH) from terrestrial isopod crustaceans (Katakura and Hasegawa 1983; Suzuki and Yamasaki 1998). Both AGH and IAG have been known as important regulators in male sex differentiation (Martin et al. 1999; Katayama et al. 2014; Alfaro-Montoya et al. 2016; Harlıoğlu and Farhadi 2017; Shi et al. 2019; Ge et al. 2020; Tan et al. 2020; Tsutsui et al. 2020; Katayama et al. 2022). Practical application of a sexual manipulation technique, specially targeting the IAG, is currently being used in certain decapod species (Nguyen et al. 2023). For example, the administration of *IAG*-specific dsRNA has been reported to induce complete and functional sex change in juvenile male *M. rosenbergii* resulting in the conversion of males into neo-females. Similarly, RNAi technology was successful in *P. monodon* and *C.*

quadricarinatus (Banks et al. 2020). However, little is known about the functions of other ILPs in these animals. Four insulin family peptides have been identified in *M. japonicus*, including three male-dominant peptides (IAG, GON, and ILP2) and one female-dominant peptide (Maj-ILP1), which is primarily expressed in the ovary and may play a role in female reproduction (Tsutsui et al. 2020; Tsutsui et al. 2022).

Therefore, the primary objective of this chapter was to investigate the potential involvement of Maj-ILP1 in vitellogenesis. To achieve this, the function of the chemically synthesized Maj-ILP1 was evaluated in *ex-vivo* tissue culture systems by examining the expression levels of two egg-yolk protein precursor genes: *Maj-Vg1,* expressed in both the ovary and hepatopancreas, and the recently identified *Maj-Vg2* expressed in the hepatopancreas (See Chapter 1).

3.2. Materials and methods

3.2.1. Chemical synthesis of Maj-ILP1

The ovarian-specific Maj-ILP1 was chemically synthesized using a combination of solid-phase peptide synthesis and regioselective disulfide bond formation reactions under the expertise of Professor Hidekazu Katayama of Tokai University. He graciously granted me access to utilize it in this study.

3.2.2. *Ex-vivo* incubation experiment

In this chapter, both adolescent female prawns and immature adult female prawns were used for *ex-vivo* incubation. The adolescent prawns were obtained from Mie prefectural fish farming

center (Mie, Japan) in December 2022 and purchased from a prawn farm (Okinawa, Japan) in February 2023. The immature adult females were purchased from a prawn farm (Okinawa, Japan) in January 2023. All prawns were promptly used upon procurement. The average body weight and gonadosomatic index (GSI) of 16adolescent prawns used for the hepatopancreas tissue incubation were 13.37 ± 0.27 g and 0.38 ± 0.02% (mean ± SEM), respectively, while those of the 27 adolescent prawns used for the incubation of the ovary were 13.56 ± 0.40 g and 0.46 ± 0.03, respectively.

Seven immature adult females (average body weight of 22.44 ± 0.80 g (mean ± SEM); average GSI of 0.98 ± 0.08%) (mean ± SEM) were also promptly used in both experiments upon procurement. However, due to the success or failure of the tissue fragment preparation, the final number of samples used in the experiments may differ from the numbers described in this section.

3.2.3. Preparation of the incubation culture medium 199

The culture medium 199 was used for *ex-vivo* incubation in the hepatopancreas. 1 x medium 199 containing Earle's Salts (Thermo Fisher Scientific, Waltham, MA, USA) was modified to be used without a CO_2 incubator. 1% antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA, USA), 50 μg/mLbovine serum albumin (BSA, Thermo Fisher Scientific), and 350 mg/L $NaHCO_3$ (Sigma-Aldrich Japan, Tokyo, Japan) were added to the medium, and the solution was buffered to pH 7.4 with 25 mM HEPES (Dojindo Laboratories, Kumamoto,

Japan). The osmolarity of the medium was adjusted to 800 - 830 mOsm with the addition of 4 M NaCl.

3.2.4. Incubation of hepatopancreatic explants

The hepatopancreas of female prawns was carefully dissected and rinsed in the cold Medium 199 (Fig. 3.1). The hepatopancreas was cut from the middle, trimmed using scissors then small fragments (8~10 pieces) from the middle part of tissue, approximately 50 mg in weight/fragment, were placed in 24-well culture plates filled with 1.5 mL medium containing 0 (control), 0.1, and 1.0 M Maj-ILP1. The fragments were incubated for 6 hours at 20°C in constant darkness while gently agitated (30 rpm). After 6 hours of hormone treatment, all tissue fragments were stored in 400 µL RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA) at -20°C in preparation for RNA extraction and subsequent quantitative real-time PCR (qRT-PCR).

3.2.5. *Ex-vivo* ovarian assay

For the ovarian tissue fragment incubation, the previously established protocol was followed (Tsutsui et al. 2005a; Tsutsui et al. 2013a; Tsutsui et al. 2020). Specifically, 100 mL of incubation solution with the following components were prepared: 336.8 mM NaCl, 10.7 mM KCl, 19.3 mM $MgSO_4$, 16.9 mM $MgCl_2$, 8 mM CaCl2, 1 mM NaH_2PO_4, 5.4 mM $NaHCO_3$ (Sigma-Aldrich Japan), 0.4 mM taurine (Thermo Fisher Scientific), 2 mM glutamine (Thermo Fisher Scientific), 1.7 mM glucose, 0.01% GlutaMAX supplement (Thermo Fisher Scientific) ,

2 mL of MEM amino acid solution (Thermo Fisher Scientific), 1 mL of MEM nonessential amino acids solution (Thermo Fisher Scientific), 1 mL of MEM vitamin solution, 1 mL of antibiotic-antimycotic (Thermo Fisher Scientific), and 100 µL of chemically defined lipid concentrate (Thermo Fisher Scientific). The medium was adjusted to pH 7.8 with 10 mM Hepes and osmolarity to 874 mOsm using 4 M NaCL. The abdominal part of the ovary was divided into right and left tubes (Fig. 3.2). One tube was placed in a 48-well plate containing 250 µL of the medium (control), while the other tube was placed in a well with the same amount of medium containing either 0.1 or 1 M Maj-ILP1 (treatment). If there was room on the length of the abdominal ovaries, an additional pair of ovarian fragments was similarly prepared. Neighboring fragments of the ovary were kept for subsequent histological analysis to determine the vitellogenic stage. The ovarian fragments were then incubated for 20 hours at 20°C in darkness with gentle agitation (50 rpm). After the incubation, all fragments were stored in 400 µL RNA later and kept at −20°C until use in total RNA extraction for the quantification of the expression levels of *Vg* mRNA.

3.2.6. Total RNA isolation and qRT-PCR

Total RNA was extracted from the incubated hepatopancreas and ovarian fragments using the NucleoSpin RNA kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The RNA samples were diluted to a concentration of nearly 20 ng/µL and were afterward used to quantify the expression levels of *Maj-Vg1* and *Maj-Vg2*

using the Luna Universal Probe One-Step RT-qPCR Kit (New England BioLabs, MA, USA) according to the manufacturer's instructions. The *arginine kinase* gene (*Maj-AK*) was utilized as an internal control. The sequences of the primers and TaqMan probes used for *Maj-Vg1,* and *Maj-AK* were the same as in previous report (Tsutsui et al. 2018), and those for *Maj-Vg2* were also included in (Chapter 2). All sequences of these three genes were collected in a single table in this chapter (Table 3.1).

The quantitative real-time PCR (qRT-PCR) was carried out using an ABI 7300 real-time system (Thermo Fisher Scientific). The procedure included a reverse transcription step at 55ºC for 10 minutes, an initial denaturation step at 95ºC for 1 minute, and 40 cycles of denaturation at 95ºC for 10 s and extension at 60ºC for 1 minute. Relative gene expression levels of *Maj-Vg1* and *Maj-Vg2* were calculated by the $2^{-\Delta\Delta Ct}$ method using *Maj-AK* expression as a reference (Livak and Schmittgen 2001).

3.2.7. Statistical analysis

The expression levels of the two *Vg* genes in both the ovary and hepatopancreas were evaluated using the Wilcoxon matched-pair signed-rank test. The data of *Vg* gene expression was presented as the mean ± standard error of the mean (SEM).

3.3. Results

3.3.1. Effects of ILP1 on the expression levels of *Vg* genes in the hepatopancreas

The expression levels of *Maj-Vg1* and *Maj-Vg2* genes were significantly increased in

hepatopancreatic fragments from adolescent prawns following treatment with chemically synthesized Maj-ILP1, as shown in (Fig. 3.3). Treatment with 1 μM Maj-ILP1 significantly increased *Maj-Vg1* expression, the average relative value was approximately 1.3-fold to control (Fig. 3.3B), while treatment with 0.1 μM did not exhibit a significant change. In addition, treatment with both 0.1 and 1 μM significantly increased *Maj-Vg2* expression levels, approximately 1.2- and 1.3-fold change increase respectively, as compared to the control based on the average relative value (Fig. 3.3C and D). However, no significant change in *Vg* expression were observed in the hepatopancreas of immature adult prawns treated with Maj-ILP1 (Fig. 3.4).

3.3.2. Effects of ILP1 on vitellogenin expression in the ovary

The results observed in the ovary indicate that the lower dose of treatment (0.1 μM) significantly upregulated the expression levels of *Maj-Vg1* in both adolescent and immature adult prawns (Fig. 3.5A and C). Comparisons of the average relative values revealed a substantial upregulation in gene expression, with approximately 1.5-fold and 2.2-fold higher *Maj-Vg1* gene expression observed in adolescent and immature adult prawns, respectively. Nevertheless, no statistically significant difference was observed in the expression levels of *Maj-Vg1* in both adolescent and immature adult prawns when treated with 1 μM ILP1 compared to the control group (Fig. 3.5B and D).

3.4. Discussion

The result of this chapter suggest that Maj-ILP1 is likely involved in regulating vitellogenesis in *M. japonicus*, particularly during puberty. This is the first report of a hormonal factor potentially involved in the regulation of Vg gene expression in the hepatopancreas. Similar studies in other invertebrates have also shown a positive correlation between ILP and vitellogenesis. For instance, in the cuttlefish *S. japonica*, RNA interference-mediated silencing of ILP reduced the expression levels of four ovarian-development-related genes, including two *Vg* genes, indicating the involvement of ILP in the regulation of vitellogenesis (Lü et al. 2022). Similarly, in the desert locust *Scistocerca gregaria*, silencing of the insulin-related peptide gene decreased the size of oocytes (Badisco et al. 2011). Considering these findings, it appears plausible to explore the development of a new vitellogenesis-promoting technique of female broodstock of *M. japonicus* in captivity.

These results demonstrate that Maj-ILP1 has a stimulatory effect on *Vg* expression mainly during the adolescent stage. This provides valuable insights into the endocrine regulation of puberty in *M. japonicus.* Since the expression of Maj-ILP1 remains constant in the adult stage (Tsutsui et al. 2022), it is plausible that it is acting as an inter-peripheral signaling molecule in a paracrine-like manner during puberty, thereby regulating *Vg* expression in the hepatopancreas. However, no clear dose-response of Maj-ILP1 on *Vg* expression was observed in this study, which is in line with previous reports that observing a stimulatory effect of *Vg* expression *in vitro* may be challenging (Tiu and Chan 2007; Tsutsui et al. 2020).

Furthermore, in *M. ensis*, the administration of the peptide MeMIH-B exhibited a notable and significant stimulatory effect on the expression of *Vg* gene in an *in vivo* assay (Tiu and Chan 2007). However, when the same peptide subjected to *in vitro* assay, it did not demonstrate a clear-dose response relationship. In addition, both *in vitro* and *in vivo* investigation on *F. merguiensis* reported that the recombinant glass bottom boat protein which is belonging to the TGF- β superfamily showed only *in vivo* stimulatory effect on *Vg* gene expression (Sathapondecha and Chotigeat 2019). These results suggest that *in vivo* assay may be more suitable than *in vitro* or *ex-vivo* assays for clearly observation of stimulatory effects of hormone-like substances and dose-response relationship. Furthermore, measuring the levels of newly transcribed mRNA may be feasible to sensitively detect the stimulatory effect on *Vg* gene expression (Zmora et al. 2009). It may be useful to improve the *ex-vivo* culture system and set a more appropriate incubation period of the hepatopancreas based on the half-life of *Vg* mRNA. Further investigation is warranted to fully elucidate the precise function of this peptide as well as other potential vitellogenesis-regulated hormones in the physiology of reproduction by combining both e*x-vivo* and *in vivo* assays. In the following chapter (Chapter 4), the potential role of both MF and 17β-estradiol will be examined and discussed by using the newly-established *ex-vivo* hepatopancreatic assay in *M. japonicus*.

Maj-IAG was reported previously to suppress the ovarian *Maj-Vg1* expression *in vitro* (Katayama et al. 2014). This study also revealed that the female-specific Maj-ILP1 upregulates

the expression of *Vg* genes in both the ovary and hepatopancreas. Taken together, these results indicate the possibility of the existence of sex-specific ILPs with essential functions in regulating male-sex differentiation and female ovarian development in this species. In vertebrates, sex steroids are sex-specific hormones, whereas, in crustaceans, the corresponding factors have not been clarified. It may be very interesting to confirm and further investigate the role of ILPs from this perspective. Moreover, elucidating the potential role of some non-peptide hormones such as MF and vertebrate-type sex steroid 17-β estradiol, in the regulation of vitellogenesis and reproduction in *M. japonicus* represents an important topic for further exploration. Furthermore, the construction of hepatopancreas culture system likely provides the foundation for further research to explore the potential effect of other peptide and non-peptide hormones on the regulation of vitellogenesis in *M. japonicus*.

Therefore, in the next chapter 4, I will address the potential effect of some non-peptide hormones on *Vg* mRNA expression in the hepatopancreas to expand the knowledge of the endocrine regulation of vitellogenesis.

Table 3.1 Sequences for primers and probes of Maj-Vg1, Maj-Vg2 and Maj-AK

Primer (qPCR)[1]	Sequence
Maj-Vg1 qF	GTGGACTCCAGAAGAACATCTTGTAG
Maj-Vg1 qR	ACGCAATCATGAGAGGCATCT
Maj-Vg1 qPr	AAGGTGGGTGCTAAGGCTCCAGTGCT
Maj-Vg2 qF	TGGTTACAAGGCTACACACGA
Maj-Vg2 qR	TGACCAGCTGTGTACTGAAGT
Maj-Vg2 qPr	ACCATCCAGTTCTTCCCAAAGCCATGCAG
Maj-Ak qF	CCATCATCGAGGACTACCATGTT
Maj-Ak qR	AAGGAGCTGACATCACCGAAGT
Maj-Ak qpr	TTCAAGCAGACTGACAAGCACCCCAAC

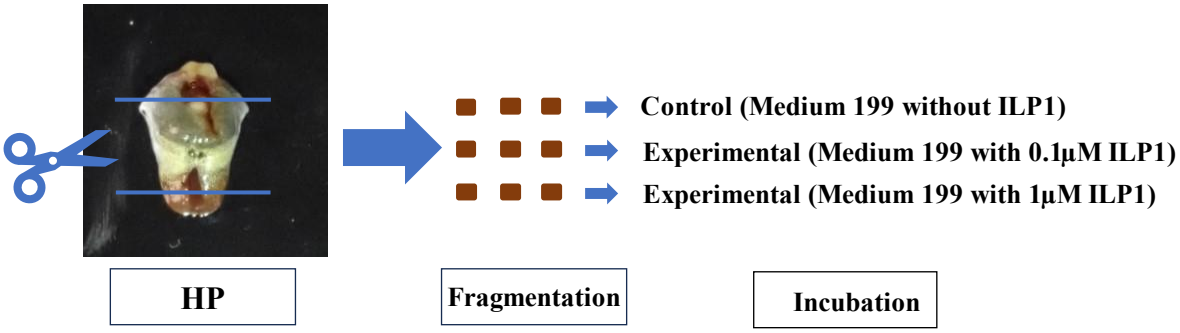

Fig. 3.1. Incubation of hepatopancreas explants. For culture experiments, the peripheral parts of the hepatopancreas were meticulously trimmed and the core portion was fragmented into smaller fragments then incubated in the medium.

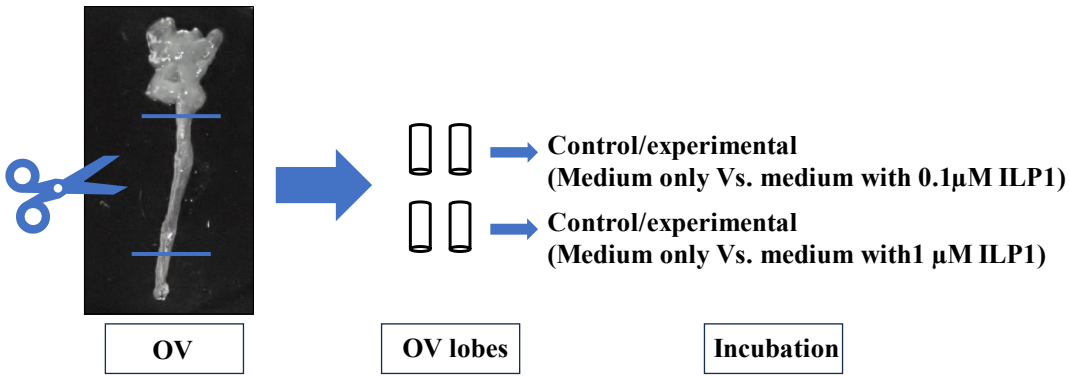

Fig. 3.2. Incubation of ovarian explants. The lines demarcate the specific fragment of the abdominal portion chosen for incubation experiment.

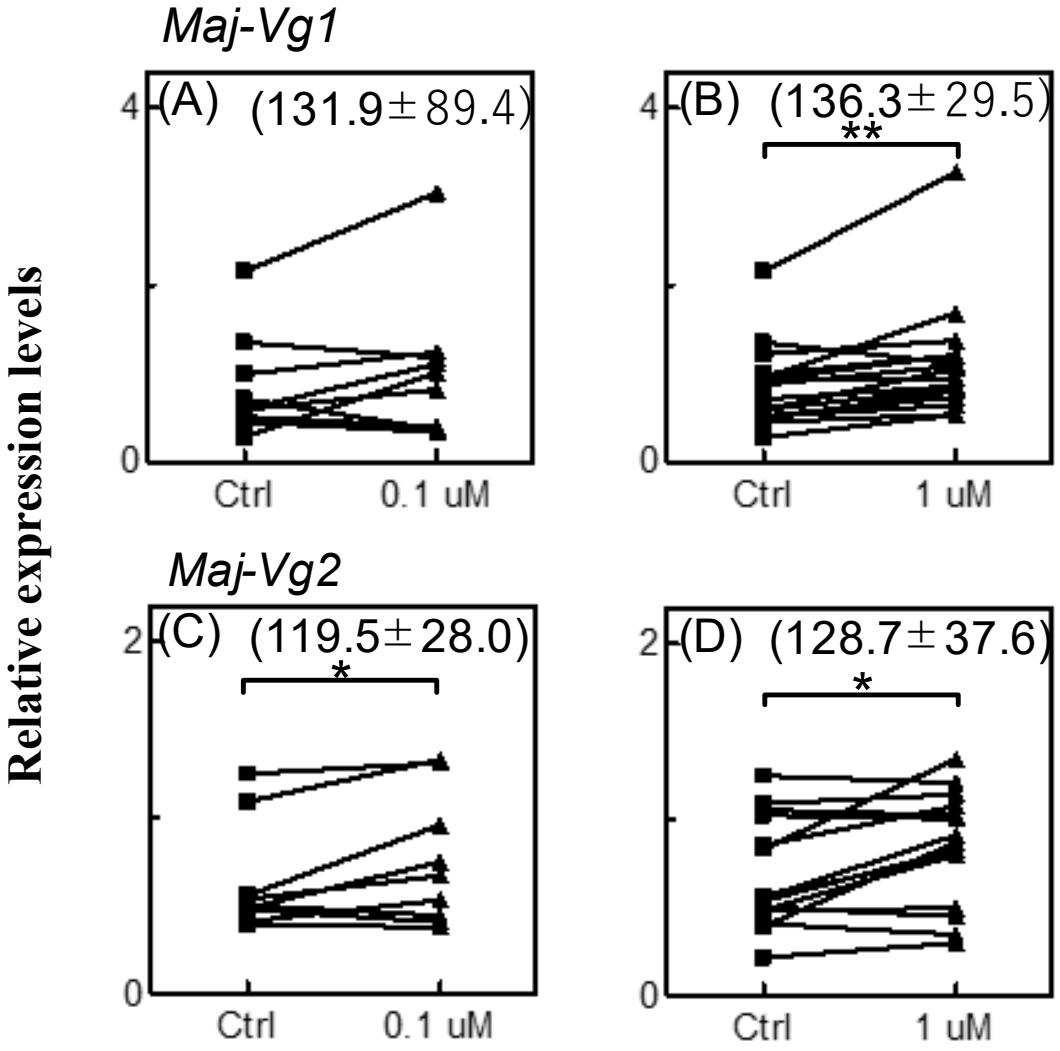

Fig. 3.3. Effect of synthetic Maj-ILP1 on mRNA expression levels of *Maj-Vg1* (A & B) and *Maj-Vg2* (C & D) in the hepatopancreas of adolescent prawns. The data are represented as before-after (control-treatment) dot plot (n = 10 prawns for A and C: n = 16 prawns for B and D), and the difference between control and hormone-treated samples are tested for significance using the Wilcoxon signed rank test (**, P <0.01; *, P <0.05).The gene expression level for the control group was artbitrarily set at 100. The gene expression level for the treated group was calculated, and the mean value was presented as mean ± standard deviation (in parenthesis) in each graph.

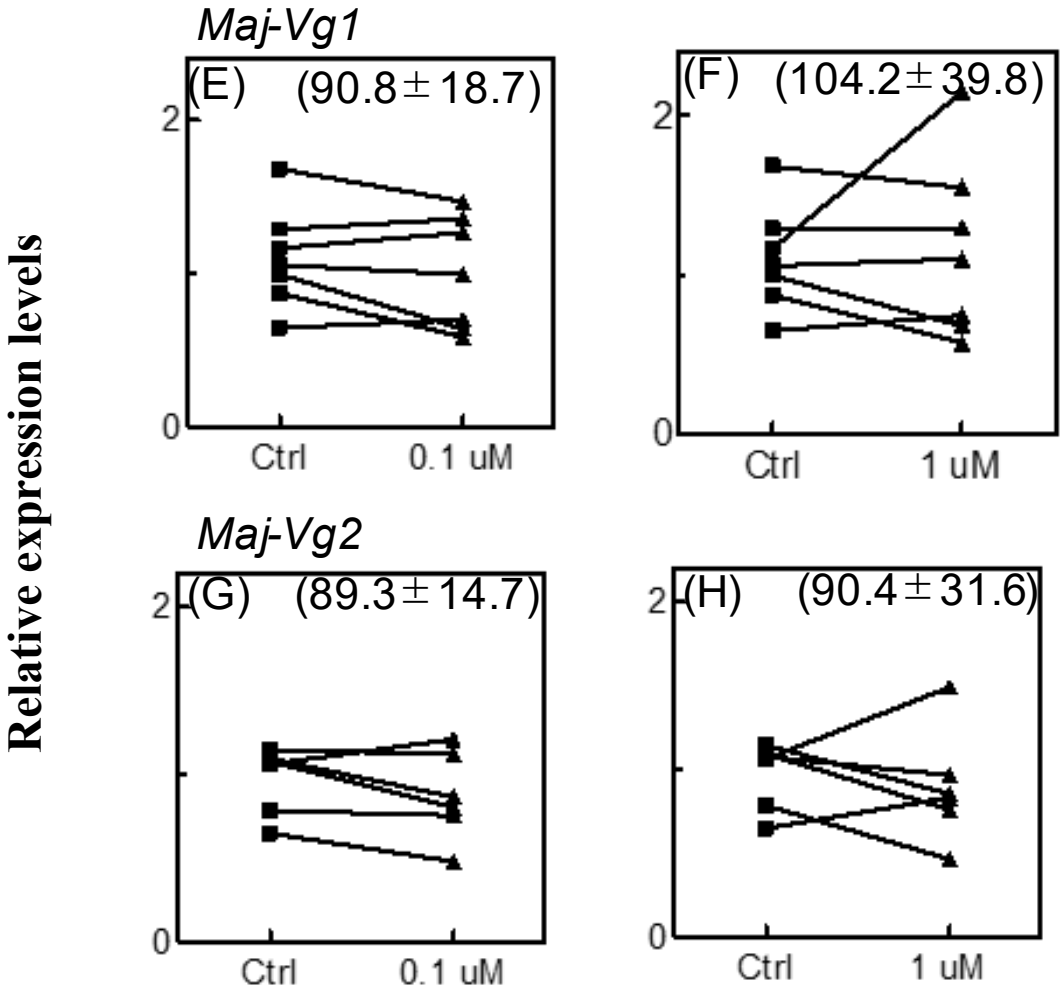

Fig. 3.4. Effect of synthetic Maj-ILP1 on mRNA expression levels of *Maj-Vg1* (E& F) and *Maj-Vg2* (G & H) in the hepatopancreas of immature adult prawns. The data are represented as before-after (control-treatment) dot plot (n = 7 prawns), and the difference between control and hormone-treated samples are tested for significance using the Wilcoxon signed rank test. The gene expression level for the control group was artbitrarily set at 100. The gene expression level for the treated group was calculated, and the mean value was presented as mean ± standard deviation (in parenthesis) in each graph.

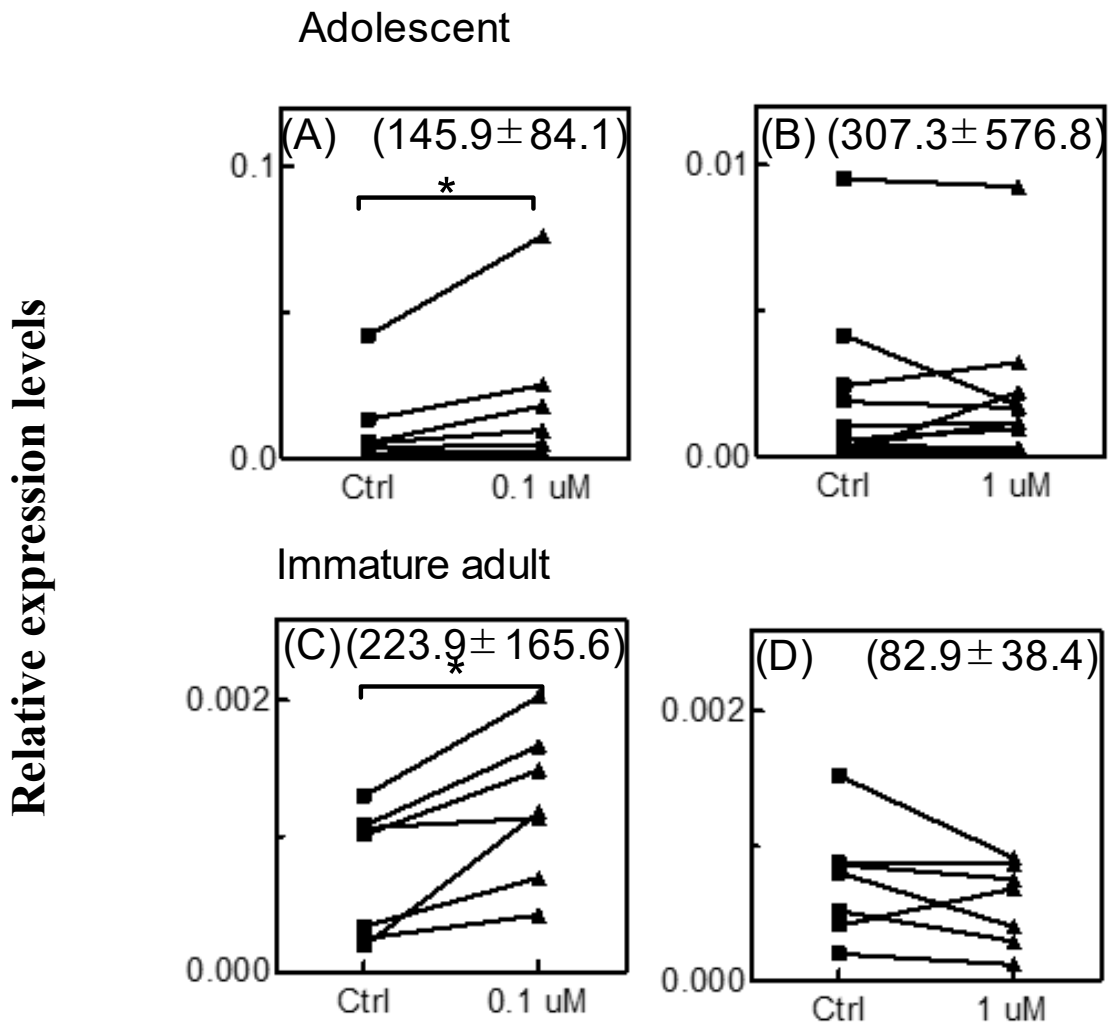

Fig. 3.5. Effect of synthetic Maj-ILP1 on *Maj-Vg1* mRNA levels in the ovary. Results of assays using adolescent prawns (A & B) and immature adult prawns (C & D) are shown. The data are represented as before-after (control-treatment) dot plot (n = 10 prawns for A: n = 17 prawns for B: n = 7 prawns for C and D). The differences between control and hormone-treated samples are tested for significance by the Wilcoxon test (*, P <0.05). The gene expression level for the control group was artbitrarily set at 100. The gene expression level for the treated group was calculated, and the mean value was presented as mean ± standard deviation (in parenthesis).

Chapter 4. Impact of some non-peptide hormones on vitellogenesis in intact and eyestalk ablated prawns

4.1. Background

Endocrine regulation of reproduction in crustaceans is complex that involves the eyestalk peptides VIH synthesized by XOSG of eyestalk and GSH/VSH synthesized by the brain and thoracic ganglia (Pamuru 2019). Thus, the central nervous system is likely a bipartite in decapod crustaceans producing inhibitory hormones in the optic ganglia and gonad-stimulating hormones in the brain and thoracic ganglia. Crustacean hyperglycemic hormones and molt-inhibiting hormones have positively influenced Vg synthesis in different decapod species, including lobsters, penaeids, and crabs (Subramoniam 2017; Jayasankar et al. 2020).

In addition to these neuropeptides, several non-peptide hormones can stimulate gonads. Methyl farnesoate (MF) is sesquiterpenoid hormone that stimulates ovarian development, and it is produced by the mandibular organ. This gland demonstrates biosynthesis of MF during vitellogenesis, whereas its activity diminishes during non-reproductive periods (Laufer et al. 1986). MF is considered as the homolog of insect juvenile hormone, JH III, which involve in the reproduction in some insects (Borst et al. 1987). Therefore, MF has been thought to be involved in the regulation of maturation in crustaceans (Reddy et al. 2004; Nagaraju 2011). MF synthesis is under the control of mandibular organ inhibiting hormone (MOIH) secreted from XOSG complex in the eyestalk (Borst et al. 2001). MF plays a stimulatory role in regulating

reproduction in both male and female crustaceans (Nagaraju 2007; Nagaraju 2011). MF can induce reproduction either by directly stimulating Vg synthesis in the ovary and/or hepatopancreas or by inducing the secretion of ecdysteroids from Y-organs, or through a combination of both mechanisms (Wang and LeBlanc 2009; Reddy and Reddy 2015; Buchi et al. 2016). The levels of MF fluctuate in the haemolymph in response to the ovarian developmental stage indicating its involvement in ovarian in ovarian development (Borst et al. 1987). This correlation has been observed in crayfish (Laufer et al. 1998) and crabs (Medesani et al. 2015). Administration of MF has been shown to stimulate vitellogenesis in certain crustaceans such as *L. vannamei* (Tsukimura and Kamemoto 1991), *P. indicus* (Saikrithi et al. 2019), *Libinia emarginata* (Borst et al. 1987), *Macrobrachium malcolmsoni* (Nagaraju et al. 2003), *N. granulata* (Medesani et al. 2015), *Oziotelphusa. senex senex* (Reddy et al. 2004), and *P. clarkii* (Rodríguez et al. 2002). MF was found to upregulated the expression levels of *Vg* in the *in vitro* hepatopancreas explants culture of *O. senex senex* (Buchi et al. 2016). Only 10 μM MF significantly increased *Vg* levels in the hepatopancreas of the red crab *Charybdis feriatus in vitro* (Mak et al. 2005b). Furthermore, ESA increased the levels of MF and promoted maturation in the spider crab *L. emarginata* (Laufer et al. 1897). However, contrary to these findings, no significant increase in the expression levels of *Vg* was observed in the hepatopancreas explant cultures of the American lobster *H. americanus* (Tiu et al. 2010). In addition, Tiu et al. (2006a) reported no significant increase in the expression levels of *Vg* in

both the ovarian and hepatopancreas explant cultures of *M. ensis.* However, it is worth nothing that the farnesoic acid, the precursor of MF, exhibited the ability to stimulate the expression levels of *Vg* in the hepatopancreatic and ovarian explants in *M. ensis* (Tiu et al. 2006a). These contradictory results suggesting either the stimulatory effect or no effect of MF on decapod crustacean reproduction are summarized in (Table 4.1).

The vertebrate-type steroids, such as 17β-estradiol (E2) and progesterone, from the ovary or hepatopancreas can also stimulate gonads in decapod crustaceans (Subramoniam 2000; Subramoniam 2011). It was reported previously that their levels in hemolymph, ovary, and hepatopancreas fluctuate during the gonadal maturation, suggesting their role in the control of reproduction in crustaceans (Subramoniam 2011). The presence of vertebrate-type steroid hormones such as 17β-estradiol, progesterone, pregnenolone and testosterone has been identified in various crustaceans (Subramoniam 2011)**.** The level of this hormone fluctuate in the hemolymph, hepatopancreas, and ovaries in relation to the reproductive stage, suggesting a functional role in regulating reproduction similar to that in vertebrates (Subramoniam 2000)**.** However, the results of both *in vivo* and *in vitro* assays administering these hormones have been contradictory. For example, treatment with 17β-estradiol did not stimulate ovarian development in tiger prawns *Penaeus esculentus* (Koskela et al. 1992), *L. vannamei* (Tsukimura and Kamemoto 1991)**,** *S. ingentis* (Bender 1996). On the other hand, steroid administration stimulated vitellogenesis and ovarian maturation in *P. monodon* (Merlin et al.

2015), *M. japonicus* (Summavielle et al. 2003), and the female swimming crab *P. trituberculatus* (Summavielle et al. 2003). This discrepancy may be attributed to ovarian stages or dose-dependent effects (Jayasankar et al. 2020). All these contradictory results are summarized in (Table 4.2). It has been previously reported that ESA increases the level of these sex hormones in the hemolymph of *P. monodon* (Merlin et al. 2015).

Thus, the aim of this chapter is to determine the impact of both MF and 17β-estradiol on *Vg* expression levels in the hepatopancreas of intact and ESA *M. japonicus* using *in vitro* hepatopancreas culture system.

4.2. Materials and Methods

4.2.1. *In vitro* incubation of hepatopancreas fragments of intact prawns

Immature adult female *M. japonicus* (weighting from 20 to 22.8 g; average: 21 ± 0.89 g; *n* =3) were purchased from local prawn farms in Okinawa Prefecture, Japan, in June 2022. These prawns were transported to the Mie University and acclimatized for approximately one week in a large tank containing filtered seawater at 20°C. Prawns were fed a commercial diet (Vitalprawn, Higashimaru Co., Kagoshima, Japan). After acclimatization, prawns were dissected and their hepatopancreas was used for *ex-vivo* assay using medium 199, following the same protocol as described in (chapter 3). Briefly, the hepatopancreas was washed in the medium and cut into nearly 50 mg fragments, which were placed in 24-well culture plates containing medium (control), or medium with either MF (2 nM, 20 and 200 nM) or 17β-

estradiol (0.1, 1 and 10 μM). After incubation for 6 hours, all fragments were preserved in RNAlater solution (Thermo Fisher Scientific) and stored at −20° until use in RNA extraction.

4.2.2. *In vitro* incubation of hepatopancreas fragments of ESA prawns

Immature adult female prawns (weighing from 21 to 30 g; average: 25.4 ± 0.88 ; n =7) were obtained from Kumamoto Prefecture, Japan, in August to be used for this experiment. After 5 days acclimatization, the prawns underwent bilateral ESA and were kept individually in cages inside the main tank. The prawns were then dissected (on day 8) and hepatopancreas was trimmed then cut into small fragments (8 ~ 10 fragments/each 50 mg) and used for incubation with either MF (0.1, 1 and 10) or 17β-estradiol (0.1, 1 and 10 μM). After 6 hours incubation, the hepatopancreas fragments were stored in 400 μL RNA*later* solution at −20°C for RNA extraction.

4.2.3. Total RNA extraction and qRT-PCR

Total RNA was extracted from the incubated tissue fragments using the NucleoSpin RNA kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany). The total RNA samples were diluted to a concentration of nearly 20 ng/μl and were used to quantify the expression levels of *Maj-Vg1* and *Maj-Vg2* using the Luna Universal Probe One-Step RT-qPCR Kit (New England BioLabs, MA, USA). The *arginine kinase* gene (*Maj-AK*) was used as an internal control. The primers and TaqMan probe sequences for *Maj-Vg1, Maj-Vg2,* and *Maj-AK* were previously reported (see chapter 3). Relative gene expression levels of *Maj-Vg1* and *Maj-Vg2* were

calculated by the $2^{-\Delta\Delta Ct}$ method using the expression levels of *Maj-AK* as a reference gene.

4.2.4. Statistical analysis

The expression levels of the two *Vg* genes in the hepatopancreas were assessed using the Kruskal Wallis test. The gene expression levels were presented as the mean ± SEM.

4.3. Results

4.3.1. Effects of MF on the expression levels of *Vg* genes in the hepatopancreas of prawns

The analysis of the expression levels of both *Maj-Vg1* and *Maj-Vg2* in the hepatopancreas of intact (Fig. 4.1A and B) and ESA prawns (Fig. 4.1C and D) revealed that treatment with MF did not elicit any significant alternation ($p > 0.05$). While there was a trend towards increased expression levels of *Maj-Vg2* (Fig. 4.1B) in the treated intact prawns relative to the control group, this difference was not statistically significant.

4.3.2. Effects of 17β-estradiol on the expression levels of *Vg* genes in the hepatopancreas of prawns

Similarly, to the results of MF, treatment with 17β-estradiol did not show significant change in the expression levels of *Vg* genes in the hepatopancreas of intact prawns (4.2A and B) as well as ESA prawns (4.2C and D) ($P > 0.05$).

4.4. Discussion

This is the first report on the effect of two non-peptide hormones (MF and E2) on the

synthesis of Vg by the hepatopancreas of *M. japonicus*. The results of the current chapter suggest that MF may not play a direct role in the regulation of vitellogenesis in *M. japonicus*. These findings are consistent with previous reports on other crustacean species such as *M. ensis* (Tiu et al. 2006a), *M. rosenbergii* (Wilder et al. 1994), and *H. americanus* (Tiu et al. 2010). Furthermore, MF has been reported to stimulate synthesis of protein in the ovary of *Cherax quadricarinatus*, but not the vitellogenin of the hepatopancreas (Medesani et al. 2012). Since the *in vivo* stimulatory role of MF in the development of reproduction has been reported previously in various decapods, but its stimulatory effect is absent in some species (Table 4.1). It is likely that MF works indirectly, meaning it stimulates other hormones (Gonad-stimulating hormone), that thereby stimulate ovarian function (Fingerman 1997; Medesani et al. 2015). Overall, the conflicting results observed in these earlier studies (Jayasankar et al. 2020) may be attributed to various factors that include species-specific differences, the mode of action of MF in crustacea (direct or indirect through other hormones/substances), the pre-capture habitat of these species, variations in ovarian stages and the utilization of different biomarkers (GSI, hemolymph Vg levels or *Vg* expression levels) (Fingerman 1997; Tsukimura 2001; Paran et al. 2010). Additionally, the differences in study methodologies (*in vivo* and *in vitro* approaches) may also contribute to the observed disparities (Table 4.1).

The results of *in vitro* assay using 17β-estradiol indicate that it is likely does not have a significant stimulatory role during early stages of vitellogenesis in *M. japonicus* and its role

may be restricted to advanced or late developmental stages. Administration 17β-estradiol at the end of the pre-reproductive period resulted in an increase in GSI and diameter of oocytes in *P. clarkii* (Rodríguez et al. 2002). It is also possible that stimulating vitellogenesis depends on the utilized dosage (Jayasankar et al. 2020). The current results are in line with the previous study on *P. esculentus* (Koskela et al. 1992), where there was no significant effect on growth or ovarian development *in vivo* in this tiger prawns. In addition, 17β-estradiol (1 pM to 100 nM) didn't enhance the increase of *L. vannamei* oocytes *in vitro* (Tsukimura and Kamemoto 1991), but the high concentrations of 17β-estradiol (10^{-3} to 10^{-5} M) stimulated the synthesis of yolk protein in the same species (Quackenbush 1992). The injection of 17β-estradiol into non reproductive *S. ingentis* resulted in no significant change in the Vg levels in the hemolymph (Bender 1996). These disparities may be related to the specific stage of ovarian development in these species and dosage of 17β-estradiol administered (Jayasankar et al. 2020). Overall, the role of the steroid hormone 17β-estradiol in crustaceans is still unclear and need more research to better understand the mechanisms of sex manipulation and vitellogenesis in this species.

Table 4.1 Effect of MF on decapod crustacean reproduction

Species	Assay	Response	References
L. vannamei	*In vitro*	Stimulation of reproduction	(Tsukimura and Kamemoto 1991)
	In vivo	Enhancement of reproductive efficiency	(Laufer 1992; Alnawafleh et al. 2014)
M. ensis	*In vitro*	No effect	(Tiu et al. 2006a)
M. rosenbergii	*In vivo*	No effect	(Wilder et al. 1994)
P. indicus	*In vitro*	Stimulation of reproduction	(Saikrithi et al. 2019)
M. japonicus	*In vitro*	No effect	This study
Sicyonia ingentis	*In vivo*	Stimulation of reproduction	(Paran et al. 2010)
P. clarkii	*In vivo*	Stimulation of reproduction	(Laufer et al. 1998; Rodríguez et al. 2002)
M. malcolmsoni	*In vivo*	Stimulation of reproduction	(Nagaraju et al. 2003)
H. americanus	*In vitro*	No effect	(Tiu et al. 2010)
N. granulata	*In vivo*	Stimulation of reproduction	(Medesani et al. 2015)
C. feriatus	*In vitro*	High concentrations stimulated vitellogenesis	(Mak et al. 2005b)
L. emargiata	*In vivo*	Stimulation of reproduction	(Borst et al. 1987)
O. senex senex	*In vivo*	Stimulation of reproduction	(Reddy et al. 2004)

Table 4.2 Effect of 17β-estradiol on decapod crustacean reproduction

Species	Assay	Response	References
L. vannamei	*In vitro*	No effect	(Tsukimura and Kamemoto 1991)
	In vitro	Stimulation of yolk protein synthesis (High Conc.)	(Quackenbush 1992)
S. ingentis	*In vivo*	No effect	(Bender 1996)
P. monodon	*In vitro*	Stimulation of Vg synthesis (yellow ovary)	(Merlin et al. 2015)
M. japonicus	*In vitro*	Stimulation of vitellogenesis	(Summavielle et al. 2003)
P. trituberculatus	*In vivo*	Promotion of ovarian development	(Lu et al. 2018)
P. esculentus	*In vivo*	No effect	(Koskela et al. 1992)

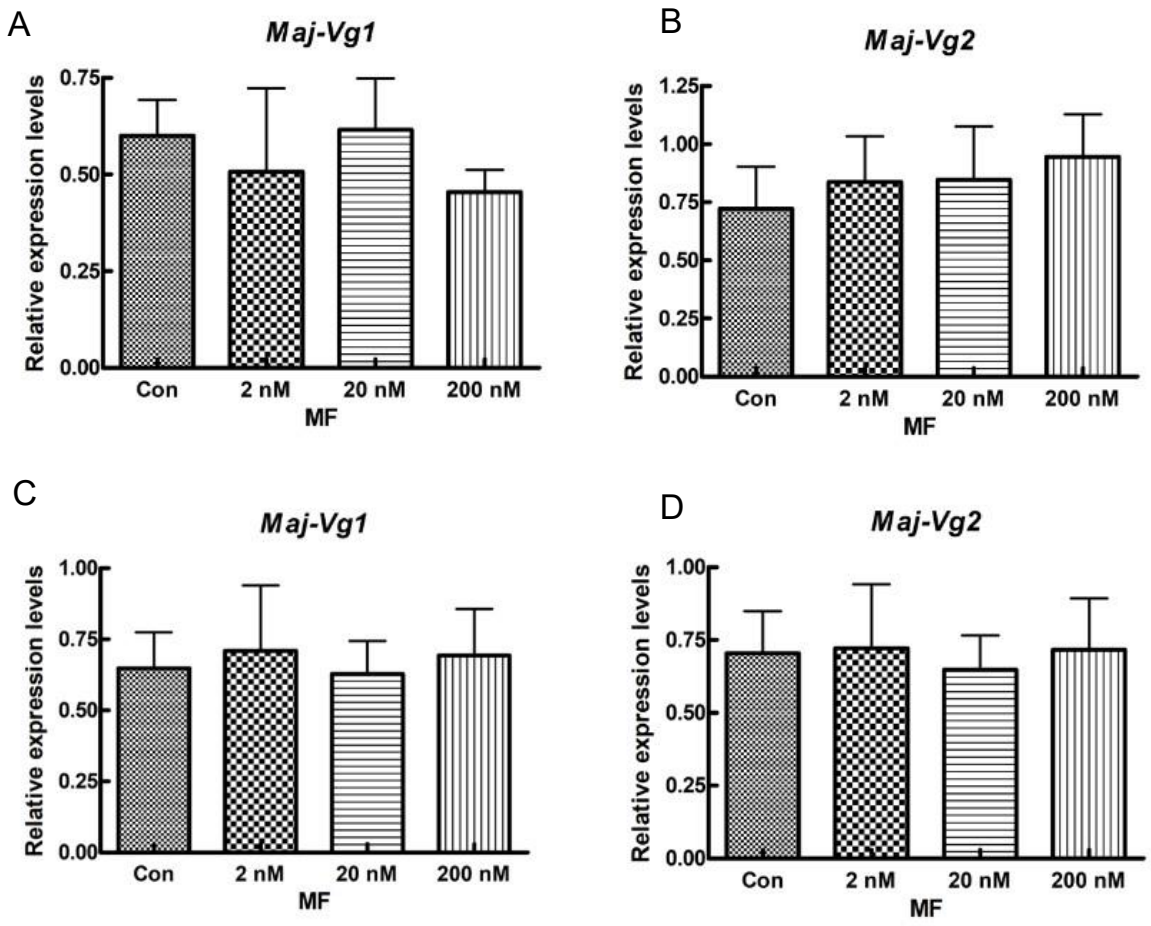

Fig. 4.1. Effect of methyl farnesoate (MF) on the expression levels of *Maj-Vg1* and *Maj-Vg2* in the hepatopancreas of intact prawns and ESA prawns. Results of assay using intact prawns (A and B) and ESA prawns (C and D) are shown. The data are represented as mean ± SEM (n = 3 individuals for intact prawns and n = 7 individuals for ESA prawns). The differences between control and hormone-treated samples were tested for significance using Kruskal-Wallis test ($p > 0.05$).

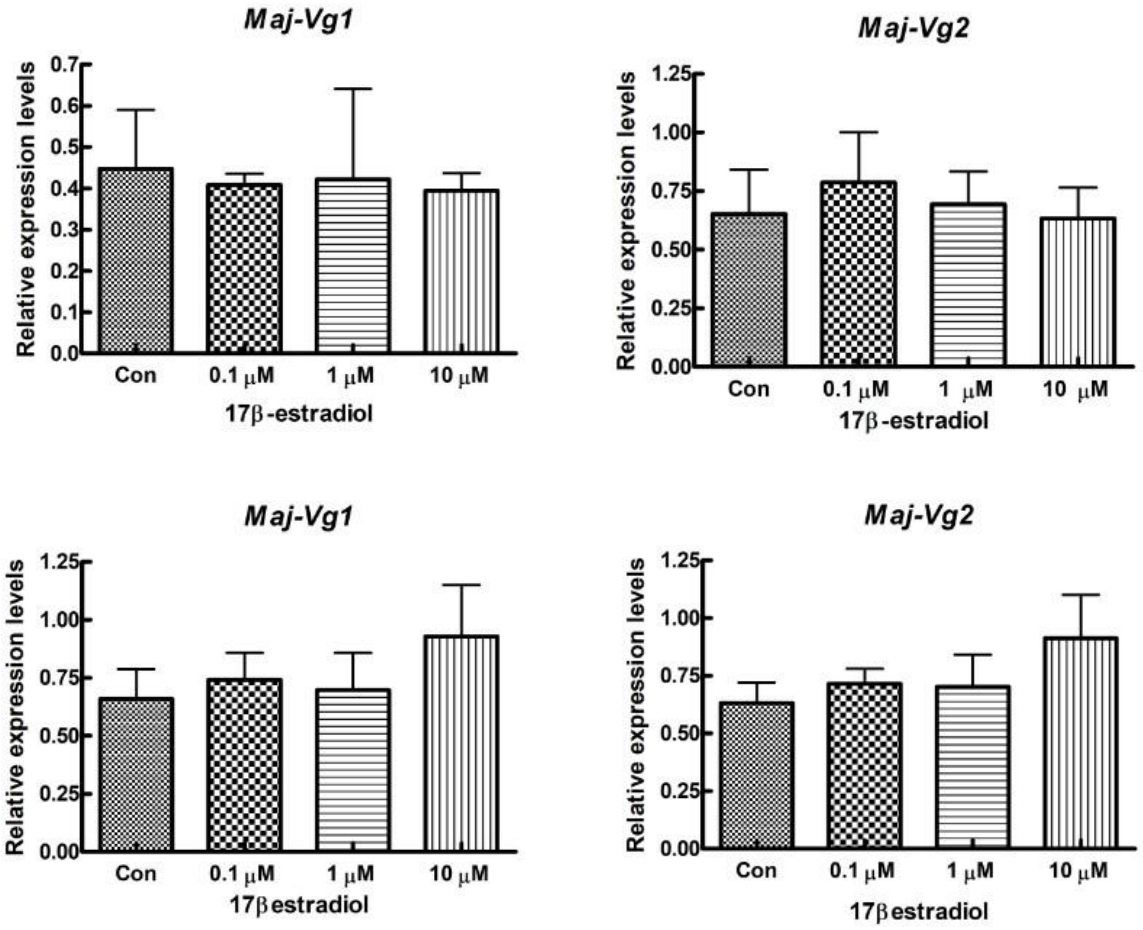

Fig. 4.2. Effect of 17β-estradiol on the expression levels of *Maj-Vg1* and *Maj-Vg2* in the hepatopancreas of intact prawns and ESA prawns. Results of assay using intact prawns (A and B) and ESA prawns (C and D) are shown. The data are represented as mean ± SEM (n = 3 individuals for intact prawns and n = 7 individuals for ESA prawns). The differences between control and hormone-treated samples were tested for significance using Kruskal-Wallis test ($p > 0.05$).

General Discussion and Conclusion

Crustaceans hold paramount economic value as a vital food and protein source. With the world's population surging, meeting the growing demand for shrimp/prawn production has become imperative. However, achieving this goal while maintaining cost-effectiveness, efficiency, and profitability requires addressing the challenges in shrimp aquaculture. One of the significant obstacles lies in successfully producing larvae from long-term-reared broodstocks under domestic conditions, largely due to a limited understanding of the endocrine regulation during reproduction. Traditional methods, such as ESA, have been employed to induce rapid vitellogenesis and ovulation in *M. japonicus* and other economically important decapod species. Unfortunately, these methods have drawbacks, including potential mortality of spawners and negative physiological effects on female broodstock. Therefore, exploring alternative approaches to promote sustainable shrimp aquaculture, without resorting to ESA, becomes crucial.

Recent research has investigated the use of double-stranded RNA of VIH for seedling production of shrimps/prawns (Treerattrakool et al. 2008; Treerattrakool et al. 2011; Feijó et al. 2016; Kang et al. 2019; Duangprom et al. 2022). However, the effectiveness of VIH silencing in enhancing ovarian maturation has proven less efficient compared to the traditional ESA technique. To overcome this limitation, a comprehensive understanding of the vitellogenesis process, with a specific focus on multiple Vgs in penaeid species, is necessary. By focusing on

multiple Vg genes as molecular markers of Vg synthesis, we can gain valuable insights into the potential roles of certain hormones in vitellogenesis.

In this study, a novel Vg molecular species was identified_ Maj-Vg2_ in the hepatopancreas of *M. japonicus* through transcriptomic analysis and cDNA cloning. The presence of both Maj-Vg1 and -Vg2, exhibiting 54% sequence similarity and a minimal variation of 1% in essential amino acids, suggests their complementary roles as sources of nutrition during early development in this species. Furthermore, the investigation of tissue-specific expression showed that *Maj-Vg2* was predominantly expressed in the hepatopancreas, while *Maj-Vg1* was expressed in both the hepatopancreas and ovary, emphasizing the critical role of the hepatopancreas as an extraovarian site in Vg synthesis. Nevertheless, the exact biological functions of the multiple Vg molecular species produced by different tissues remain elusive, warranting further investigation in the hepatopancreas of *M. japonicus* to better comprehend vitellogenesis.

To understand the involvement of various hormones in the decapod reproductive process, other than VIH, opens avenues for further research (Guan et al.2014; Zeng et al. 2016; Chen et al. 2018). Investigating the functions of such hormones will contribute to a comprehensive understanding of the regulatory mechanisms governing vitellogenesis in penaeid species, which will prove beneficial in promoting healthy seedling production. Thereby, an *ex-vivo* assay specific to the hepatopancreas of *M. japonicus* was developed in the current study to

explore the hormonal effects of some peptide (Maj-ILP1) and non-peptide (MF and E2) hormones on the expression levels of *Vg* genes. Our findings suggest that Maj-ILP1 likely plays a role in regulating vitellogenesis, particularly during puberty, and may function as an inter-peripheral signaling molecule, acting in a paracrine-like manner to regulate *Vg* gene expression in the hepatopancreas. This finding holds great promise in advancing sustainable shrimp aquaculture and seedling production practices, contributing significantly to global food security and economic growth. By administering Maj-ILP1, it may be possible to induce vitellogenesis in female broodstock and facilitating spawning. Furthermore, by using ILP1, fisheries can move towards more humane, ethical, and sustainable approaches in stimulating vitellogenesis without resorting to the invasive harmful ESA technique. In addition, the administration of ILP1 may lead to better larval quality, higher survival rate and increased hatchery efficiency. The exploration of the role of ILP1 in inducing vitellogenesis in *M. japonicus* can provide insights into aquaculture approaches for various decapod crustaceans. However, it is essential to acknowledge that the current study focused on investigating the role of Maj-ILP1 using an *ex-vivo* assay. While the findings provide valuable insights into the potential stimulatory effect of ILP1 on vitellogenesis, further research utilizing *in vivo* assay is warranted to precisely understand the physiological. Employing *in vivo* assays will allow for a more comprehensive examination of the direct and indirect effects of Maj-ILP1 on vitellogenesis in *M. japonicus*, providing a deeper understanding of its role in the reproductive

process.

On the other hand, it is plausible to infer that neither MF nor E2 exerted a direct role in regulating vitellogenesis in *M. japonicus*. Notably, no discernible effect on the expression levels of *Vg* genes was observed in the hepatopancreas of prawns, encompassing both intact prawns and those subjected to ESA, following the respective treatments.

Overall, this study has unveiled the presence of an additional hepatopancreas-specific *Maj-Vg2* involved in vitellogenesis of *M. japonicus* and highlighted the pivotal role of the hepatopancreas as an extraovarian site for Vg synthesis. Moreover, we provided evidence of potential hormonal regulation of *Vg* expression in both the hepatopancreas and ovary. However, to achieve a comprehensive understanding of vitellogenesis in decapod crustaceans, further exploration of the hormonal regulation in *M. japonicus* is essential (Fig. 5).

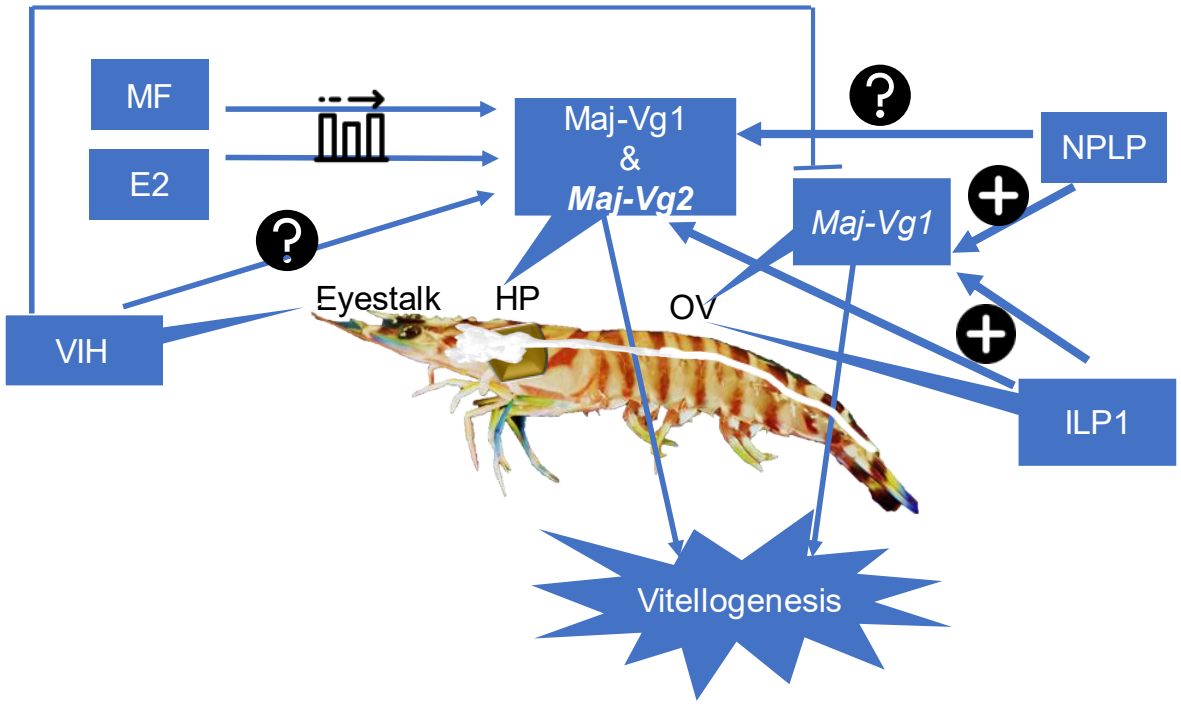

Fig. 5. Schematic view of the hormonal regulation of *Vg* expression levels in *M. japonicus*. The inhibitory effect of VIH is denoted by a blunt-ended arrow, whereas the stimulatory influence of neuroparasin-like peptide (NPLP) and ILP1 are indicated by a (+) symbol. The absence of an effect from MF and E2 is represented by a stable icon. The influence of VIH and NPLP on Vg gene expression in the hepatopancreas is represented by an arrow with a question mark, signifying that this influence has yet to be determined.

References

Abdu U, Davis C, Khalaila I, Sagi A (2002) The vitellogenin cDNA of *Cherax quadricarinatus* encodes a lipoprotein with calcium binding ability, and its expression is induced following the removal of the androgenic gland in a sexually plastic system. Gen Comp Endocrinol 127:263–272. https://doi.org/10.1016/S0016-6480(02)00053-9.

Alfaro-Montoya J, Braga A, Umaña-Castro R (2019) Research frontiers in penaeid shrimp reproduction: Future trends to improve commercial production. Aquaculture 503:70–87. https://doi.org/10.1016/j.aquaculture.2018.12.068.

Alfaro-Montoya J, Hernández-Noguera L, Vega-Alpízar L, Umaña-Castro R (2016) Effects of androgenic gland ablation on growth, sexual characters and spermatogenesis of the white shrimp, *Litopenaeus vannamei* (Decapoda: Penaeidae) males. Aquac Res 47:2768–2777. https://doi.org/10.1111/are.12727.

Alnawafleh T, Kim BK, Kang HE, Yoon TH, Kim HW (2014) Stimulation of molting and ovarian maturation by methyl farnesoate in the pacific white shrimp *Litopenaeus vannamei* (Boone, 1931). Fish Aquat Sci 17:115–121. https://doi.org/10.5657/FAS.2014.0115.

Avarre J-C, Michelis R, Tietz A, Lubzens E (2003) Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. Biol Reprod 69:355–364.

https:// doi.org/ 10. 1095/ biolr eprod. 102. 011627.

Babin PJ (2008) Conservation of a vitellogenin gene cluster in oviparous vertebrates and identification of its traces in the platypus genome. Gene 413:76–82. https://doi.org/10.1016/j.gene.2008.02.001

Badisco L, Marchal E, Wielendaele P Van, Verlinden H, Vleugels R, Broeck J Vanden (2011) RNA interference of insulin-related peptide and neuroparsins affects vitellogenesis in the desert locust *Schistocerca gregaria*. Peptides 32:573–580. https://doi.org/10.1016/j.peptides.2010.11.008.

Banks TM, Wang T, Fitzgibbon QP, Smith GG, Ventura T (2020) Double-stranded rna binding proteins in serum contribute to systemic rnai across phyla—towards finding the missing link in achelata. Int J Mol Sci 21:6967. https://doi.org/10.3390/ijms21186967.

Barr PJ (1991) Mammalian subtilisins: The long-sought dibasic processing endoproteases. Cell 66:1–3. https://doi.org/10.1016/0092-8674(91)90129-M.

Bathgate RAD, Samuel CS, Burazin TCD, Layfield S, Claasz AA, Reytomas IGT, Dawson NF, Zhao C, Bond C, Summers RJ, Parry LJ, Wade JD, Tregear GW (2002) Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene: Novel members of the relaxin peptide family. J Biol Chem 277:1148–1157. https://doi.org/10.1074/jbc.M107882200.

Bender, JS (1996). Regulation of gonadal development in the ridgeback shrimp, *Sicyonia ingentis*. California State University, Fresno ProQuest Dissertations. 1382032.

Böcking D, Dircksen H, Keller R (2002) The Crustacean Neuropeptides of the CHH/MIH/GIH Family: Structures and Biological Activities. Crustac Nerv Syst 84–97. https://doi.org/10.1007/978-3-662-04843-6_6.

Boenish R, Kritzer JP, Kleisner K, Steneck RS, Werner KM, Zhu W, Schram F, Rader D, Cheung W, Ingles J, Tian Y, Mimikakis J (2022) The global rise of crustacean fisheries. Front Ecol Environ 20:102–110. https://doi.org/10.1002/fee.2431

Borst DW, Laufer H, Landau M, Chang ES, Hertz WA, Baker FC, Schooley DA (1987) Methyl farnesoate and its role in crustacean reproduction and development. Insect Biochem 17:1123–1127. https://doi.org/10.1016/0020-1790(87)90133-8.

Borst DW, Ogan J, Tsukimura B, Claerhout T, Holford KC (2001) Regulation of the Crustacean Mandibular Organ1. Am Zool 41:430–441. https://doi.org/10.1668/0003-1569(2001)041[0430:ROTCMO]2.0.CO;2.

Brown FA, Jones GM (1949) Ovarian inhibition by a sinus-gland principle in the fiddler crab. biolBull 96:228–232. https://doi.org/10.2307/1538356.

Buchi SR, Vaadala S, Hosamani N, Pamuru RR, Pamanji SR (2016) Regulation of vitellogenesis by selected endocrine modulators in crab *Oziothelphusa senex senex*, with special reference to methyl farnesoate. Aquac Reports 3:24–30. https://doi.org/10.1016/j.aqrep.2015.11.006.

Chan SM, Rankin SM, Keeley LL (1988) Characterization of the molt stages in *Penaeus*

vannamei: setogenesis and hemolymph levels of total protein, ecdysteroids, and glucose. Biol Bull 175:185–192. https://doi.org/10.2307/1541558.

Chen HY, Kang BJ, Sultana Z, Wilder MN (2018) Molecular cloning of red pigment-concentrating hormone (RPCH) from eyestalks of the whiteleg shrimp (*Litopenaeus vannamei*): Evaluation of the effects of the hormone on ovarian growth and the influence of serotonin (5-HT) on its expression. Aquaculture 495:232–240. https://doi.org/10.1016/j.aquaculture.2018.04.027.

Chen T, Zhang LP, Wong NK, Zhong M, Ren CH, Hu CQ (2014) Pacific white shrimp (*Litopenaeus vannamei*) vitellogenesis-inhibiting hormone (VIH) is predominantly expressed in the brain and negatively regulates hepatopancreatic vitellogenin (VTG) gene expression. Biol Reprod 90:1–10. https://doi.org/10.1095/biolreprod.113.115030.

Christie AE (2011) Crustacean neuroendocrine systems and their signaling agents. Cell Tissue Res 345:41–67. https://doi.org/10.1007/s00441-011-1183-9.

Duangprom S, Saetan J, Phanaksri T, Songkoomkrong S, Surinlert P, Tamtin M, Sobhon P, Kornthong N (2022) Acceleration of Ovarian Maturation in the Female Mud Crab With RNA Interference of the Vitellogenesis-Inhibiting Hormone (VIH). Front Mar Sci 9:1–13. https://doi.org/10.3389/fmars.2022.880235.

FAO (2022) The State of World Fisheries and Aquaculture 2022. Towads Blue Transformation. Rome, FAO. https://doi.org/10.4060/cc0461en.

Feijó RG, Braga AL, Lanes CFC, Figueiredo MA, Romano LA, Klosterhoff MC, Nery LEM, Maggioni R, Wasielesky WJ, Marins LF (2016) Silencing of gonad-inhibiting hormone transcripts in *Litopenaeus vannamei* females by use of the RNA interference technology. Mar Biotechnol 18:117–123. https://doi.org/10.1007/s10126-015-9676-2.

Fernandes de Abreu DA, Caballero A, Fardel P, Stroustrup N, Chen Z, Lee KH, Keyes WD, Nash ZM, López-Moyado IF, Vaggi F, Cornils A, Regenass M, Neagu A, Ostojic I, Liu C, Cho Y, Sifoglu D, Shen Y, Fontana W, Lu H, Csikasz-Nagy A, Murphy CT, Antebi A, Blanc E, Apfeld J, Zhang Y, Alcedo J, Ch'ng QL (2014) An insulin-to-insulin regulatory network orchestrates phenotypic specificity in development and physiology. PLoS Genet 10:17–19. https://doi.org/10.1371/journal.pgen.1004225.

Fingerman M (1997) Roles of neurotransmitters in regulating reproductive hormone release and gonadal maturation in decapod crustaceans. Invertebr Reprod Dev 31:47–54.

Fingerman M, Nagabhushanam R (1992) Control of the release of crustacean hormones by neuroregulators. Comp Biochem Physiol Part C, Comp 102:343–352. https://doi.org/10.1016/0742-8413(92)90125-Q.

Finn RN, Kristoffersen BA (2007) Vertebrate vitellogenin gene duplication in relation to the "3R hypothesis": Correlation to the pelagic egg and the oceanic radiation of teleosts. PLoS One 2:e169. https://doi.org/10.1371/journal.pone.0000169.

Garelli A, Gontijo AM, Miguela V, Caparros E, Dominguez M (2012) Imaginal discs secrete

insulin-like peptide 8 to mediate plasticity of growth and maturation. Science336:579–582. https://doi.org/10.1126/science.1216735.

Ge HL, Tan K, Shi LL, Sun R, Wang WM, Li YH (2020) Comparison of effects of dsRNA and siRNA RNA interference on insulin-like androgenic gland gene (IAG) in red swamp crayfish *Procambarus clarkii*. Gene 752:144783. https://doi.org/10.1016/j.gene.2020.144783.

Grönke S, Clarke DF, Broughton S, Andrews TD, Partridge L (2010) Molecular evolution and functional characterization of *Drosophila* insulin-like peptides. PLoS Genet 6. https://doi.org/10.1371/journal.pgen.1000857.

Guan Z-B, Shui Y, Liao X-R, Xu Z-H, Zhou X (2014) Primary structure of a novel gonadotropin-releasing hormone (GnRH) in the ovary of red swamp crayfish *Procambarus clarkii*. Aquaculture 418–419:67–71. https://doi.org/10.1016/j.aquaculture.2013.10.010.

Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B, Lieber M, Macmanes MD, Ott M, Orvis J, Pochet N, Strozzi F, Weeks N, Westerman R, William T, Dewey CN, Henschel R, Leduc RD, Friedman N, Regev A (2013) De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc 8:1494–1512. https://doi.org/10.1038/nprot.2013.084.

Hajarnavis A, Korf I, Durbin R (2004) A probabilistic model of 3′ end formation in *Caenorhabditis elegans*. Nucleic Acids Res 32:3392–3399. https://doi.org/10.1093/nar/gkh656.

Harlıoğlu MM, Farhadi A (2017) Feminization strategies in crustacean aquaculture. Aquac Int 25:1453–1468. https://doi.org/10.1007/s10499-017-0128-z.

Hayashi K (1992) Dendrobranchiata Crustaceans from Japanese Waters, Seibutsu Kenkyusha, Tokyo, 300 pp (in japanese).

Hopkins PM (2012) The eyes have it: A brief history of crustacean neuroendocrinology. Gen Comp Endocrinol 175:357–366. https://doi.org/10.1016/j.ygcen.2011.12.002.

Huberman A (2000) Shrimp endocrinology. A review. Aquaculture 191:191–208. https://doi.org/10.1016/S0044-8486(00)00428-2.

Jayasankar V, Tomy S, Wilder MN (2020) Insights on molecular mechanisms of ovarian development in decapod crustacea: focus on vitellogenesis-stimulating factors and pathways. Front Endocrinol 11:577925. https://doi.org/10.3389/fendo.2020.577925.

Jeon JM, , Kim BK, , Kim YJ, & Kim HW (2011) Structural similarity and expression differences of two PjVg genes from the pandalus shrimp *Pandalopsis japonica*. Fish Aquat Sci 14:22–30. https://doi.org/10.5657/FAS.2011.14.1.022.

Jia X, Chen Y, Zou Z, Lin P, Wang Y, Zhang Z (2013) Characterization and expression profile of Vitellogenin gene from *Scylla paramamosain*. Gene 520:119–130.

https://doi.org/10.1016/j.gene.2013.02.035.

Jimenez-Gutierrez S, Cadena-Caballero CE, Barrios-Hernandez C, Perez-Gonzalez R, Martinez-Perez F, Jimenez-Gutierrez LR (2019) Crustacean vitellogenin: A systematic and experimental analysis of their genes, genomes, mRNAs and proteins; and perspective to Next Generation Sequencing. Crustaceana 92:1169–1205. https://doi.org/10.1163/15685403-00003930.

Kanda S (2019) Evolution of the regulatory mechanisms for the hypothalamic-pituitary-gonadal axis in vertebrates–hypothesis from a comparative view. Gen Comp Endocrinol 284:113075. https://doi.org/10.1016/j.ygcen.2018.11.014.

Kang BJ, Nanri T, Lee JM, Saito H, Han CH, Hatakeyama M, Saigusa M (2008) Vitellogenesis in both sexes of gonochoristic mud shrimp, *Upogebia major* (Crustacea): Analyses of vitellogenin gene expression and vitellogenin processing. Comp Biochem Physiol - B Biochem Mol Biol 149:589-598. https://doi.org/10.1016/j.cbpb.2007.12.003.

Kang BJ, Bae SH, Suzuki T, Niitsu S, Wilder MN (2019) Transcriptional silencing of vitellogenesis-inhibiting hormone (VIH) subtype-I in the whiteleg shrimp, *Litopenaeus vannamei*. Aquaculture 506:119–126. https://doi.org/10.1016/j.aquaculture.2019.03.028.

Katakura Y, Hasegawa Y (1983) Masculinization of females of the isopod crustacean, Armadillidium vulgare, following injections of an active extract of the androgenic gland. Gen Comp Endocrinol 48:57–62. https://doi.org/10.1016/0016-6480(83)90007-2.

Katayama H, Kubota N, Hojo H, Okada A, Kotaka S, Tsutsui N, Ohira T (2014) Direct evidence for the function of crustacean insulin-like androgenic gland factor (IAG): Total chemical synthesis of IAG. Bioorganic Med Chem 22:5783–5789. https://doi.org/10.1016/j.bmc.2014.09.031.

Katayama H, Toyota K, Tanaka H, Ohira T (2022) Chemical synthesis and functional evaluation of the crayfish insulin-like androgenic gland factor. Bioorg Chem 122:105738. https://doi.org/10.1016/j.bioorg.2022.105738.

Kawato S, Nishitsuji K, Arimoto A, Hisata K, Kawamitsu M, Nozaki R, Kondo H, Shinzato C, Ohira T, Satoh N, Shoguchi E, Hirono I (2021) Genome and transcriptome assemblies of the kuruma shrimp, *Marsupenaeus japonicus*. G3 11:jkab268. https://doi.org/10.1093/g3journal/jkab268.

Koskela RW, Greenwood JG, Rothlisberg PC (1992) The influence of prostaglandin E2 and the steroid hormones, 17α-hydroxyprogesterone and 17β-estradiol on moulting and ovarian development in the tiger prawn, *Penaeus esculentus* Haswell, 1879 (Crustacea: Decapoda). Comp Biochem Physiol - Part A Physiol 101:295–299. https://doi.org/10.1016/0300-9629(92)90535-X.

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/MOLBEV/MSW054.

Kung SY, Chan SM, Hui JHL, Tsang WS, Mak A, He JG (2004) Vitellogenesis in the sand shrimp, *Metapenaeus ensis*: the contribution from the hepatopancreas-specific vitellogenin gene (MeVg2). Biol Reprod 71:863–870. https://doi.org/10.1095/biolreprod.103.022905.

Laufer H (1992) Methods for increasing crustacean larval production. United States Pat 5,161,481.

Laufer H, Biggers WJ, Ahl JSB (1998) Stimulation of ovarian maturation in the crayfish *Procambarus clarkii* by methyl farnesoate. Gen Comp Endocrinol 111:113–118. https://doi.org/10.1006/gcen.1998.7109.

Laufer, H., Landau, M., Borst, D., and Homola, E. (1986). The synthesis and regulation of methyl farnesoate, a new juvenile hormone for crustacean reproduction. In: M. Porchet, J.C. Andries, and A. Dhainaut (Eds) Advances in Invertebrate Reproduction 4. Elsevier, Amsterdam, PP.143–153.

Laufer H, Borst D, Baker FC, Carrasco C, Sinkus M, Reuter CC, Tsai LW, Schooley DA (1897) Identification of a juvenile hormone-like compound in a crustaceans. Science 235:202–205. https://doi.org/10.1126/science.5.124.760.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Lu Y, Liu M, Gong J, Cheng Y, Wu X (2018) Effect of exogenous estrogen on the ovarian

development and gene expression in the female swimming crab *Portunus trituberculatus* (Miers, 1876) (Decapoda: Brachyura: Portunidae). J Crustac Biol 38:367–373. https://doi.org/10.1093/jcbiol/ruy013.

Lü Z, Yao C, Zhao S, Zhang Y, Gong L, Liu B, Liu L (2022) Characterization of insulin-like peptide (ILP) and its potential role in ovarian development of the cuttlefish *Sepiella japonica*. Curr Issues Mol Biol 44:2490–2504. https://doi.org/10.3390/cimb44060170.

Mak ASC, Choi CL, Tiu SHK, Hui JHL, He JG, Tobe SS, Chan SM (2005a) Vitellogenesis in the red crab *Charybdis feriatus*: Hepatopancreas-specific expression and farnesoic acid stimulation of vitellogenin gene expression. Mol Reprod Dev 70:288–300. https://doi.org/10.1002/mrd.20213.

Mak ASC, Choi CL, Tiu SHK, Hui JHL, He JG, Tobe SS, Chan SM (2005b) Vitellogenesis in the red crab *Charybdis feriatus*: Hepatopancreas-specific expression and farnesoic acid stimulation of vitellogenin gene expression. Mol Reprod Dev 70:288–300. https://doi.org/10.1002/mrd.20213.

Martin G, Sorokine O, Moniatte M, Bulet P, Hetru C, Van Dorsselaer A (1999) The structure of a glycosylated protein hormone responsible for sex determination in the isopod, *Armadillidium vulgare*. Eur J Biochem 262:727–736. https://doi.org/10.1046/j.1432-1327.1999.00442.x.

Matsunaga Y, Kawano T (2018) The *C. elegans* insulin-like peptides (ILPs). AIMS Biophys

5:217–230. https://doi.org/10.3934/biophy.2018.4.217.

Medesani DA, Ferré LE, Canosa IS, Silveyra GR, Rodríguez EM (2015) Induction of vitellogenesis by 17-hydroxyprogesterone and methyl farnesoate during post-reproductive period, in the estuarine crab *Neohelice granulata*. Invertebr Reprod Dev 59:104–110. https://doi.org/10.1080/07924259.2015.1019014.

Medesani DA, Ferré LE, Grodzielski M, Rodríguez EM (2012) In vitro effect of methyl farnesoate on the vitellogenin content of ovary and hepatopancreas, in the crayfish *Cherax quadricarinatus*. Invertebr Reprod Dev 56:138–143. https://doi.org/10.1080/07924259.2011.582691.

Merlin J, Mohanlal DL, Balasubramanian CP, Sherly T, Subramoniam T, Syamadayal J, Ravichandran P, Ponniah AG, Gopal C, Vijayan KK (2015) Induction of vitellogenesis and reproductive maturation in tiger shrimp, *Penaeus monodon* by 17ß-estradiol and 17α-hydroxyprogesterone: *In vivo* and *in vitro* studies. Invertebr Reprod Dev 59:166–175. https://doi.org/10.1080/07924259.2015.1051192.

Meusy JJ (1980) Vitellogenin, the extraovarian precursor of the protein yolk in Crustacea : A review. Reprod Nutr Dev 20:1-21. https://doi.org/10.1051/rnd:19800101

Mita M, Yoshikuni M, Ohno K, Shibata Y, Paul-Prasanth B, Pitchayawasin S, Isobe M, Nagaham Y (2009) A relaxin-like peptide purified from radial nerves induces oocyte maturation and ovulation in the starfish, *Asterina pectinifera*. Proc Natl Acad Sci U S A

106:9507–9512. https://doi.org/10.1073/pnas.0900243106.

Nagamine C, Knight AW, Maggenti A, Paxman G (1980) Effects of androgenic gland ablation on male primary and secondary sexual characteristics in the Malaysian prawn, *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae), with first evidence of induced feminization in a nonhermaphroditic decapod. Gen Comp Endocrinol 41:423–441. https://doi.org/https://doi.org/10.1016/0016-6480(80)90048-9.

Nagaraju GPC (2011) Reproductive regulators in decapod crustaceans: An overview. J Exp Biol 214:3–16. https://doi.org/10.1242/jeb.047183.

Nagaraju GPC (2007) Is methyl farnesoate a crustacean hormone? Aquaculture 272:39–54. https://doi.org/10.1016/j.aquaculture.2007.05.014.

Nagaraju GPC, Suraj NJ, Reddy PS (2003) Methyl farnesoate stimulates gonad development in *Macrobrachium malcolmsonii* (H . Milne Edwards) (Decapoda, Palaemonidae). Crustaceana 76:1171–1178. https://doi.org/10.1163/156854003773123401.

Nguyen AHT, Glendinning S, Ventura T (2023) A refined roadmap to decapod sexual manipulation. Rev Aquac 1–10. https://doi.org/10.1111/raq.12808.

Nose Y, Lee JM, Ueno T, Hatakeyama M, Oishi K (1997) Cloning of cDNA for vitellogenin of the parasitoid wasp, *Pimpla nipponica* (Hymenoptera: Apocrita: Ichneumonidae): vitellogenin primary structure and evolutionary considerations. Insect Biochem Mol Biol 27:1047–1056. https://doi.org/10.1016/S0965-1748(97)00091-X.

Okamoto N (2016). In Handbook of Hormones: Comparative Endocrinology for Basic and Clinical Research, (Eds: Y. Takei, H. Ando, K. Tsutsui), Elsevier, Waltham, MA, USA, Ch.45. https://doi.org/10.1016/B978-0-12-801028-0.00045-3.

Okumura T, Kim YK, Kawazoe I, Yamano K, Tsutsui N, Aida K (2006) Expression of vitellogenin and cortical rod proteins during induced ovarian development by eyestalk ablation in the kuruma prawn, *Marsupenaeus japonicus*. Comp Biochem Physiol Part A 143:246–253. https://doi.org/10.1016/j.cbpa.2005.12.002.

Okumura T, Yamano K, Sakiyama K (2007) Vitellogenin gene expression and hemolymph vitellogenin during vitellogenesis, final maturation, and oviposition in female kuruma prawn, *Marsupenaeus japonicus*. Comp Biochem Physiol Part A 147:1028–1037. https://doi.org/10.1016/j.cbpa.2007.03.011.

Okuno A, Yang WJ, Jayasankar V, Saido-Sakanaka H, Huong DTT, Jasmani S, Atmomarsono M, Subramoniam T, Tsutsui N, Ohira T, Kawazoe I, Aida K, Wilder MN (2002) Deduced primary structure of vitellogenin in the giant freshwater prawn, *Macrobrachium rosenbergii*, and yolk processing during ovarian maturation. J Exp Zool 292:417–429. https://doi.org/10.1002/JEZ.10083.

Palacios E, Perez-Rostro CI, Ramirez JL, Ibarra AM, Racotta IS (1999) Reproductive exhaustion in shrimp (*Penaeus vannamei*) reflected in larval biochemical composition, survival and growth. Aquaculture 171:309–321. https://doi.org/10.1016/S0044-

8486(98)00393-7.

Pamuru RR (2019). Comparative endocrinology of animals: endocrinology of reproduction in crustaceans, (Eds: E. Narayan). IntechOpen https://www.intechopen.com/chapters/65460.

Paran BC, Fierro IJ, Tsukimura B (2010) Stimulation of ovarian growth by methyl farnesoate and eyestalk ablation in penaeoidean model shrimp, *Sicyonia ingentis* Burkenroad, 1938. Aquac Res 41:1887–1897. https://doi.org/10.1111/j.1365-2109.2010.02612.x.

Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, Buchman AR, Ferguson KC, Heller J, Platt DM, Pasquinelli AA, Liu LX, Doberstein SK, Ruvkun G (2001) Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse *C. elegans* insulin gene family. Genes Dev 15:672–686. https://doi.org/10.1101/gad.867301.

Qiao H, Xiong Y, Zhang W, Fu H, Jiang S, Sun S, Bai H, Jin S, Gong Y (2015) Characterization, expression, and function analysis of gonad-inhibiting hormone in oriental river prawn, *Macrobrachium nipponense* and its induced expression by temperature. Comp Biochem Physiol Part A 185:1–8. https://doi.org/10.1016/j.cbpa.2015.03.005.

Quackenbush LS (1992) Yolk synthesis in the marine shrimp, *Penaeus vannamei*. Comp Biochem Physiol - Part A Physiol 103:711–714. https://doi.org/10.1016/0300-9629(92)90170-U.

Raviv S, Parnes S, Segall C, Davis C, Sagi A (2006) Complete sequence of *Litopenaeus*

vannamei (Crustacea: Decapoda) vitellogenin cDNA and its expression in endocrinologically induced sub-adult females. Gen Comp Endocrinol 145:39–50. https://doi.org/10.1016/j.ygcen.2005.06.009.

Reddy BS, Reddy PR (2015) Methyl farnesoate induced ovarian vitellogenin syntehsis in freshwater rice field crab *Oziothelphusa senex senex* Fabricious. J Infertil Reprod Biol 3:237–244. https://dormaj.org/index.php/JIRB/article/view/343.

Reddy PR, Nagaraju GPC, Reddy PS (2004) Involvement of methyl farnesoate in the regulation of molting and reproduction in the freshwater crab *Oziotelphusa senex senex*. Journal Crustac Biol 24:511–515. https://doi.org/10.1651/C-2478.

Rodríguez EM, López Greco LS, Medesani DA, Laufer H, Fingerman M (2002) Effect of methyl farnesoate, alone and in combination with other hormones, on ovarian growth of the red swamp crayfish, *Procambarus clarkii*, during vitellogenesis. Gen Comp Endocrinol 125:34–40. https://doi.org/10.1006/gcen.2001.7724.

Saikrithi P, Balasubramanian CP, Otta SK, Tomy S (2019) Characterization and expression profile of farnesoic acid O-methyltransferase gene from Indian white shrimp, *Penaeus indicus*. Comp Biochem Physiol Part - B Biochem Mol Biol 232:79–86. https://doi.org/10.1016/j.cbpb.2019.03.004.

Saitou N, Nei M (1987) The neighbor-joining method: new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

https://doi.org/10.1093/oxfordjournals.molbev.a040454.

Sathapondecha P, Chotigeat W (2019) Induction of vitellogenesis by glass bottom boat in the female banana shrimp, *Fenneropenaeus merguiensis* de Man. Gen Comp Endocrinol 270:48–59. https://doi.org/10.1016/j.ygcen.2018.10.005.

Semaniuk U, Piskovatska V, Strilbytska O, Strutynska T, Burdyliuk N, Vaiserman A, Bubalo V, Storey KB, Lushchak O (2021) *Drosophila* insulin-like peptides: from expression to functions – a review. Entomol Exp Appl 169:195–208. https://doi.org/10.1111/eea.12981.

Sheppard S, Lawson ND, Zhu LJ (2013) Accurate identification of polyadenylation sites from 3` end deep sequencing using a naive Bayes classifier. Bioinformatics 29:2564–2571. https://doi.org/10.1093/bioinformatics/btt446.

Shi L, Han S, Fei J, Zhang L, Ray JW, Wang W, Li Y (2019) Molecular characterization and functional study of insulin-like androgenic gland hormone gene in the red swamp crayfish, *procambarus clarkii*. Genes (Basel) 10:1–18. https://doi.org/10.3390/genes10090645.

Speck D, Kleinau G, Meininghaus M, Erbe A, Einfeldt A, Szczepek M, Scheerer P, Pütter V (2022) Expression and Characterization of Relaxin Family Peptide Receptor 1 Variants. Front Pharmacol 12:1–14. https://doi.org/10.3389/fphar.2021.826112.

Subramoniam T (2000) Crustacean ecdysteriods in reproduction and embryogenesis. Comp Biochem Physiol Part C 125:135–156. https://doi.org/10.1016/S0742-8413(99)00098-5.

Subramoniam T (2011) Mechanisms and control of vitellogenesis in crustaceans. Fish Sci

77:1–21. https://doi.org/10.1007/s12562-010-0301-z.

Subramoniam T (2017) Steroidal control of vitellogenesis in crustacea: A new understanding for improving shrimp hatchery production. Proc Indian Natl Sci Acad 83:595–610. https://doi.org/10.16943/ptinsa/2017/48969.

Summavielle T, Monteiro PRR, Reis-Henriques MA, Coimbra J (2003) *In vitro* metabolism of steroid hormones by ovary and hepatopancreas of the crustacean Penaeid shrimp *Marsupenaeus japonicus*. Sci Mar 67:299–306. https://doi.org/10.3989/scimar.2003.67n3299.

Suzuki S, Yamasaki K (1998) Sex reversal by implantations of ethanol-treated androgenic glands of female isopods, *Armadillidium vulgare* (Malacostraca, Crustacea). Gen Comp Endocrinol 111:367–375. https://doi.org/10.1006/gcen.1998.7121.

Tan K, Zhou M, Jiang H, Jiang D, Li Y, Wang W (2020) siRNA-mediated MrIAG silencing induces sex reversal in *Macrobrachium rosenbergii*. Mar Biotechnol 22:456–466. https://doi.org/10.1007/s10126-020-09965-4.

Teshima S, Alam MS, Koshio S, Ishikawa M, Kanazawa A (2002) Assessment of requirement values for essential amino acids in the prawn, *Marsupenaeus japonicus* (Bate). Aquac Res 33:395–402. https://doi.org/10.1046/j.1365-2109.2002.00684.x.

Thompson JD, HigginsGibson DGTJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific

gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680. https://doi.org/10.1093/nar/22.22.4673.

Tian B, Hu J, Zhang H, Lutz CS (2005) A large-scale analysis of mRNA polyadenylation of human and mouse genes. Nucleic Acids Res 33:201–212. https://doi.org/10.1093/nar/gki158.

Tiu SHK, Chan SM (2007) The use of recombinant protein and RNA interference approaches to study the reproductive functions of a gonad-stimulating hormone from the shrimp *Metapenaeus ensis*. FEBS J 274:4385–4395. https://doi.org/10.1111/j.1742-4658.2007.05968.x.

Tiu SHK, Chan SM, Tobe SS (2010) The effects of farnesoic acid and 20-hydroxyecdysone on vitellogenin gene expression in the lobster, *Homarus americanus*, and possible roles in the reproductive process. Gen Comp Endocrinol 166:337–345. https://doi.org/10.1016/j.ygcen.2009.11.005.

Tiu SHK, Hui HL, Tsukimura B, Tobe SS, He JG, Chan SM (2009) Cloning and expression study of the lobster (*Homarus americanus*) vitellogenin: Conservation in gene structure among decapods. Gen Comp Endocrinol 160:36–46. https://doi.org/10.1016/J.YGCEN.2008.10.014.

Tiu SHK, Hui JHL, He JG, Tobe SS, Chan SM (2006a) Characterization of vitellogenin in the shrimp *Metapenaeus ensis*: Expression studies and hormonal regulation of MeVg1

transcription *in vitro*. Mol Reprod Dev 73:424–436. https://doi.org/10.1002/mrd.20433.

Tiu SHK, Hui JHL, Mak ASC, He JG, Chan SM (2006b) Equal contribution of hepatopancreas and ovary to the production of vitellogenin (PmVg1) transcripts in the tiger shrimp, *Penaeus monodon*. Aquaculture 254:666–674. https://doi.org/10.1016/j.aquaculture.2005.11.001.

Treerattrakool S, Boonchoy C, Urtgam S, Panyim S, Udomkit A (2014) Functional characterization of recombinant gonad-inhibiting hormone (GIH) and implication of antibody neutralization on induction of ovarian maturation in marine shrimp. Aquaculture 428–429:166–173. https://doi.org/10.1016/j.aquaculture.2014.03.009.

Treerattrakool S, Panyim S, Chan SM, Withyachumnarnkul B, Udomkit A (2008) Molecular characterization of gonad-inhibiting hormone of Penaeus monodon and elucidation of its inhibitory role in vitellogenin expression by RNA interference. FEBS J 275:970–980. https://doi.org/10.1111/j.1742-4658.2008.06266.x.

Treerattrakool S, Panyim S, Udomkit A (2011) Induction of Ovarian Maturation and Spawning in Penaeus monodon Broodstock by Double-Stranded RNA. Mar Biotechnol 13:163–169. https://doi.org/10.1007/s10126-010-9276-0.

Tsang WS, Quackenbush LS, Chow BKC, Tiu SHK, He JG, Chan SM (2003) Organization of the shrimp vitellogenin gene: Evidence of multiple genes and tissue specific expression by the ovary and hepatopancreas. Gene 303:99–109.

https://doi.org/10.1016/S0378-1119(02)01139-3.

Tsukimura B (2001) Crustacean vitellogenesis: its role in oocyte development. Am Zool 41:465–476. https://doi.org/10.1668/0003-1569(2001)041[0465:CVIRIO]2.0.CO;2.

Tsukimura B, Kamemoto FI (1991) *In vitro* stimulation of oocytes by presumptive mandibular organ secretions in the shrimp, *Penaeus vannamei*. Aquaculture 92:59–66. https://doi.org/10.1016/0044-8486(91)90008-U.

Tsutsui N, Katayama H, Ohira T, Nagasawa H, Wilder M N, Aid K (2005a) The effects of crustacean hyperglycemic hormone-family peptides on vitellogenin gene expression in the kuruma prawn, Marsupenaeus japonicus. Gen Comp Endocrinol 144:232–239. https://doi.org/10.1016/j.ygcen.2005.06.001.

Tsutsui N, Kawazoe I, Ohira T, Jasmani S, Yang WJ, Wilder MN, Aida K (2000) Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. Zoolog Sci 17:651–660. https://doi.org/10.2108/zsj.17.651.

Tsutsui N, Kim YK, Jasmani S, Ohira T, Wilder MN, Aida K (2005b) The dynamics of vitellogenin gene expression differs between intact and eyestalk ablated kuruma prawn *Penaeus* (*Marsupenaeus*) *japonicus*. Fish Sci 71:249–256. https://doi.org/10.1111/j.1444-2906.2005.00957.x.

Tsutsui N, Kobayashi Y, Izumikawa K, Sakamoto T (2020) Transcriptomic analysis of the

kuruma prawn *Marsupenaeus japonicus* reveals possible peripheral regulation of the ovary. Front Endocrino 11:541. https://doi.org/10.3389/fendo.2020.00541.

Tsutsui N, Kotaka S, Ohira T, Sakamoto T (2018) Characterization of distinct ovarian isoform of crustacean female sex hormone in the kuruma prawn *Marsupenaeus japonicus*. Comp Biochem Physiol Part A Mol Integr Physiol 217:7–16. https://doi.org/10.1016/j.cbpa.2017.12.009.

Tsutsui N, Nagakura-Nakamura A, Nagai C, Ohira T, Wilder MN, Nagasawa H (2013a) The *ex vivo* effects of eyestalk peptides on ovarian vitellogenin gene expression in the kuruma prawn *Marsupenaeus japonicus*. Fish Sci 79:33–38. https://doi.org/10.1007/s12562-012-0566-5.

Tsutsui N, Ohira T, Kawazoe I, Takahashi A, Wilder MN (2007) Purification of sinus gland peptides having vitellogenesis-inhibiting activity from the whiteleg shrimp *Litopenaeus vannamei*. Mar Biotechnol 9:360–369. https://doi.org/10.1007/s10126-006-6151-0.

Tsutsui N, Ohira T, Okutsu T, Shinji J, Bae SH, Kang BJ, Wilder MN (2013b) Molecular cloning of a cDNA encoding vitellogenesis-inhibiting hormone in the whiteleg shrimp *Litopenaeus vannamei* and preparation of its recombinant peptide using an *E. coli* expression system. Fish Sci 79:357–365. https://doi.org/10.1007/s12562-013-0603-z.

Tsutsui N, Yamane F, Kakinuma M, Yoshimatsu T (2022) Multiple insulin-like peptides in the gonads of the kuruma prawn *Marsupenaeus japonicus*. Fish Sci 88:387–396.

https://doi.org/10.1007/s12562-022-01596-z.

Tsutsui N, Saido-Sakanaka H, Yang WJ, Jayasankar V, Jasmani S, Okuno A, Ohira T, Okumura T, Aida K, Wilder MN (2004) Molecular Characterization of a cDNA encoding vitellogenin in the coonstriped shrimp, *Pandalus hypsinotus* and site of vitellogenin mRNA expression. J Exp Zool Part A Comp Exp Biol 301A:802-814. https://doi.org/10.1002/JEZ.A.53.

Uawisetwathana U, Leelatanawit R, Klanchui A, Prommoon J, Klinbunga S, Karoonuthaisiri N (2011) Insights into eyestalk ablation mechanism to induce ovarian maturation in the black tiger shrimp. PLoS One 6:e24427. https://doi.org/10.1371/journal.pone.0024427.

Veenstra JA (2020) Gonadulins, the fourth type of insulin-related peptides in decapods. Gen Comp Endocrinol 296:113528. https://doi.org/10.1016/j.ygcen.2020.113528.

Wang YH, LeBlanc GA (2009) Interactions of methyl farnesoate and related compounds with a crustacean retinoid X receptor. Mol Cell Endocrinol 309:109–116. https://doi.org/10.1016/j.mce.2009.05.016.

Wang H, Yan T, Tan JTT, Gong Z (2000) A zebrafish vitellogenin gene (vg3) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. Gene 256:303–310. https://doi.org/10.1016/S0378-1119(00)00376-0.

Wang W, Li B, Zhou T, Wang C, Kyei AB, Shi L, Chan S (2020) Investigation of gene sequence divergence, expression dynamics, and endocrine regulation of the vitellogenin

gene family in the whiteleg shrimp *Litopenaeus vannamei*. Front Endocrinol 11:577745. https://doi.org/10.3389/fendo.2020.577745.

Webster SG, Keller R, Dircksen H (2012) The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. Gen Comp Endocrinol 175:217–233. https://doi.org/10.1016/j.ygcen.2011.11.035.

Wilder M, Okumura T, Suzuki Y, Fusetani N, Aida K (1994) Vitellogenin production induced by eyestalk ablation in juvenile giant freshwater prawn *Macrobrachium rosenbergii* and trial methyl farnesoate administration. Zoolog Sci 11:45–53.

Wilder M, Okumura T, Tsutsui N (2010) Reproductive mechanisms in crustacea focusing on selected prawn species: vitellogenin structure, processing and synthetic control. Aqua-BioSci Monogr 3:73–110. https://doi.org/10.5047/absm.2010.00303.0073.

Wong QWL, Mak WY, Chu KH (2008) Differential gene expression in hepatopancreas of the shrimp *Metapenaeus ensis* during ovarian maturation. Mar Biotechnol 10:91–98. https://doi.org/10.1007/S10126-007-9042-0.

Xie S, Sun L, Liu F, Dong B (2009) Molecular characterization and mRNA transcript profile of vitellogenin in Chinese shrimp, *Fenneropenaeus chinensis*. Mol Biol Rep 36:389–397. https://doi.org/10.1007/s11033-007-9192-1.

Yang F, Xu HT, Dai ZM, Yang WJ (2005) Molecular characterization and expression analysis

of vitellogenin in the marine crab *Portunus trituberculatus*. Comp Biochem Physiol - B Biochem Mol Biol 142:456–464. https://doi.org/10.1016/j.cbpb.2005.09.011.

Yang Y, Zheng B, Bao C, Huang H, Ye H (2016) Vitellogenin2: Spermatozoon specificity and immunoprotection in mud crabs. Reproduction 152:235–243. https://doi.org/10.1530/REP-16-0188.

Zeng H, Bao C, Huang H, Ye H, Li S (2016) The mechanism of regulation of ovarian maturation by red pigment concentrating hormone in the mud crab *Scylla paramamosain*. Anim Reprod Sci 164:152–161. https://doi.org/10.1016/j.anireprosci.2015.11.025.

Zhao J, Wang W, Wang C, Shi L, Wang G, Sun C, Chan SF (2021) The presence of multiple copies of the vitellogenin gene in *Fenneropenaeus merguiensis* (De Man, 1888) (Decapoda: Dendrobranchiata: Penaeidae): Evidence for gene expansion and functional diversification in shrimps. J Crustac Biol 41:1–12. https://doi.org/10.1093/jcbiol/ruaa100.

Zmora N, Trant J, Chan SM, Chung JS (2007) Vitellogenin and its messenger RNA during ovarian development in the femaleblue crab, *Callinectes sapidus*: gene expression, synthesis, transport, and cleavage. Biol Reprod 77:138–146. https://doi.org/10.1095/BIOLREPROD.106.055483.

Zmora N, Trant J, Zohar Y, Chung JS (2009) Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 1: An ovarian stage dependent involvement. Saline Systems 5:1–11.

https://doi.org/10.1186/1746-1448-5-7.

List of Publications

This doctoral dissertation encompasses two pivotal scientific manuscripts which serves as the main focal points of Chapters 1,2 and 3 of the current study.

The first paper, reproduced with permission from Springer Nature, has been judiciously employed as the foundational framework for the formulation of Chapters 1 and 2.

1. El-Desoky MS, Jogatani T, Yamane F, Izumikawa K, Kakinuma M, Sakamoto T, Tsutsui N. (2023) Identification of an additional vitellogenin gene showing hepatopancreas-specific expression in the kuruma prawn *Marsupenaeus japonicus*. Fish Sci. https://doi.org/10.1007/s12562-023-01705-6.

2. El-Desoky MS, Takeuchi R, Katayama H, Tsutsui N (2023). Chemical synthesis of insulin-like peptide 1 and its potential role in vitellogenesis of the kuruma prawn *Marsupenaeus japonicus*. Journal of Peptide Science, e3529. https://doi.org/10.1002/psc.3529.

ACKNOWLEDGEMENTS

*First and foremost, prayerful thanks to "**Allah**" who leads my success in my entire life.*

*I'm profoundly grateful to **Prof. Naoaki Tsutsui**, my supervisor, for his invaluable supervision, unwavering encouragement, generous support, and expert guidance throughout the thesis. Without his dedication, this research would not have been possible.*

*I extend my deepest thanks to **Prof. Hirokazu Matsuda** and **Prof. Akira Komaru** for their meticulous editing, valuable discussions and insightful advice, which greatly contributed to the quality of this dissertation.*

*I also extend my deepest thanks to **Prof. Tadashi Isshiki** and **Prof. Makoto Kakinuma** for granting me the access to utilize the experimental equipment necessary for completing this study.*

*I would also like to express my sincere appreciation to **Prof. Khaled Mohammed Geba** and **Prof. Shaymaa Hussein** of Menoufia University for their constant advice and support during my journey.*

*I'm profoundly grateful to **my family**, especially **my parents**, **my husband Abdelmageed**, and **my siblings**, for their unwavering patience, and encouragement throughout my study in Japan.*

I also extend my heartfelt thanks to my children ***Dai Abdelmageed*** *and* ***Nour Abdelmageed****, for their endless love, understanding, and prayers.*

Also, warm words of gratitude go to ***all members of the Laboratory of Shallow Sea Aquaculture*** *for their kindness, continuous support and help during my study in Japan.*

Last, but not least, I would like to thank the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan for the financial support of my research and life in Japan.

Marwa Said El-Desoky