Reproductive Mechanisms in Crustacea Focusing on Selected Prawn Species: Vitellogenin Structure, Processing and Synthetic Control

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Abstract
Shrimp culture is a significant world-wide industry, with current production levels reaching over 3 million tons per year. The expansion of the industry has given rise to the problems of environmental deterioration due to intensive-scale culture, and the outbreak of disease. While many of these issues are now being sufficiently addressed, the establishment of sustainable seed production technology is an area that should be given continued attention. In this regard, it remains difficult to control reproduction under hatchery conditions for a large number of commercially-important species. At present, an understanding of reproductive mechanisms in Crustacea is not complete, although in recent years, a great deal of knowledge has accumulated on vitellogenin structure, processing, and synthetic site in a number of economically-important species. This monograph will cover the current status of research on vitellogenin in decapod crustaceans, especially prawns and shrimp, and discuss mechanisms of vitellogenin synthetic control, both demonstrated and postulated. The monograph will also present current knowledge of crustacean vitellogenin receptors, and cover related facets of reproductive development, such as mechanisms of cortical rod formation and the utilization of vitellin during embryogenesis. Finally, future directions for this research and potential applications to aquaculture will be discussed.

1. Introduction

In recent years, shrimp culture has become a significant world-wide industry. Current production levels reach over 3 million tons per year, corresponding to a market volume of over 10 billion U.S. dollars (FAO 2008). Especially in Southeast Asia, where more than 75% of the world’s shrimp culture occurs, shrimp farming has been considered to be the cause of myriad environmental problems such as the destruction of mangrove forest, and deterioration of the coastal environment due to efflux from intensive shrimp farms. Thankfully, much effort has been extended by governments and researchers/technical specialists to address these concerns, and there has been a significant amelioration of the adverse affects of shrimp farming (SEAFDEC 2004).

In addition to environmentally-related issues, the control of disease (especially viral) and artificial seed production, remain areas of importance in relation to the sustainability of the industry. Regarding the latter, while the life cycle has been closed (e.g., the ability to obtain larvae from parent spawners, which are in turn reared to maturity and used as parent shrimp) in the whiteleg shrimp, *Litopenaeus vannamei*, it remains difficult to control reproduction under hatchery conditions for a large number of commercially-important species. Indeed, for *Li. vannamei*, not more than 30% of all females in a given hatchery will develop mature ovaries at a given time (personal communication, the Oceanic Institute, Hawaii, USA); it would be desirable to gain a better understanding of reproductive mechanisms in target species, and use this knowledge to improve efficiency in hatchery operations.

The understanding of reproductive mechanisms in Crustacea lags behind that of fish species, with the re-
sult that there are few technologies to control reproduction in the former. Yet, a great deal of knowledge has accumulated in the past 15 years, especially regarding the structure and processing of vitellogenin, and the physiological functioning of eyestalk hormones, especially those that control the synchronous processes of ovarian development and molting. While vitellogenesis commonly occurs in oviparous animal groups, it has been shown that crustacean vitellogenins differ a great deal even from those of insects, which are also arthropods. However, the general process, in which vitellogenin is produced in specific tissues as a yolk protein precursor, undergoes processing into smaller molecules, and is subsequently accumulated in developing oocytes as vitellin to serve as a source of nutrients during embryogenesis, is a feature of Crustacea as much as it is in insects, amphibians, chickens, and all other groups studied thus far. In addition to their nutritive roles in reproduction/embryogenesis, vitellogenin and vitellin are also apparently involved in the importation of minerals, lipids, and other materials into developing oocytes essential to embryogenic development.

The elucidation of the complete primary structure of vitellogenin in insects preceded that of crustaceans by many years (Chen et al., 1997; Sappington and Raikhel 1998; Tufail et al., 2000), and the induction of vitellogenin gene expression via hormonal action (ecdysteroids and/or juvenile hormone), has been conclusively demonstrated in much earlier studies (Davey 1983; Wyatt 1988). The first report of a complete primary sequence in a crustacean species was by the authors of this monograph, Tsutsui et al. (2000), in the kuruma prawn, _Macrobrachium rosenbergii_, and the induction of vitellogenin/vitellin, with the precise molecular weight and composition of the various vitellogenins were based on electrophoretic or immunological techniques to examine molecular weight and subunit composition.

2. Structural and biochemical characterization of vitellogenin and vitellin

2-1. Structural aspects: molecular weight, physical properties and amino acid sequence; processing mechanisms of vitellogenin to vitellin

The earliest studies on vitellin/vitellogenin structure utilized immunological and electrophoretic techniques to examine molecular weight and subunit composition. In addition, before molecular biological techniques started to be used in this field, various workers characterized amino acid composition, and lipid and carbohydrate content. Many of the species that have been studied are commercially important targets of fisheries and aquaculture activity, while other species serve as valuable research models. The first studies examining yolk protein characteristics in Crustacea focused on shrimp and prawns and of the genus _Penaeus_ (which has since been divided into several separate genera), the genera _Metapenaeus_ and _Parapenaeus_ (Tom et al., 1987a, b; Chang et al., 1993a; Qiu et al., 1997; Longyant et al., 2000), and the _Macrobrachium_ sp, especially the giant freshwater prawn _Macrobrachium rosenbergii_ (Derecle et al. 1986; Chang et al. 1993b; Wilder et al., 1994), an economically significant species cultured widely throughout Southeast Asia. A number of crab species, some lobsters, and certain crayfishes have been studied.

Initially, various strategies and methodologies had been employed to determine molecular weight and subunit composition of vitellogenin/vitellin, with the result that although many groups obtained similar results, certain discrepancies were found in regard to precise molecular weight and composition of the various subunits. Nevertheless, such information served as the basis for further studies elucidating partial amino acid sequence (Yang et al. 2000); with the commencement of studies using gene cloning techniques, a wealth of information has thus been obtained on full cDNA.
Fig. 1. Deduced amino acid sequence and nucleotide sequences of the 5’ and 3’ UTRs of vitellogenin in the kuruma prawn, *Marsupenaeus japonicus*. The putative signal sequence is shown in italics (–18~–1). Amino acid sequences elucidated in older studies (fragments obtained by digestion with lysyl-endopeptidase) are underlined with a solid line (LE3, LE19, LE21, and LE46); those obtained in Tsutsui et al. (2000) are underlined with a dotted line (LE101–104; fraction 2). Ser or Thr residues that are possibly phosphorylated by casein kinase II are shaded. Two consensus cleavage sequences targeted by endoproteases of the subtilisin family are shown in white lettering with black background. Positions of nucleotides and amino acids are indicated respectively by numbers of the left and right sides of the figures. The polyadenylation signal is doubly underlined. Accession number AB033719 (GenBank/EMBL Data Bank). Reprinted with permission from *Zoological Science*, 17, Tsutsui et al., Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*, 651–660, Fig. 3, © 2000, Zoological Society of Japan.
Table 1. Amino acid composition of vitellin (molar percentage) in selected crustacean species. ND: not done or undetectable.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peneaus monodon</th>
<th>Litopenaeus semisulcatus</th>
<th>Litopenaeus vannamei</th>
<th>Macrobrachium rosenbergii</th>
<th>Procambarus clarkii</th>
<th>Pacifichypophaus sp.</th>
<th>Artemia salina</th>
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<tr>
<td>Axx</td>
<td>7.28</td>
<td>6.67</td>
<td>6.24</td>
<td>9.03</td>
<td>8.26</td>
<td>8.5</td>
<td>9.9</td>
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<tr>
<td>Threonine</td>
<td>6.08</td>
<td>5.63</td>
<td>6.08</td>
<td>0.14</td>
<td>6.35</td>
<td>5.7</td>
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<td>Serine</td>
<td>5.96</td>
<td>7.27</td>
<td>7.37</td>
<td>5.07</td>
<td>8.83</td>
<td>8.8</td>
<td>10.6</td>
</tr>
<tr>
<td>Glx</td>
<td>13.06</td>
<td>12.11</td>
<td>11.39</td>
<td>13.77</td>
<td>12.07</td>
<td>8.2</td>
<td>12.1</td>
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<td>Proline</td>
<td>4.83</td>
<td>6.27</td>
<td>5.36</td>
<td>7.98</td>
<td>5.40</td>
<td>4.5</td>
<td>6.3</td>
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<tr>
<td>Glycine</td>
<td>6.04</td>
<td>8.30</td>
<td>7.05</td>
<td>6.62</td>
<td>8.83</td>
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<td>Alanine</td>
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<td>10.62</td>
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<td>13.21</td>
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<td>Valine</td>
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<td>7.64</td>
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<td>9.84</td>
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<td>Methionine</td>
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<td>2.45</td>
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<td>2.00</td>
<td>0.76</td>
<td>2.4</td>
<td>0.0</td>
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<td>Isoleucine</td>
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<td>6.82</td>
<td>4.89</td>
<td>6.1</td>
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<td>Leucine</td>
<td>7.16</td>
<td>7.27</td>
<td>7.35</td>
<td>8.25</td>
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<td>Tyrosine</td>
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<td>2.01</td>
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<td>Phenylalanine</td>
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<td>3.70</td>
<td>3.81</td>
<td>4.07</td>
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<td>Histidine</td>
<td>2.52</td>
<td>1.89</td>
<td>2.50</td>
<td>2.08</td>
<td>2.67</td>
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<td>Lysine</td>
<td>6.12</td>
<td>7.33</td>
<td>6.26</td>
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<td>7.56</td>
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<td>4.2</td>
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<td>Arginine</td>
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<td>4.80</td>
<td>4.65</td>
<td>0.96</td>
<td>6.35</td>
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<td>Cysteine</td>
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<td>Tryptophan</td>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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*Quintino et al. 1990; Tom et al. 1992; Qiu et al. 1997; Lee et al. 1997; Lui and O’Connor 1976; Fyffe and O’Connor 1974; De Chaffoy de Courcelles and Kondo 1980*

Ass includes aspartate and asparagines; Glx includes glutamate and glutamine.
acids of vitellin and vitellogenin. For example, in the giant freshwater prawn, *Mac. rosenbergii*, Yang et al. (2000) purified the 102 kDa and 90 kDa vitellogenin bands of this species using reversed-phase HPLC, and examined their N-terminal amino acid sequences. The 102 kDa band (named as VnB), had the sequence SIDLRQ- - -, while the 90 kDa vitellin band was a mixture of two subunits of APWPSG- - - (named as VnA) and RREEQKV- - - (named as VnC). Vitellogenin was known to have three subunit components of 199 kDa, 102 kDa and 90 kDa, but at that time, it was not possible to examine their N-terminal sequences. However, in a follow-up study also by the authors of this monograph, a method was developed enabling vitellogenin in the hemolymph to be concentrated in sufficient quantity to carry out sequence analysis (Okuno et al. 2002). It was then seen that the 102 kDa and 199 kDa vitellogenin bands shared the same N-terminal sequence, being identical to that of the 102 kDa vitellin band (Fig. 2B). The sequences of the 90 kDa vitellogenin and vitellin bands were also the same.

However, the first breakthrough in terms of elucidation of a full-length cDNA for a crustacean vitellogenin was accomplished by Tsutsui et al. (2000), for the kuruma prawn, *Mar. japonicus*. In this species, vitellogenin is comprised of subunits of 186 kDa, 128 kDa and 91 kDa (Kawazoe et al. 2000), for which the N-terminal amino acid of the 91 kDa subunit had been determined. Tsutsui et al. (2000) used this information and the method of “library walking”, to obtain a full-length cDNA encoding 2,587 amino acids (Fig. 1). This vitellogenin precursor contained 2 consensus cleavage sites R-X-K/R-R targeted by enzymes of the subtilisin family. It was postulated that cleavage occurs at the first of these sites, producing the 91 kDa subunit (Tsutsui et al. 2000). This was later confirmed by N-terminal amino acid sequence analysis of the 186 kDa subunit (Tsutsui et al. 2002).

Following the above studies in the kuruma prawn, the next report elucidating the full cDNA sequence and deduced primary structure of a crustacean species was for *Mac. rosenbergii* (Okuno et al. 2002). This cDNA was 7,800 bp, and encoded a protein corresponding to 2,537 amino acids containing 3 R-X-K/R-R consensus cleavage sites. It was found that the subunits VnA, VnB and VnC discussed above were connected in a large vitellogenin precursor in that order (VnA-B-C, postulated to be 284 kDa; Fig. 28). Of note, a subunit referred to as VnD is also shown in Fig. 2B, but VnC and VnD were determined to be identical ovarian subunits that showed slight separation on reversed-phase high performance liquid chromatography in Yang et al. 2000 due to supposed glycosylation; hence, subsequent discussion refers to VnC only for purposes of simplicity. This precursor (VnA-B-C) is initially cleaved at the first of these sites, most likely at the hepatopancreas prior to secretion into the hemolymph.

Fig. 2. Vitellogenin processing in the giant freshwater prawn *Macrobrachium rosenbergii* (daily changes in subunit composition and schematic diagram). (A) Examination of daily changes in subunit composition of vitellogenin (Vg) in hemolymph and vitellin (Vn) in ovary based on SDS-PAGE and Western blotting in eyestalk ablated female *Mac. rosenbergii*. Lanes 1–12 (top) correspond to 1–12 days after eyestalk ablation, and show the order of the appearance of Vg bands in the hemolymph. Lanes 1, 4, 8 and 12 (bottom) show corresponding Vn band composition in the ovary during ovarian maturation. (B) Schematic representation of synthesis and processing of vitellogenin in *Mac. rosenbergii*. Vitellogenin is synthesized as a single precursor molecule, A–B–C/D, in the hepatopancreas, which is then cleaved into two subunits A and proB. Subunits A and proB are released into the hemolymph, where proB is cleaved to form two subunits B and C/D. The three processed subunits A, B and C/D are incorporated into the ovary. Note: as described in the text, VnC and VnD are identical ovarian subunits that showed slight separation on reversed-phase high performance liquid chromatography due to supposed glycosylation. Reprinted with permission of John Wiley & Sons, Inc. from *Journal of Experimental Zoology Part A*, 292, Okuno et al., © 2002 Wiley-Liss, Inc., a Wiley Company.
producing a 90 kDa and a 199 kDa protein, equivalent in terms of sequence to VnA and VnB-C, respectively. The 199 kDa protein then undergoes a second cleavage, yielding a 102 kDa protein equivalent to VnB and an additional 90 kDa protein corresponding to VnC, after which all 3 proteins are incorporated into the ovaries as VnA, B, and C (Okuno et al. 2002). Furthermore, it was seen that the N-terminal sequence of VnA in *Macrobrachium rosenbergii* was 60% identical to that of the 91-kDa vitellin in *Marsupenaeus japonicus*, while that of VnB was highly similar to the sequence of the N-terminal region of the 186 kDa subunit in *Marsupenaeus japonicus*. Thereafter, a full vitellogenin cDNA sequence has been elucidated in numerous prawn and shrimp species, and other decapod species as well, such as crayfish and crabs. Thus far, all species have shown high levels of identity, many of them having the starting characteristic sequence APW- - - (see also Auttarat et al. 2006). Furthermore, the degree of actual identity among species reflects phylogenetic classification, with members of the same family (in the case of Penaeidae) having identity levels of around 90% throughout the entire molecule, for example, the comparison of *Penaeus semisulcatus* and *Litopenaeus vannamei* (Raviv et al. 2006). In less-related species, the degree of identity is especially high in the first part of the molecule, but somewhat lower in the middle and latter parts of the molecule.

For example, *Macrobrachium rosenbergii* vitellogenin harbors a fairly high degree of identity (52%) to that of *Marsupenaeus japonicus* in the VnA region, but is just over 30% in the VnB-C regions (Okuno et al. 2002). *Macrobrachium rosenbergii* vitellogenin shows higher identity to that of the hermaphroditic coonstripe shrimp, *Pandalus hypsinotus*, not surprisingly, as these two species are of the same infraorder, i.e., Caridea (Tsutsui et al. 2004). Figure 3 shows a phylogenetic tree based on vitellogenin sequence identity of currently known species. Even in decapods other than prawns and shrimp, such as that of the crayfish *Cherax quadricarinatus* (Abdu et al. 2002), a certain level of identity (overall 37% with *Marsupenaeus japonicus*) is conserved. Since the report of Tsutsui et al. (2000), numerous data in other species have been obtained, and have indicated that shrimp, prawns and other decapod crustacean harbor similar vitellogenin structure. These reports have also revealed that almost all species examined thus far undergo similar mechanisms of processing during ovarian maturation. The results of these reports are too numerous to detail, and are therefore summarized in Table 2 in an attempt to consolidate knowledge concerning vitellogenin structure and processing in known prawn and shrimp species. Other decapod species (crayfish, lobster and crabs) are not shown in the table for purposes of simplicity, but full cDNA sequences
Table 2. Summary of data for molecular weight, subunit composition, N-terminal amino acid sequence and processing mechanisms for selected decapod species. ND: not done; bp: base pairs; aa: amino acid residues; Native: determined by native page; Polypeptides: determined by SDS-PAGE unless noted as postulated (*). Polypeptides determined as vitellogenin denoted as Vg; those determined as vitellin denoted as Vn. Note: amino acid length represents that of the ORF.

<table>
<thead>
<tr>
<th>Species (Prawns and shrimp)</th>
<th>Vitellogenin/vitellin (kDa)</th>
<th>cDNA length (bp); (amino acid length)</th>
<th>N-terminal amino acid sequence</th>
<th>Processing mechanisms; gene structure; other information</th>
<th>References (Authors and year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penaeus monodon</td>
<td>ND</td>
<td>78.6, 106, 86 (Vg)*</td>
<td>Approx. 7800 bp (2589 aa)</td>
<td>Sequencing ND for subunits; however, APWGADLPRC and SIDSSVISDF equivalent to A and B in other species found from gene cloning.</td>
<td>Tiu et al. 2006b</td>
</tr>
<tr>
<td>Fenneropenaeus chinensis</td>
<td>ND</td>
<td>85, 91 (Vg)</td>
<td>7761 bp (2587 aa)</td>
<td>Sequencing ND for subunits; however, APWGADLPRC and SIDSSVISDF equivalent to A and B in other species found from gene cloning. Processing mechanisms not elucidated, but likely similar to other species (first cleavage after aa 710 if putative 18-amino acid-length signal peptide is excluded).</td>
<td>Chang and Jeng 1995 Xie et al. 2009</td>
</tr>
<tr>
<td>Fenneropenaeus merguensis</td>
<td>398</td>
<td>A: 78, B: 87, C: 104 (Vn)</td>
<td>7961 bp (2586 aa)</td>
<td>Vn consists of doublets of A and B on SDS-PAGE. Mature protein (283 kDa) cleaved to 78 kDa and 203 kDa subunits (conserved site as in Mar. rosenbergii). 203 kDa subunit likely processed to 87 and 104 kDa subunits.</td>
<td>Auttarat et al. 2006 Phiriyangkul and Utarabhand 2006 Phiriyangkul et al. 2007</td>
</tr>
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Table 2. (continued).

<table>
<thead>
<tr>
<th>Species (Prawns and shrimp)</th>
<th>Vitellogenin/vitellin (kDa)</th>
<th>cDNA length (bp); (amino acid length)</th>
<th>N-terminal amino acid sequence</th>
<th>Processing mechanisms; gene structure; other information</th>
<th>References (Authors and year)</th>
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<tr>
<td><em>Litopenaeus vannamei</em> ND</td>
<td>A: 78, B: 179, C: 113, D: 61, E: 42 (Vn) *</td>
<td>7970 bp (2587 aa)</td>
<td>A: Postulated to be APWGAD B, C, D, E: Postulated to be SIDASV</td>
<td>Mature protein (257 kDa) cleaved to 78 kDa and 179 kDa subunits (conserved site as in <em>Mar. rosenbergii</em>). 179 kDa subunit considered to be processed to 113, 61 and 42 kDa subunits.</td>
<td>Raviv et al. 2006</td>
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<tr>
<td><em>Marsupenaeus japonicus</em> 530</td>
<td>A: 91, B: 128, C: 186 (Vn)</td>
<td>7970 bp (2587 aa)</td>
<td>A: APWGADLPRCB, C: SIDSSVI</td>
<td>Mature protein (287 kDa) cleaved to 91 kDa and 186 kDa (from aa sequence predicted as 78 kDa and 207 kDa, respectively) subunits (conserved site as in <em>Mar. rosenbergii</em>). 187 kDa subunit likely processed to smaller subunits, harboring B and C sequences at N-termini.</td>
<td>Tsutsui et al. 2000; Kawazo et al. 2000; Tsutsui et al. 2002</td>
</tr>
<tr>
<td><em>Metapenaeus ensis</em> ND</td>
<td>A: 76, B: 35, other (Vg1)</td>
<td>7677 bp (2559 aa)</td>
<td>A: APYGESTECPB: similar to above</td>
<td>76 kDa subunit is likely secreted into hemolymph after being processed into smaller subunits, and is then taken into the ovary. Multiple genes have been found.</td>
<td>Tia et al. 2006a; Kung et al. 2004; Tsang et al. 2003</td>
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<tr>
<td><em>Infraorder Caridea</em> 1700</td>
<td>A: 90, B: 102, C/D: 90 (Vn)</td>
<td>7800 bp (2537 aa)</td>
<td>A: APWPSGTNLCB: similar to above</td>
<td>A-B-C (284 kDa, predicted) produced at hepatopancreas, then cleaved to A (90 kDa), B-C (199 kDa). A, B-C secreted into hemolymph, processed to A, B (102 kDa), C (90 kDa). Then, oocytes take up A, B, C. Note: C and D are identical; see text.</td>
<td>Derelle et al. 1986; Wilder et al. 1994; Lee et al. 1997; Okano et al. 2002</td>
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<tr>
<td>Species (Prawns and shrimp)</td>
<td>Vitellogenin/vitellin (kDa)</td>
<td>cDNA length (bp); amino acid length</td>
<td>N-terminal amino acid sequence</td>
<td>Processing mechanisms; gene structure; other information</td>
<td>References (Authors and year)</td>
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<tr>
<td><em>Macrobrachium borellii</em></td>
<td>440 A: 94, B: 112 (Vn) ND</td>
<td>N-terminal sequences ND, but peptide mass fingerprinting of 94 kDa subunit revealed 42 peptide fragments identical with the N-terminal region of <em>Mar. rosenbergii</em> Vg (VnA region)</td>
<td>Processing mechanisms not elucidated, but vitellin shows quasi-spherical morphology under electron microscopy.</td>
<td>Garcia et al. 2006</td>
<td></td>
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<tr>
<td><em>Pandalus hypsinotus</em></td>
<td>ND A: 85, B: 100, C/D: 85 (Vn)</td>
<td>7827 bp (2534 aa)</td>
<td>A: APWPSNLPRC B: SIDFSLSLH C/D: AQYTRNEQRI</td>
<td>A-B-C (282 kDa, predicted) produced at hepatopancreas, then cleaved to A (85 kDa), B-C (190 kDa). A, B-C secreted into hemolymph. Taken into oocytes as A (85 kDa), B-C (190 kDa), C (85 kDa), but site of B-C cleavage unclear (in hemolymph as in <em>Mar. rosenbergii</em>, or ovary as in <em>Pe. semisulcatus</em>).</td>
<td>Tsutsui et al. 2004</td>
</tr>
<tr>
<td><em>Infraorder Anomura</em></td>
<td>ND A: 82, B: 115, C: 100 (Vn)</td>
<td>7799 bp (2568 aa)</td>
<td>A: VPVWPEAPLC B: SIDFDLRLS C: VVQYISTQGS</td>
<td>A-B-C (289 kDa) produced at hepatopancreas, then cleaved to A (82 kDa), B-C (210 kDa). A, B-C secreted into hemolymph, processed to A, B (115 kDa), C (100 kDa). Oocytes then take up A, B, C (same as <em>Mar. rosenbergii</em>).</td>
<td>Kang et al. 2008</td>
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</tbody>
</table>
have been obtained for the crayfish, *Che. quadricarinatus* (Abdu *et al.* 2002), the lobster, *Homarus americanus* (Tiu *et al.* 2000), the red crab, *Charybdis feriatus* (Chan *et al.* 2005; Mak *et al.* 2005), the blue crab, *Callinectes sapidus* (Zanora *et al.* 2007), and the marine crab, *Portunus trituberculatus* (Yang *et al.* 2005). All show overall high identity, similar N-terminal amino acid sequences in their subunits, and potentially similar processing mechanisms compared with the shrimp and prawn species.

Interestingly, crustacean vitellogenins do not show much similarity to vertebrate vitellogenin in the kuruma prawn, less than 20% identity to sturgeon and killifish vitellogenin and 23% identity to tobacco hornworm apolipophorin were observed in the N-terminal region (Tsutsui *et al.* 2000), and similar results were obtained for *Pan. hypsinotus* (Tsutsui *et al.* 2004). Most surprising is that very little identity exists between crustacean and insect vitellogenins (Sappington et al. 2002; Wilder *et al.* 2002), and of note, decapod vitellogenins differ yet from those thus identified in other crustacean groups such as isopods (Okuno *et al.* 2000). Crustacean vitellogenins (meaning decapod crustaceans) are characterized by the absence of polyserine domains that feature in many of the known invertebrate and vertebrate vitellogenins. In such species, processing sites targeted by enzymes of the subtilisin family are flanked by a series of serine residues (Barr 1991; Sappington and Raikhel 1998). This is thought to create a beta-pleat arrangement thus exposing the processing site in a conformation that can be more easily cut by the enzyme (Chen *et al.* 1997). Nevertheless, decapod vitellogenins do contain 2–3 consensus cleavage sites, e.g., R-X-K/R-R, with actual cleavage occurring at the first of these sites. This feature is generally conserved throughout decapoda; for *Mac. rosenbergii*, the first cleavage site occurs from Arg707–Arg710, with cleavage occurring between amino acid residues 710 and 711 (Okuno *et al.* 2002). This is exactly the same in several other species, for example, *Macrobrachium* (*Tsutsui et al.* 2002) and *Pe. semisulcatus* (Avare et al. 2003), while other species exhibit slight variation in precise location of the cleavage site. A conceptual scheme for processing of vitellogenin in *Mac. rosenbergii*, along with actual SDS-PAGE data that served as a basis for this elucidation, are shown in Figs. 2A, B, reproduced from Okuno *et al.* (2002). That for other species is not described in detail in the text, but can be found summarized in Table 2. Furthermore, several studies have examined the secondary structure of native vitellogenin, and have published data on beta-pleat and alpha-helix composition using circular dichroism (Garcia *et al.* 2006). However, the focus on such studies related more to how such structure may relate to uptake at the oocytes by the vitellogenin receptor rather than to processing mechanisms.

### 2-2. Biochemical modifications of vitellogenin and vitellin and secondary structure

Crustacean vitellin was first characterized as a lipoprotein by Wallace *et al.* (1967). It is now known that various modifications occur to vitellogenin and vitellin (especially vitellin), where the molecules may be conjugated to lipids, carbohydrates, carotenoids, hormones, and metals. This area was reviewed extensively by Wilder *et al.* (2002), and since then, much work in this field has focused more on the elucidation of gene structure and amino acid differences in various species of crustaceans and other invertebrates. Differences in structure can be found related to uptake of lipids at the oocytes and their association with proteins. A conceptual scheme for processing of vitellogenin at its synthetic site. One of the first studies on the glycosylation of vitellogenin was compiled by Wallace *et al.* (1967) examined mature ovaries and eggs in 6 decapod families, e.g., the crabs *Pagurus, Sarsara, Uca, Libinia* and *Cancer*, and the lobster *Homarus*, finding that that yolk proteins contained approximately 30% lipid and were of molecular weights of around 350 kDa. More recently, Tirumalai and Subramoniam (1992) analyzed phospholipid content of vitellin in the crab *Emerita asiatica*, with phosphatidyl choline and phosphatidyl serine being the major species present. Lubzens *et al.* (1997) analyzed lipid content in detail in *Pe. semisulcatus*, finding that lipid profiles differed somewhat between vitellogenin and vitellin. Vitellin contained considerable amounts of triacylglycerols and negligible amounts of diacylglycerols, differing from vitellogenin in this respect. The physiological significance of lipid conjugation is still unclear, but this may be a means of providing lipids to the embryo via the ovary. The manner of how lipids physically associate with vitellogenin and vitellin also remains unclarified.

Carotenoids are another type of modification found in crustacean vitellogenin and vitellin. Early studies showed that they are either directly linked to protein chains through amino acid residues or are esterified to the fatty acids of the lipovitellin molecules (Cheesman *et al.* 1967). In terms of function, carotenoids linked to vitellin molecules may protect developing ovaries and embryos from visible wave-lengths of light or over-radiation (Hirston 1976, 1979; Sagi *et al.* 1995). Late vitellogenic ovaries in matur- ing crayfish *Che. quadricarinatus* are known to accumu-

late large quantities of beta-carotene (Sagi *et al.* 1995). The main roles of this type of modification may be to protect the ovary from radiation, and provide a source of pigmentation during subsequent embryonic development.

Regarding carbohydrates, glycosylation is considered to be an early step in the post-translational modification of vitellogenin at its synthetic site. One of the first studies on the glycosylation of vitellogenin was con-

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ducted by Tirumalai and Subramoniam (1992). These authors found that protein-bound carbohydrates of *Emerita* lipovitellin consisted mostly of hexosamines and hexoses (5 O-linked oligosaccharides and 4 N-linked oligosaccharides), but not many similar studies had been done by other groups. However, Khalaila *et al.* (2004) performed the first extensive study on the structure of carbohydrate moieties in vitellogenin using the crayfish, *Che. quadricarinatus*.

Here, the authors had previously elucidated the full cDNA structure of *Che. quadricarinatus* (Abdu *et al.* 2002), but there were not many studies on species with high sequence identity to other decapod species as mentioned above. Based on this information, it was found that there were 10 putative glycosylation sites. Next, the authors used lectin immunoblotting, in-gel deglycosylation, and mass spectrometry to identify actual sites and determine the structure of glycan moieties. They found that there were 10 putative glycosylation sites, at Asn152, Asn160, and Asn2,491, which were glycosylated with high-mannose glycans identified as Man3GlcNAc2 (Man = mannose; Glc = glucose; GlcNAc = N-acetylglucosamine) species. Glycosylation of vitellogenin and vitellin may be important in the folding, processing, and transport of the molecules into the developing oocytes, and the conjugating moieties may also serve as a source of carbohydrates during ensuing embryogenesis (Khalaila *et al.* 2004). Roth *et al.* (2010) performed a similar study using *Mac. rosenbergii*. It was found that glycosylation of vitellogenin occurs at 6 N-glycosylation sites: Asn151, Asn159, Asn168, Asn614, Asn660 and Asn2,300. As with *Che. quadricarinatus*, the glycan moieties were also hexose type sugars rich in mannose; of note, an unusual mannose N-linked oligosaccharide with a glucose cap (Glc3Man3GlcNAc2) was contained.

Roth *et al.* (2010) also conducted a bioinformatics analysis of putative O- and N-glycosylation sites in 16 decapod species in which full vitellogenin amino acid sequence is known. Interestingly, Pleocyemata sub-order species, which are egg-releasers, were lacking in N-glycosylation sites. These authors postulate that these differences in glycosylation sites and resultant structure of vitellogenin may have implications in actual reproductive strategy. Perhaps differences in structure may have implications in how eggs are physically formed and extruded—for example, in penaeid species, cortical rod proteins present in mature oocytes form the jelly layer that occurs after egg extrusion (see also below, Sub-section 3-3B).

As for other forms of conjugation, in the crab, *Em. asiatica*, oocytes are found to be conjugated to vitellin (Subramoniam *et al.* 1999). In this way, vitellin uptake may also be a means of transporting ecdy steroids to the ovary in crustaceans; ecdy steroids are known to be accumulated in developing ovaries in both crustaceans and insects, and are thought to be utilized during early embryogenesis before the developing embryo is capable of synthesizing its own (Wilder *et al.* 1990, 1991). Metal binding to vitellogenin is less-studied, but analysis of the major lipovitellin in *Em. asiatica* revealed that copper and calcium are bound to the lipid component of the vitellin molecule, whereas iron, sodium, and phosphorus are bound directly to the protein component of lipovitellin (Tirumalai 1996). The physiological roles of metal ions bound to vitellogenin remain unclarified, but more recently, Abdu *et al.* (2002) found that vitellogenin in the crayfish *Che. quadricarinatus* has calcium-binding properties (in the amino acid residue 2,132–2,584 range), and the authors postulate that this property may be related to the transportation of calcium to the oocyte for purposes of oocyte maturation, fertilization, and embryonic development.

Finally, Garcia *et al.* (2006) have performed structural characterization of lipovitellin in *Mac. borelli* using electron microscopy, Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry, circular dichroism, and other techniques. As discussed above, most decapod crustacean vitel lins consist of subunits of around 90 kDa and 100 kDa, and it seems that in the native form, there are two of each included in the overall molecule. However, these authors found that vitellin is a hetero-trimer, comprised of two subunits of 94 kDa, and one subunit of 112 kDa (however, this does not seem to account for the observed native molecular weight of 440 kDa). Using circular dichroism, it was estimated that *Mac. borelli* vitellin contains 37.5% alpha-helix, 16.6% beta-sheets and 20% turns (Garcia *et al.* 2006). Also interesting were the electron microscopy results, showing that vitellin formed particles of quasi-spherical morphology. Regarding secondary structure, there have been very few other studies on crustaceans. In *Li. vannamei* vitellin, results were somewhat different, with 25% alpha-helix, 37% beta-sheets and 14% turns (Garcia-Orozco *et al.* 2002). Structural features such as these may have significance in conferring properties relating to its transport in the hemolymph and/or interactions with the vitellogenin receptor, but more information is required to provide insight into these areas.

3. Vitellogenin synthesis and ovarian development

3-1. Determination of vitellogenin synthetic sites

3-1A. Earlier studies targeting vitellin or vitellogenin polypeptides

There have been many attempts to explore the sites of vitellogenin synthesis in various crustacean species.
Ovarian protein, either vitellin or its precursor vitellogenin, was considered to be an important molecular marker until the late 1990s; hence, immunoprecipitation, electrophoresis, Western blotting, ELISA, immunohistochemistry, and tissue incubation were the main techniques used in this regard. The combination of tissue incubation with radiolabeled amino acids and immunoprecipitation using anti-vitellogenin serum was used mostly to define the origin of vitellogenin synthesis. Vitellogenin was detected in the fat body (adipocytes) of ovarioctomized females of the isopod 

\textit{Idotea balthica} (Yano and Chinzei 1987), 

\textit{Jasminia emarginata} (Paulus and Laufer 1987), 

\textit{Palaemon serratus} (Zmora et al. 1986), 

\textit{Palaemonetes semisulcatus} (Avarre et al. 2004), 

\textit{Li. vannamei} (Raviv et al. 2006), 

\textit{Ca. sapidus} (Mak et al. 2005), 

\textit{Pan. hypsinotus} (Tsutsui et al. 2000), and 

\textit{Scylla serrata} (Zmora et al. 1986). Whether the main source is intracellular or extracellular tissue (or both) has been a controversial subject.

3.1B. Confirmation of vitellogenin synthetic site based on vitellogenin gene expression studies

As described in Subsection 2-1, full-length cDNAs encoding vitellogenin precursor have been cloned in several crustacean species since 2000, allowing the sites of vitellogenin synthesis to be clarified. The contributions of the hepatopancreas and ovary were shown in 

\textit{Mar. japonicus} (Tsutsui et al. 2000), 

\textit{Pe. semisulcatus} (Avarre et al. 2003), 

\textit{Li. vannamei} (Raviv et al. 2006), 

\textit{Pe. monodon} (Tiu et al. 2006b), 

\textit{Fe. merguiensis} (Phiriyangkul et al. 2007), and 

\textit{P. monodon} (Yano et al. 2003b). The mRNA was also presumed to exist in 

\textit{P. monodon} (Tsutsui et al. 2005b) and 

\textit{Pe. semisulcatus} (Avarre et al. 2003), but it is likely that multiple genes exist in these species. In fact, two vitellogenin genes, \textit{MeVg1} and \textit{MeVg2}, were cloned in the sand shrimp \textit{Metapenaeus ensis}; \textit{MeVg1} was expressed in both organs, whereas \textit{MeVg2} was expressed only in the hepatopancreas (Tsang et al. 2003; 

Kung et al. 2004); and multiple vitellogenin genes are also presumed to exist in 

\textit{Fe. merguiensis} (Phiriyangkul et al. 2007). Detailed localization of vitellogenin mRNA was examined by \textit{in situ} hybridization in 

\textit{Mar. japonicus} (Tsutsui et al. 2000); the mRNA was accumulated in the parenchymal cells of the hepatopancreas and in the follicle cells of the ovary (Fig. 4). Vitellogenin mRNA signals were detected in the whole of the hepatopancreas; cells specific to vitellogenin synthesis, such as the "vitellogenocytes" of Paulus and Laufer (1987), could not be discerned. In contrast, \textit{in situ} hybridization analysis of \textit{Mac. rosenbergii} suggested that the R-cells of the hepatopancreas were responsible for vitellogenin synthesis (Jasmani et al. 2004).

It is highly likely that the hepatopancreas and ovary are responsible for vitellogenin synthesis in penaeid species, and that the hepatopancreas is the main site of vitellogenin synthesis in species of the Caridea, Astacidea, and Portunoidea infraorders. Full-length cDNAs encoding vitellogenin were cloned and tissue-specific expression of vitellogenin genes was examined by Northern blot or RT-PCR analyses in the giant freshwater prawn \textit{Mac. rosenbergii} (Yang et al. 2000), crayfish \textit{Che. quadricarinatus} (Abdu et al. 2002), blue crab \textit{Callinectes sapidus} (Zmora et al. 2007), American lobster \textit{H. americana} (Tiu et al. 2009). These gene expression analyses confirm that in decapod crustaceans, vitellogenin is synthesized in both the hepatopancreas and ovary in species of the Penaeidae, and principally in the hepatopancreas in species of the Caridea, Astacidea, and Portunoidea infraorders (Fig. 7).
The contribution of the subepidermal adipose tissue, as examined in the above manner, has not been reported thus far.

3-2. Dynamics of vitellogenin synthesis

Isolation of vitellogenin cDNA has allowed the examination of vitellogenin gene expression levels at different vitellogenic stages, the relationship between vitellogenin gene expression levels and hemolymph vitellogenin protein levels, and how the presumed multiple sites of synthesis contribute to overall vitellogenin synthesis during vitellogenesis. Northern blot and quantitative RT-PCR analyses revealed simultaneous increases of vitellogenin transcript levels in the hepatopancreas and ovary during vitellogenesis in *Marsupenaeus japonicus* (Tsutsui et al. 2000, 2005b). Levels in the hepatopancreas were low at the previtellogenic stage, increased during the endogenous vitellogenic stage, and remained high during the exogenous vitellogenic stage. Those in the ovary were also low at the previtellogenic stage and increased during the endogenous vitellogenic stage and the early exogenous vitellogenic stage, yet they decreased significantly during the late exogenous vitellogenic stage. Viewed in the whole organ, the total amounts of vitellogenin mRNA were similar between the hepatopancreas and ovary at each stage, and there was no significant dif-

![Fig. 4. Localization of vitellogenin mRNA in the hepatopancreas and ovary by in situ hybridization in *Marsupenaeus japonicus*. Vitellogenin gene expression was observed in (A) follicle cells of the ovary and (C) parenchymal cells of the hepatopancreas. Hybridization with a sense probe resulted in no significant signal in (B) ovary or (D) hepatopancreas. Bars = 20 µm in A and B; 10 µm in C and D. Reprinted with permission from Zoological Science, 17, Tsutsui et al., Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*, 651–660, Fig. 6, © 2000, Zoological Society of Japan.](image-url)
ference in ovarian vitellogenin mRNA levels between the early and late exogenous vitellogenic stages. The overall gene expression profile showed a good relationship with hemolymph vitellogenin levels (Jasmani et al. 2000). Similarly high vitellogenin gene expression patterns in both tissues during yolk accumulation have been reported in *P. semisulcatus* (Avarre et al. 2003) and *P. monodon* (Tiu et al. 2006b), although expression was higher in the ovary than in the hepatopancreas in *L. vannamei* (Raviv et al. 2006), *F. merguensis* (Phiriyangkul et al. 2007), and *M. ensis* (for *MeVg1*; Tiu et al. 2006a). Vitellogenin gene expression patterns in non-penaeid species, in which the hepatopancreas is the site of vitellogenin synthesis, were also correlated with vitellogenic stage (Mak et al. 2005; Zmora et al. 2007). In *M. rosenbergii*, changes in vitellogenin mRNA levels, gonadosomatic index (GSI), and hemolymph vitellogenin levels were examined during the reproductive molt cycle (Jayasaker et al. 2002). In intact animals, vitellogenin gene expression levels in the hepatopancreas and vitellogenin protein levels in the hemolymph showed a gradual increase during the reproductive molt cycle concomitant with increasing GSI (Figs. 5A, B). Eyestalk ablation shortened the cycle, accelerating vitellogenin gene expression and ovarian maturation, although it did not alter the overall pattern of vitellogenin mRNA expression (Figs. 5C, D). In addition, hemolymph vitellogenin levels and maximum GSI were higher in eyestalk-ablated animals than in intact animals, suggesting that eyestalk factors partially regulate vitellogenin translation, release from the hepatopancreas, and incorporation into the oocyte.

The relationship between vitellogenin gene expres-
sion and programmed sex change, which is presumed to be regulated by androgenic gland hormone (AGH), has also been studied. *Pa. hypsinotus* first matures as a functional male, and then becomes a functional female. Vitellogenin mRNA increased concomitantly with ovarian development in the hepatopancreas of vitellogenic females, whereas it was not detected either in the hepatopancreas or gonads of immature females having GSI lower than 1.0 and those of males (Tsutsui et al. 2004; Fig. 6). In *Che. quadricarinatus*, ablation of the androgenic gland induced vitellogenin gene expression in the hepatopancreas of sexually plastic intersex animals that were functional males, suggesting negative regulation by the androgenic gland on vitellogenin transcription (Abdu et al. 2002). Moreover, in the mud shrimp *Upogebia major*, vitellogenin transcripts were detected by RT-PCR in the male hepatopancreas and the ovarian part of the testis, as well as in the female hepatopancreas and ovary (Kang et al. 2008). Further studies are needed to clarify the relationship between regulatory systems for sex determination and those for vitellogenesis.

3-3. Modes of ovarian maturation

3-3A. Microscopic studies of ovarian development in representative prawn species

In crustaceans, vitellogenin is accumulated in oocytes as a major yolk protein, vitellin, during vitellogenesis. Yolk accumulation causes a rapid increase in oocyte diameter and size. This process has been histologically investigated in several crustacean species (for reviews, see Charniaux-Cotton and Payen 1988; Meusy and Payen 1988). One of the authors of this monograph has also studied ovarian development in relation to reproductive season and molt cycle in representative prawn species (Okumura et al. 1992, 2004, 2005, 2007; Okumura and Aida 2000).

Histological changes in oocytes during ovarian development in *Mac. rosenbergii* are shown in Fig. 7 (Okumura and Aida 2000). At the center of the ovary, oogonia commence meiotic division I and become oocytes. While the oocytes remain arrested in prophase I, they accumulate RNA at the previtellogenic stage, oil globules and PAS (periodic acid-Schiff)-positive vesicles at the endogenous vitellogenic stage, and eosin-positive yolk globules at the exogenous vitellogenic stage (Figs. 7A–C, E, F). The endogenous and exogenous vitellogenic oocytes are enveloped by follicle cells (Figs. 7A–C). The cytoplasm of the exogenous vitellogenic oocytes is stained with anti-vitellin antibody (Fig. 7G), indicating that the yolk globules contain vitellin. Vitellogenic ovaries are almost completely filled with synchronously developing oocytes at the exogenous vitellogenic stage. These oocytes are spawned at the next oviposition, and oogonia and oocytes at the previtellogenic and endogenous vitellogenic stages remain in the ovary (Figs. 7A, D). Ovaries similarly develop in *Macrobrachium nipponense* (Okumura et al. 1992) and *Pan. hypsinotus* (Okumura et al. 2004).

In electron microscopic observations of oogenesis in *Pan. hypsinotus* (Okumura et al. 2004), the cytoplasm of the previtellogenic oocytes contains mitochondria (Fig. 8A), and the cytoplasm of the endogenous vitellogenic oocytes is filled with rough endo-

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Fig. 6. Northern blot analysis of *Pandalus hypsinotus* vitellogenin mRNA. (A) Hepatopancreas in female prawns. (B) Gonads in female prawns. (C) Hepatopancreas and gonads in male prawns. The number at the top of each lane indicates the GSI of each prawn. PC = positive control (Total RNA from female hepatopancreas: GSI = 3.1). The exposure time for B and C was three times longer than that of membrane A in order to detect weak signals. Results of ethidium bromide staining of gels before blotting show equal loading of RNAs. Reprinted with permission of John Wiley & Sons, Inc. from *Journal of Experimental Zoology Part A*, 301A, Tsutsui et al., Molecular characterization of a cDNA encoding vitellogenin in the coonstriped shrimp, *Pandalus hypsinotus* and site of vitellogenin mRNA expression, 802–814, Fig. 6, © 2004, Wiley-Liss, Inc., a Wiley Company.
Fig. 7. Cross-sections of *Macrobrachium rosenbergii* ovaries. (A) Periodic acid-Schiff (PAS) staining at the endogenous vitellogenic stage. (B) Hematoxylin and eosin (HE) staining at the early exogenous vitellogenic stage. (C) HE staining at the late exogenous vitellogenic stage. (D) PAS staining just after oviposition. (E) PAS staining at the endogenous vitellogenic stage. (F) OsO₄ postfixation at the endogenous vitellogenic stage. (G) Immunohistochemistry at the early exogenous vitellogenic stage using anti-vitellin antibody. AO, regressing atretic oocyte; EF, empty follicle after ovulation; EN, endogenous vitellogenic oocyte; EX, exogenous vitellogenic oocyte; O, oogonium; OG, oil globule; PO, previtellogenic oocyte. Bars: 0.1 mm. Reprinted with permission from *Fisheries Science*, 66, Okumura and Aida, Hemolymph vitellogenin levels and ovarian development during the reproductive and non-reproductive molt cycles in the giant freshwater prawn *Macrobrachium rosenbergii*, 678–685, Fig. 2, © 2000, The Japanese Society of Fisheries Science.
plasmic reticulum, indicating active protein synthesis (Figs. 8B, C). The exogenous vitellogenic oocytes contain electron-dense yolk globules in addition to mitochondria, lipid droplets, and rough endoplasmic reticulum (Figs. 8D, G). Active endocytosis at the oocyte surface suggests that yolk globules are exogenously formed by the uptake of vitellogenin from hemolymph (Figs. 8E, F). The vitellin membrane is formed on the surface of the maturing oocytes (Fig. 8H).

In penaeid shrimps, ovarian development is characterized by the formation of cortical rods in the oocytes after the completion of yolk accumulation (Clark et al. 1990). As in Macrobrachium prawns and shrimp, oocyte development is classified into the previtellogenic, endogenous vitellogenic, and exogenous vitellogenic stages (Fig. 9). During the oocyte development, RNA, oil globules, PAS-positive vesicles, and yolk globules are accumulated in the ooplasm and oocyte diameter and size increase. After the completion of yolk accumulation (Fig. 10a), cortical rods are formed radially around the periphery of the oocyte plasma membrane (Fig. 10b). During their develop-
ment, the germinal vesicle begins to disintegrate at the center of the oocyte (germinal vesicle breakdown, GVBD; Fig. 10c) and subsequently migrates toward the oocyte surface. The oocytes that have cortical rods are spawned at oviposition, and oogonia and oocytes at the previtellogenic and endogenous vitellogenic stages remain in the ovary (Fig. 10d). Electron microscopy reveals that before cortical rod formation, small cortical vesicles appear in the ooplasm and fuse together to form larger vesicles (Hong 1977; Wallis et al. 1990; Carvalho et al. 1999). The fused vesicles then migrate toward the oocyte surface and fuse with the oocyte plasma membrane. The vesicles grow by continuous fusion and form a rod-like shape. The cortical rods thus develop outside the oocyte plasma membrane but under the vitellin membrane. Their content gives the appearance of tightly packed feathery elements under electron microscopic observation (Hong 1977; Wallis et al. 1990; Carvalho et al. 1999).

In species that brood an egg mass on the abdomen, spawning and molting are coordinated to avoid loss of the egg mass by untimely ecdysis. Female *Mac. rosenbergii* mate and spawn just after ecdysis, and they complete the brooding of the egg mass during the subsequent intermolt period. While the eggs are being carried, the ovaries redevelop via yolk accumulation for the next oviposition. Gonadosomatic index (GSI) increases and hemolymph vitellogenin levels are high during the molt stages C1–D3 (Okumura and Aida 2000; Fig. 11).

Species that have a defined spawning season show seasonal changes in ovarian development. In *Mar. japonicus*, which spawns from April to October in the coastal areas of Japan, ovaries develop during March to May (Fig. 12), and hemolymph vitellogenin levels and vitellogenin mRNA levels in the ovary and hepatopancreas increase (Okumura et al. 2007). In the mid-regions of the Sea of Japan, *Pan. hysinotus* inhabits cold water and requires a long duration (8–14 months) for ovarian development. The hatched larvae are released in February–March, yolk accumulation starts during April–October, GSI and hemolymph vitellogenin levels increase (Fig. 13), and oviposition occurs in June–July of the next year (Okumura et al. 2004).
3-3B. Identification of cortical rod proteins in penaeid species, and role in reproduction

In penaeid shrimps, after the completion of yolk accumulation, cortical rods are formed radially around the periphery of the oocyte plasma membrane (Clark et al. 1990; Wallis et al. 1990). Two major proteins comprising the cortical rods have been purified and characterized: shrimp ovarian peritrophin (SOP, 29–35 and 33–36 kDa) in Penaeus semisulcatus (Khayat et al. 2001) or cortical rod protein (CRP, 28.6 and 30.5 kDa) in Mar. japonicus (Kim et al. 2004, 2005); and Mar. japonicus thrombospondin (MjTSP, 130, 140, and 150 kDa; Yamano et al. 2003, 2004). Except for their molecular weights, SOP, CRP, and MjTSP show similar molecular structure in terms of repeated cysteine-rich domains, and proteolytic sites for post-translational modulation and glycosylation, as well as similar deduced amino acid sequences. Yamano et al. (2004) suggested that they are the products of a single gene or are encoded by orthologous genes. Messenger RNAs for SOP, CRP, and MjTSP are expressed in the previtellogenic oocytes, and the respective proteins are synthesized and accumulated in the vitellogenic oocytes as yolk materials (Khayat et al. 2001; Yamano et al. 2003, 2004; Kim et al. 2004, 2005). The proteins are first scattered throughout the ooplasm, and then become localized in the cortical rods during cortical rod formation.

The endocrine mechanisms regulating the synthesis of SOP, CRP, and MjTSP have been studied in relation

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Fig. 11. Hemolymph vitellogenin levels and gonadosomatic index (GSI) in Macrobrachium rosenbergii. Data indicate the mean and SE. Reprinted with permission from Japan Agricultural Research Quarterly (JARQ), 38, Okumura, Perspectives on hormonal manipulation of shrimp reproduction, 49–54, Fig. 2, © 2004, Japan International Research Center for Agricultural Sciences.

Fig. 12. Changes in (a) ovarian developmental stage and (b) gonadosomatic index in pond-reared female Marsupenaeus japonicus from December to June. Bars in (b) indicate the mean and SD of 14 animals, and bars without common letters differ significantly (P < 0.05). Reprinted from Comparative Biochemistry and Physiology Part A, 147A, Okumura et al., Vitellogenin gene expression and hemolymph vitellogenin during vitellogenesis, final maturation, and ovi-position in female kuruma prawn, Marsupenaeus japonicus, 1028–1037, Copyright (2007), with permission from Elsevier.

Fig. 13. Seasonal changes in gonadosomatic index (GSI) and hemolymph vitellogenin levels in Pandalus hystrixotus. Points and bars indicate the mean and SD. Points without common letters differ significantly (P < 0.05). Reprinted with permission from Zoological Science, 21, Okumura et al., Ovarian development and hemolymph vitellogenin levels in laboratory-maintained protandric shrimp, Pandalus hystrixotus: measurement by a newly developed time-resolved fluoroimmunooassay (TR-FIA), 1037–1047, Fig. 10, © 2004, Zoological Society of Japan.
Fig. 14.
to crustacean hyperglycemic hormone-family (CHH-family) peptides. CHH-family peptides are major neuropeptides produced in the X-organ/sinus gland complex in the eyestalks. CHH, molt-inhibiting hormone (MIH), vitellogenesis-inhibiting hormone (VIIH), and mandibular organ-inhibiting hormone (MOIH) are included in this family due to their similarities in primary structure. CHH-family peptides are divided into two subtypes based on the absence (type-I) or presence (type-II) of a glycine residue at position 12 in the mature peptide. In *Mar. japonicus*, seven CHH-family peptides designated as Pej-SGP-I to -VII, were purified from the sinus glands (Nagasawa et al. 1999; Yang et al. 1995, 1996, 1997; Fig. 17). Those peptides inhibited de novo synthesis of SOP, but did not affect SOP mRNA levels in incubated ovarian fragments of *Pe. semisulcatus* (Avarre et al. 2001). One of the authors of this monograph determined that eyestalk ablation—removal of the source of CHH—increased CRP and MjTSP protein levels but did not affect their mRNA levels in the ovary of *Mar. japonicus* (Okumura et al. 2007). Taken together with the fact that SOP, CRP, and MjTSP genes are transcribed and translated at different oocyte stages, these results indicate that expression of these genes is controlled at the translational level by CHH-family peptides.

The content of the cortical rods is released around eggs and forms into a jelly layer, which envelopes the eggs on contact with sea water at spawning (Clark et al. 1990). The jelly layer starts dissipating around 45 minutes after spawning, and the hatching envelope forms on the egg surface. The jelly layer is considered to function as a barrier against polyspermy and as an environmental factor during embryogenesis (Sappington and Raikhel 1998), such studies in Crustacea remain limited. In the earliest work, Jugan and Soyez (1985) demonstrated the uptake of vitellin conjugated with colloidal gold by *Mac. rosenbergii* oocytes, while Laverdure and Soyez (1988) solubilized VgR from the oocyte membrane of *Ho. americanus*. In the latter, using an enzyme linked immunosorbent assay, it was shown that binding of vitellogenin with the solubilized receptors increased at the onset of vitellogenesis, but decreased in older oocytes. Jugan and Van Herp (1989) demonstrated that vitellogenin specifically binds to an oocyte membrane protein in the crayfish *Oncorhynchus limosus*.

In more recent work, Warrier and Subramoniam (2002) purified VgR in the mud crab, *Sc. serrata* by HPLC, revealing it to have a molecular weight of 230 kDa. These authors also employed direct binding studies using $[^{125}]$I-labelled vitellogenin, finding that the receptor possesses a high affinity for crab vitellogenin with a dissociation constant of Kd 0.8 × 10$^{-6}$ M. It was also seen that the *Sc. serrata* VgR binds mammalian low density lipoprotein (LDL); this suggested that the

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**Fig. 17.** VgR cloning strategy and sequence for *Marsupenaeus japonicus*. (A) Cloning strategy of *Mar. japonicus* ovarian LDLR cDNA. Solid black triangles indicate the primers designed based on the sequence of *Penaeus semisulcatus* putative ovarian lipoprotein receptor cDNA. Open triangles indicate the primers designed based on the sequence of *Mar. japonicus* ovarian LDLR. Arrows indicate the universal primers for 5’ and 3’-RACE. Meshwork shows the ligand binding domain (LDL-receptor class A); gray color shows the EGF precursor domain (LDL-receptor class B); The horizontal striped section shows an EGF-like domain; a capital T shows a transmembrane region. (B) Nucleotide sequence of ovarian LDLR cDNA from *Mar. japonicus* and its deduced amino acid sequence. This nucleotide sequence has been deposited in the DDBJ database (accession number AB304798). Shadowed areas indicate the ligand binding domain (LDL-receptor class A), thinly-outlined rectangles indicate the EGF precursor domain (LDL-receptor class B), the double underline indicates an EGF-like domain and the dotted underline indicates a transmembrane region. Three bold serial circles (SDE) indicate highly conserved residues in the LDL receptor family. Potentially related sequences of SDE are shown in thinly-outlined serial circles. Bold letters indicate YWTD repeats and its potentially related sequence. The single underlines indicate residues homologous to the internalization signal (NPXY). A pair of solid black arrowheads indicates the 5' upstream probe region, and open arrowheads indicate the 3' downstream probe region used for library screening. Thickly-outlined rectangles indicate the amino acid residues of the synthetic peptide sequence used for synthesizing ovarian LDLR antibody of *Mar. japonicus*. Reprinted with permission from *Zoological Science*, 25. Mekuchi et al., Characterization and expression of the putative ovarian lipoprotein receptor in the kuruma prawn, *Marsupenaeus japonicus*, 428–437, Figs. 1 and 2, © 2008, Zoological Society of Japan.
crab receptor belongs to the vertebrate low density lipoprotein receptor (LDLR) family, as do vitellogenin receptors in nematodes, insects, and vertebrates (Bujo et al. 1994; Sappington et al. 1996; Grant and Hirsh 1999). However, at this point in time, the VgR gene had not been cloned; hence sequence information of crustacean VgR could not be compared with that of other oviparous animals.

More recently, Tiu et al. (2008) carried out the molecular characterization of VgR in the tiger prawn, Penaeus monodon. This was the first cloning of a cDNA for this gene in Crustacea. It was found that the VgR cDNA was 6.8 kb in length, corresponding to a deduced protein of 1,943 amino acids and molecular weight of 211 kDa. In studies of levels of gene expression, these authors found that levels are very low in the ovary during early vitellogenesis, but increase to maximal levels in females having a GSI of 3–4. Of interest, Tiu et al. (2008) were also able to knock down VgR expression by injecting VgR double-stranded RNA, leading to decreased VgR protein content in the ovary, and higher vitellogenin content in the hemolymph, due to the decreased availability of receptor present to serve in the uptake of vitellogenin into the oocytes. Regarding sequence information, Pe. monodon VgR shared a certain degree of amino acid identity with other known invertebrate (insect) receptors, and exhibited two putative internalization signals (FANPGFG and FENPFF) (Tiu et al. 2008).

The authors of this monograph characterized a putative ovarian lipoprotein receptor in the kuruma prawn, Marsupenaeus japonicus, which was hypothesized to incorporate vitellogenin into developing oocytes (Mekuchi et al. 2008). A full-length cDNA of 3,598 bp was obtained for this molecule, referred to as LDLR, and the deduced amino acid sequence encoded 1,120 amino acid residues. This is a little over half of the size of Pe. monodon VgR discussed above; however, these two molecules have not been directly compared in terms of sequence identity. The Pe. monodon VgR sequence

Fig. 15. Molecular phylogenetic tree between ovarian LDLR of Marsupenaeus japonicus and those of other species. A molecular phylogenetic tree of VgR and/or ovarian LDLR was constructed using the Tree View program (version 1.6.6; Division of Environmental and Evolutional Biology, Institute of Biomedical and Life Science, University of Glasgow). The scale bar shows 1.0 amino acid substitutions per site. Reprinted with permission from Zoological Science, 25, Mekuchi et al., Characterization and expression of the putative ovarian lipoprotein receptor in the kuruma prawn, Marsupenaeus japonicus, 428–437, Fig. 3, © 2008, Zoological Society of Japan. (Note: throughout the main text and figure captions, scientific names are abbreviated with the first two letters, or where further specification is necessary, with the first three letters of the genus name. However, in this figure, for purposes of simplicity, all scientific names are indicated with the first letter of the genus name.)
containing characteristic domains of the LDL-receptor family, such as the ligand-binding domain, the epidermal growth factor (EGF) precursor domain, an EGF-like domain, and a transmembrane region. These structures are also found in *Mar. japonicus* and are shown in Figs. 14A, B together with a phylogenetic tree comparing LDLR in *Mar. japonicus* to those of other oviparous animals in Fig. 15. In examination of tissue-specific gene expression, it was seen that LDLR was expressed in the ovary, but not in gill, heart, intestine, muscle, or testis. Furthermore, it was seen that LDLR mRNA expression was highest in previtellogenic ovaries, decreasing in accordance with the progression of ovarian maturation (Fig. 16). As vitellogenin gene expression in the hepatopancreas/ovary generally increases with the progression of ovarian maturation (Jayasankar et al. 2002; Kim et al. 2005), these results suggested that the receptor is prepared in advance for incorporation of its ligand into the oocytes (Mekuchi et al. 2008). These results were also reflected by in situ hybridization carried out in the same study.

It remains that few studies have been carried out on shrimp vitellogenin/lipoprotein receptors; future studies using other crustacean species should contribute to enhancing knowledge of reproductive function in this regard. At the same time, the manner in which vitellogenin accesses the oocyte surface to be then taken in by pinocytosis has been extensively studied in insect. In a phenomenon called “patency”, a juvenile hormone (JH)-specific Na/K-ATPase exists in the follicle cells of the ovary, and its enzymatic activity is elevated in the presence of JH. In turn, the follicle cells based on the stoichiometry of the Na/K-ATPase exchange mechanism, become hypotonic to their surrounding medium, and subsequently “shrink”. This causes spaces between the follicle cells to enlarge, which allows vitellogenin to access the oocyte surface (Davey et al. 1993). This phenomenon, and related knowledge as it concerns Crustacea, is discussed in further detail in Subsection 4-1C below.

**4. Regulation of vitellogenin synthesis and uptake; utilization of vitellin**

**4-1. Endocrine control of vitellogenin synthesis**

4-1A. Eyestalk neurohormones and vitellogenesis inhibition

The existence of a vitellogenesis-or gonad-inhibiting factor originating in the eyestalks was shown by means of eyestalk ablation in *Pal. serratus* (Panouse 1943), and by sinus gland implantation into eyestalk-ablated *Uc. pagilator* (Brown and Jones 1948), over 50 years ago. The acceleration of vitellogenesis by eyestalk ablation was observed in a large number of crustaceans (Adiyodi and Adiyodi 1970). Since then, the factor became a target of investigation in many crustacean species. As indices of vitellogenesis these studies used GSI (Klej-Kawinska and Bomirski 1975), oocyte diameter (Soyez et al. 1987), frequency of reproductive molt (Grève et al. 1999), hemolymph vitellogenin protein levels (Vincent et al. 2001), and amounts of total protein or vitellogenin synthesized (Quackenbush and Keeley 1988; Quackenbush 1989; Aguilar et al. 2001), and in vivo assays have been used in preference to assays for hyperglycemic or molt-inhibiting activities. An in vivo bioassay with *Palaemonetes varians* to characterize *Ho. americanus* VIH (Soyez et al. 1991), and second in the terrestrial isopod *Armadillidium vulgare* (Grève et al. 1999; Fig. 17).

Knowledge of the VIH molecule was originally very limited because of difficulties in preparing a suitable bioassay for vitellogenesis-inhibiting activity. To overcome those difficulties, heterologous *in vivo* or *in vitro* assays have been used in preference to assays for hyperglycemic or molt-inhibiting activities. An in vivo bioassay with *Palaemonetes varians* to characterize *Ho. americanus* VIH (Soyez et al. 1987) confirmed that anti-lobster VIH serum cross-reacted with sinus gland extract of several crustacean species, including *Pal. varians* (Meusy et al. 1987). VIH of the Mexican crayfish *Procambarus bouvieri* was characterized by incubation of ovarian tissue of *Li. vannamei* (Aguilar et al. 1992) and partially sequenced (Huberman et al. 1995). Incubation experiments using *Pe. semisulcatus* ovary contents.
were used to investigate the vitellogenesis-inhibiting activity of sinus gland peptides in terms of the synthesis of protein or vitellogenin. Six CHHs of *M. japonicus* (type-I peptides: Pej-SGP-I, -II, -III, -V, -VI, and -VII; Fig. 17) strongly inhibited protein synthesis including that of vitellogenin. Pej-SGP-IV, a type-II peptide with molt-inhibiting activity also had an inhibitory effect, but its efficacy was weaker than those of type-I peptides (Khayat et al. 1998).

Similar results were obtained in an assay of CHH-family peptides from the South African spiny lobster *Jasus lalandii*: two type-I peptides (CHH-I and -II) inhibited protein synthesis, but MIH did not. Additionally, C-terminally shortened peptides of CHH-I and -II did not inhibit synthesis (Marco et al. 2002). Dose-response analysis of sinus gland extracts of *Mar. japonicus* and *Penaeus* species suggested that homologous sinus gland extracts were more potent than heterologous ones (Khayat et al. 1998). Similarly, sinus gland extract from *Procambarus clarkii* reduced protein synthesis in *Pr. clarkii* ovaries, but that from *Penaues* species did not (Chaves 2000).

Accumulated information on vitellogenin gene expression has allowed the effects of vitellogenesis-regulating factors to be observed more precisely. In *Mar. japonicus*, the hepatopancreas and ovary are responsible for vitellogenin synthesis, and vitellogenin mRNA levels in those tissues and hemolymph vitellogenin protein levels increase significantly during vitellogenesis (Jasmani et al. 2000; Tsutsui et al. 2000). Therefore, ovarian tissue incubation is often used to search for factors affecting vitellogenin gene expression, which could be VIH or vitellogenesis-stimulating hormone (VSH). Vitellogenin mRNA lev-
els in ovarian fragments spontaneously increased during the incubation period in this assay system (Tsutsui et al. 2005a; Fig. 18A), and homologous sinus gland extract reduced expression to 20–45% of control levels at concentrations of 0.001 to 10 sinus gland equivalents/mL (Fig. 18B). Pej-SGP-III significantly inhibited vitellogenin expression in a dose-dependent manner, whereas recombinant Pej-SGP-IV, as well as Pej-MIH-B for which transcript is much more abundant in the thoracic and abdominal ganglia than in the eyestalks, reduced vitellogenin expression at 100 nM. However, those effects were not significant (Tsutsui et al. 2005a; Fig. 19). The other five type-I peptides of Mar. japonicus also inhibited vitellogenin expression with nearly the same efficacy as Pej-SGP-III (Tsutsui, unpublished data), but no peptide inhibited GAPDH expression. Together, these results show that vitellogenin expression is suppressed by type-I CHH-family peptides in previtellogenic ovaries and starts to increase when liberated from inhibition in Mar. japonicus.

The authors of this monograph also used this incubation assay to examine the effects of seven CHH-family peptides purified from sinus glands of Li. vannamei on vitellogenin expression (Tsutsui et al. 2007). Type-I peptides (Liv-SGP-A, -B, -C [Pev20 of Wang et al. 2000], -E [Pev27], -F [Pev28], and -G) inhibited vitellogenin expression, although with different efficacies, whereas type-II Pev-SGP-D (recently designated as Liv-MIH-I; Chen et al. 2007) did not (Fig. 20). Interestingly, the inhibitory activity of SGP-E, a C-terminally truncated form of SGP-G, was lower than that of SGP-G by approximately two orders of magnitude. These results suggest that the molecular characteristics required for vitellogenesis-inhibiting activity in penaeid shrimp species are present in type I

Fig. 19. Effects of Pej-SGP-III, -SGP-IV, and -MIH-B on vitellogenin (VG) mRNA levels in Marsupenaeus japonicus. Levels are expressed as percentage changes relative to control values (mean ± SEM; n = 4–5). *P < 0.05; **P < 0.01. Modified from General and Comparative Endocrinology, 144, Tsutsui et al., The effects of crustacean hyperglycemic hormone family peptides on vitellogenin gene expression in the kuruma prawn, Marsupenaeus japonicus, 232–239, Copyright (2005), with permission from Elsevier.

Fig. 20. Effects of CHH-family peptides from Litopenaeus vannamei on vitellogenin (VG) mRNA levels in Marsupenaeus japonicus ovary. Levels are expressed as averages of percentage changes relative to each control group (n = 4–5). Modified with kind permission from Springer Science + Business Media: Marine Biotechnology, Purification of sinus gland peptides having vitellogenesis-inhibiting activity from the whiteleg shrimp Litopenaeus vannamei, 9, 2007, 360–369, Tsutsui et al., Figs. 4B, C, D, E, F, G and H, © 2007, Springer-Verlag.
peptides having a carboxamide C-terminal moiety.

_Homo americanus_ VIH (Hoa-VIH; Fig. 17) has an amidated C-terminus (Soyez 1997). The biological significance of this amide moiety was examined by the assay system employed above (Ohira et al. 2006). A recombinant Hoa-VIH with a free C-terminus (rHoa-VIH-OH), showed no effect on vitellogenin gene expression at 4 to 400 nM, whereas a carboxy-terminal-amidated rHoa-VIH (rHoa-VIH-amide) significantly inhibited vitellogenin mRNA levels at 400 nM (50.9%), compared with the control (Fig. 21), suggesting the importance of C-terminal amidation in Hoa-VIH. Its efficacy was definite, but much lower than those of sinus gland extracts and Pej-SGP-III from _Mesta japonicus_ (VTG) mRNA levels in previtellogenic and vitellogenic ovaries. Relative expression level of VTG (% control VTG/GAPDH) indicate significant difference from control (P < 0.05). Reprinted from General and Comparative Endocrinology, 148, Okumura, Effects of cyclic nucleotides, calcium ionophore, and phorbol ester on vitellogenin mRNA levels in incubated ovarian fragments of the kuruma prawn _Marsupenaeus japonicus_, 245–251, Copyright (2006), with permission from Elsevier.

Recently, the gene silencing effect of double-stranded RNA (dsRNA) has been utilized to clarify the biological functions of various genes, including those encoding crustacean neuropeptides. In _Me. ensis_, this technique showed the gonad stimulatory effects of MeMIH-B. MeMIH-B is expressed in nervous tissues, and its expression pattern in the eyestalk was correlated with the reproductive cycle (Gu et al. 2002). Injection of recombinant MeMIH-B increased vitellogenin gene expression in the hepatopancreas and ovary, and vitellogenin protein in hemolymph and ovary; injection of MeMIH-B-dsRNA decreased them (Tiu and Chan 2007). In _Penaeus monodon_, a cDNA encoding PenGIH was cloned, and the administration of GIH-dsRNA increased vitellogenin gene transcript levels (Treerattrakool et al. 2008).

4-1B. Mode of action of VIH on the ovary: experiments using secondary messengers in ovarian incubation to examine vitellogenin gene expression

Vitellogenin synthesis in the ovary is directly inhibited by vitellogenesis-inhibiting hormone (VIH) (Tsutsui et al. 2005a). Because actions of peptide hormones are mediated by intracellular signaling pathways, the actions of VIH are expected to be mediated via secondary messengers in the ovary. Thus, Okumura (2006) determined whether cyclic nucleotides, Ca²⁺, and protein kinase C are involved in the regulation of vitellogenin mRNA levels in the ovary of _Mar. japonicus_.

Ovarian fragments of _Mar. japonicus_ were incubated...
with pharmacological agents, and vitellogenin mRNA levels in the fragments were determined after 24 hours by quantitative RT-PCR. A23187 (calcium ionophore), dibutyl-cAMP (cAMP analogue), dibutyl-cGMP (cGMP analogue), forskolin (adenylate cyclase activator), 3-isobutyl-1-methylxanthine (IBMX, phosphodiesterase inhibitor), and phorbol 12-myristate 13-acetate (PMA, protein kinase C activator) significantly decreased vitellogenin mRNA levels in a dose-dependent manner (Fig. 22). Addition of IBMX in combination with cyclic nucleotides dramatically reduced vitellogenin mRNA levels. These results suggest that cyclic nucleotides, Ca2+, and protein kinase C are involved in the signaling pathways that regulate vitellogenin mRNA levels in the ovaries (Figs. 23, 24).

The signaling pathways of peptide hormones involved in ecdysteroid synthesis in Y-organs have been intensively investigated (for reviews, see Mattson 1986; Spaziani et al. 2001). By analogy with the cellular signaling in Y-organ cells, cAMP and cGMP in the ovarian follicle cells probably mediate the action of VIH, and Ca2+ and protein kinase C probably inhibit vitellogenin synthesis independently of VIH. Further tests on signal transduction in vitellogenin synthesis using pharmacological inhibitors (e.g., protein kinase A or C inhibitors, phosphatidylinositol 3-kinase inhibitors) will clarify the specific pathways.

4.1C. Role of vitellogenesis-promoting hormones: putative vitellogenesis-stimulating hormone, biogenic amines, and juvencoids

While the existence of inhibitory hormones in Crustacea has been conclusively demonstrated and their characteristics elucidated, an understanding of positive mechanisms controlling crustacean reproduction remains elusive. There is a history of several decades of work in this regard, but in contrast to the above discussed for eyestalk hormones, a definitive factor that induces vitellogenesis, analogous to the role of juvenile hormone/ecdysteroids in insects, has not been fully identified.

The earliest work in this area suggests that a putative vitellogenesis-stimulating factor originates in the brain and thoracic ganglion. Hinsch and Bennett (1979) found that thoracic ganglion implants stimulated vitellogenesis in immature spider crabs Libinia emarginata. In the shrimp, Paratya compressa, brain and thoracic ganglia extracts stimulated ovarian development both in vitro and in vivo (Takayanagi et al. 1986). Similar results were obtained in implantation experiments in the whiteleg shrimp, Li. vannamei (Yano 1988; Yano...
and Wyban 1992), and crab, *Potamon koolooense* (Joshi 1989). At this time, the putative VSH could not be fully chemically characterized, but appeared to be a 10 kDa peptide that is inactivated by trypsin (Yano 1990).

There is also evidence that the neurotransmitter serotonin, also referred to as 5-hydroxytryptamine (5-HT), is responsible for triggering the relevant neuroendocrine organs to release VSH. In the red swamp crayfish, *Procambarus clarkii*, the effects of brain or thoracic ganglia on ovarian maturation *in vitro* were enhanced by the administration of 5-HT (Sarojini 1995). Similar results were obtained in the fiddler crab, *Uc. pugilator* (Landau et al. 1991; Wildet et al. 1994), among other species. However, to date, there are no reports of JH in a crustacean species.

In some of the most recent work in this area, Meeratana et al. (2006) injected 5-HT intramuscularly into adult female *Mac. rosenbergii*, or alternatively, 5-HT-primed *Artemia salina* larvae, and methoprene in brain, thoracic ganglion, muscle, and eyestalk. At a dose of 1 µg 5-HT/g body weight, ovarian GSI was increased to 5.79% compared to 1.59% for the control group. However, doses of 5-HT above 1 µg/g up to 50 µg/g did not cause any further increases in GSI. At the same time, regarding the injection of tissue medium, it was seen that culture medium of 5-HT-primed thoracic ganglion caused significant advancement of ovarian stage, both in terms of GSI and histological examination (with brain culture medium having some effect as well) (Meeratana et al. 2006). Such research strongly indicates the presence of a vitellogenesis-stimulating factor in the thoracic ganglion, and perhaps the brain, the release of which is brought about by 5-HT. Of note, Sosa et al. (2004) have cloned a crustacean serotonin receptor for three species, e.g., the giant freshwater prawn, *Mac. rosenbergii*, the crayfish, *Pr. clarkii*, and the Pacific spiny lobster, *Panulirus interruptus*. Rodríguez-Sosa et al. (1997) have demonstrated the presence of 5-HT in the eyestalk ganglia of *Pr. clarkii*, suggesting its role as a neurotransmitter.

On the other hand, there is a body of thought that methyl farnesoate (MF), the precursor of juvenile hormone III in insects, may stimulate reproduction in Crustacea. Methoprene, a JH mimic, seemed to promote methoprene in *Artemia salina* larvae, while 0.001, 0.01, and 0.1 µM concentrations stimulated expression above the control, expression decreased at concentrations over 0.1 µM (Mak et al. 2005). The same group has investigated the effects of these substances in the shrimp *Me. ensis* (Tiu et al. 2006a), and the lobster, *Ho. americanus* (Tiu et al. 2009, 2010). While results differ according to species and reproductive size, a similar trend is seen, especially with respect to the effects of FA. Of note in *Tiu et al. (2010)*, the molting hormone, 20-hydroxyecdysone, when administered together with FA, synergistically increased vitellogenin gene expression. More research of this nature is needed to characterize the effects of juvenoids in other crustacean species; moreover, to date, it appears that no research has demonstrated any conclusive effect of juvenoids on vitellogenin gene expression *in vivo*.
Fig. 25. Correlation between gonadosomatic index (GSI) and hemolymph levels of estradiol-17β, estriol, progesterone, testosterone, and 11-ketotestosterone in female *Marsupenaeus japonicus* under the natural reproductive cycle. Undetectable levels are shown as 0 pg/mL. Dashed lines indicate the limit of detection. Reprinted with permission from *Fisheries Science*, 70, Okumura and Sakiyama, Hemolymph levels of vertebrate-type steroid hormones in female kuruma prawn *Marsupenaeus japonicus* (Crustacea: Decapoda: Penaeidae) during natural reproductive cycle and induced ovarian development by eyestalk ablation, 372–380, Fig. 1, © 2004, The Japanese Society of Fisheries Science.

Fig. 26. Correlation between gonadosomatic index (GSI) and hemolymph levels of estradiol-17β, estriol, and progesterone in control and eyestalk-ablated female *Marsupenaeus japonicus*. Dashed lines indicate the limit of detection. Reprinted with permission from *Fisheries Science*, 70, Okumura and Sakiyama, Hemolymph levels of vertebrate-type steroid hormones in female kuruma prawn *Marsupenaeus japonicus* (Crustacea: Decapoda: Penaeidae) during natural reproductive cycle and induced ovarian development by eyestalk ablation, 372–380, Fig. 7, © 2004, The Japanese Society of Fisheries Science.

4-1D. Possible roles of vertebrate-type steroid hormones in vitellogenesis

In oviparous vertebrates, steroid hormones play important roles in the regulation of vitellogenesis. By analogy with the function of steroid hormones in vertebrates, the occurrence of vertebrate-type steroid hormones in crustaceans have been examined, and estradiol-17β, progesterone, 17α-hydroxyprogesterone, and testosterone have been found (for reviews, see Fingerman et al. 1993; Huberman 2000; Subramoniam 2000).

To determine the functions of vertebrate-type steroid hormones in female shrimp reproduction, one of the authors of this monograph examined the correlation between hemolymph hormone levels and ovarian development in female *Mar. japonicus* (Okumura and Sakiyama 2004). Hemolymph samples were taken at different ovarian developmental stages, and steroid levels were measured. Levels of estradiol-17β, estriol, progesterone, testosterone, and 11-ketotestosterone were not significantly related to ovarian development (GSI, 0.80–9.39; Fig. 25). Furthermore, levels of estradiol-17β, estriol, and progesterone did not differ significantly between control non-vitellogenic female prawns and female prawns under vitellogenesis induced by eyestalk ablation (removal of the source of VIH; Fig. 26). These results suggest that these vertebrate-type steroid hormones do not play an important role in ovarian development in *Mar. japonicus*.
The above results are supported by recent advances in genome-wide surveys. Genes encoding estrogen receptors are lacking in the fruit fly *Drosophila melanogaster* and the ascidian *Ciona intestinalis*. This absence suggests that innovations in steroid hormone receptors occurred in the vertebrate lineage (Yagi et al. 2003). On the other hand, there are several reports that the administration of vertebrate-type steroid hormones stimulates vitellogenesis in crustaceans (for reviews, see Fingerman et al. 1993; Huberman 2000; Subramoniam 2000). To be able to elucidate the roles of vertebrate-type and crustacean-type vitellogenin molecules in the crustacean ovary, further research is necessary.

## 4.2. Role and utilization of vitellin during embryogenesis

Penaeid shrimp and prawns are an exception among decapod Crustacea. Their species broadcast their eggs into the surrounding medium following spawning, and eggs generally hatch within about 1 day. However, most female decapods brood their eggs externally, with the egg mass being attached to reproductive setae for a length of time that varies according to species. For example, the shrimp *Palaemon serratus* exhibits a 110-day embryonic period (Spindler et al. 1987), while in *Mac. rosenbergii*, this period lasts for only 18 days (Wilder et al. 1990). In the crabs, *Cancer magister* and *Cancer anthonyi*, eggs are brooded for 90 and 40 days, respectively (Okazaki and Chang 1991).

Therefore, it is necessary that the newly-spawned egg contains a reserve of nutrient sources which serve the developing embryo until it hatches and is able to feed. Vitellin is the main provider of nutrition during this time, and is often referred to as lipovitellin when discussed in this context. The egg should also possess hydrolytic enzymes and proteases to break down the lipovitellin and make lipids, proteins, and amino acids available for usage. In the brine shrimp, *Artemia salina* (which is in the Class Branchiopoda, not Malacostraca to which decapods belong), the utilization of lipovitellin in eggs has been studied extensively; egg lipovitellin contains primarily two apoprotein subunits having molecular weights of 68 kDa and 190 kDa. During embryonic development, these subunits undergo proteolytic cleavage to give rise to smaller peptides (De Chaffoy de Courcelles and Kondo 1980). Under unfavorable conditions, *Ar. salina* produces dormant gastrulae capable of resuming development when incubated under appropriate conditions (Vallejo et al. 1981). A vitellin-bound trypsin-like protease (Ezquieta and Vallejo 1985) is activated concomitantly with the yolk granule dissolution that takes place during hydration of the dry cyst. In the case of *Artemia* yolk utilization, protease activity appears to be a programmed developmental event with possible control mechanisms conferred by its association with lipovitellin.

In the mole crab, *Em. asiatica*, lipovitellins I and II are progressively cleaved proteolytically into their constituent polypeptides during embryonic development until they are finally utilized as a source of amino acids (Subramoniam 1991). This is also a time of intense esterase activity in this species (Subramoniam 1991). Of note, the proteolytic products of the vitellins gradually lose their PAS staining properties; this suggests that carbohydrate prosthetic groups have become disassociated from vitellin (Tirumalai 1996). Furthermore, the activity of two glycosidases, glucosidase and lactosidase, increases in embryos as PAS staining subsides in vitellin fractions (Gunamalai 1993). These glycosidases may be required to release bound glucose and galactose from the glycolipid and oligosaccharide components of the major yolk proteins during embryogenesis in *Em. asiatica*.

More recently, the utilization of lipovitellin in the eggs and embryos of *Mac. borelli* has been extensively studied by Garcia et al. (2008). This species broods an egg mass for approximately 39 days, and this time frame is divided into seven embryonic stages. The authors found that the lipovitellin discussed above exists in its native form as a 440 kDa protein in ovaries, and is also found in developing eggs. During early embryogenesis, this protein decreases in quantity, but is consumed slowly, after which it is consumed rapidly during stages 6 and 7 (Garcia et al. 2008). Furthermore, during the early embryonic stages, lipovitellin consists of the 112 kDa and 94 kDa subunits also identified in the ovary, but during the late stages, is broken down to smaller subunits of 70 kDa, and finally 43 kDa. A similar study was also conducted for the blue crab, *C. sapidus* (Walker et al. 2006), revealing that lipovitellin is used in much the same way, although the embryonic period is shorter (10–13 days). Such studies at present have not been conducted for other shrimp and prawn species, but it is likely that other *Macrobrachium* genus species exhibit similar patterns of vitellin usage during embryonic development.

## 5. Conclusions and future perspectives

In decapod Crustacea, the process of vitellogenesis, where yolk proteins are synthesized, transformed into smaller molecules, and taken into maturing oocytes, is a central feature of reproduction. As discussed above, the vitellogenic site is principally the hepatopancreas, or both the hepatopancreas and ovary. While vitellogenin molecules in some animal groups exhibit a certain degree of homology, such as those proteins found in animal groups such as nematodes, insects and amphibians (Chen et al. 1997), it is now known that crustacean vitellogenins differ somewhat from those of other animals. Most notably, crustacean vitellogenins are lacking in polyserine domains. However, in the
numerous decapod species for which full cDNA sequence is known, all vitellogenins show a characteristic length of around 7,800 bp and an ORF with a typical size of about 2,534–2,589 amino acid residues (see Table 2).

Vitellogenesis is under the negative control of eyestalk hormones of the CHH family. Vitellogenesis-inhibiting hormone (VIH) has been isolated and characterized, and conclusively demonstrated to inhibit vitellogenin mRNA expression in vitro (Tsutsui et al. 2007). On the other hand, while a great deal of research has demonstrated evidence for a putative vitellogenesis-stimulating hormone (VSH), perhaps a peptide or protein hormone originating from the brain and/or thoracic ganglion, a factor having these properties remains to be isolated and fully identified. The work of Sarojini et al. (1995) using the crayfish *P. clarkii*, strongly indicates that serotonin has a role in promoting vitellogenesis via its effects as a neurotransmitter causing the release of a putative VSH. However, this type of work has not been extensively conducted by other research groups or in other species. Such experiments should be attempted by others in order to fully reveal the nature of the positive mechanisms affecting reproduction in decapod Crustacea. The same may be put forth for the other categories of work on positive factors described in this monograph—e.g., on juvenoids, second messenger substances, even vertebrate-type steroids.

Nevertheless, a great deal of progress has been made in understanding vitellogenesis in Crustacea. In addition to the knowledge concerning vitellogenin structure and processing, much has been learned about its biochemical features—e.g., conjugating moieties, and so forth, as discussed in Subsection 2-2. There is also a great deal of new information available on the crustacean vitellogenin receptor, and how vitellin is utilized during embryogenesis after the egg mass is spawned up until hatching. Of particular note, vitellogenin is a high-density lipoprotein and has the additional role of lipid transport; obviously, lipid accumulation is a primary feature of crustacean eggs, where it serves as the source of energy during embryogenesis (Pandian 1970; Subramoniam 1991).

In terms of basic science, the full elucidation of crustacean reproductive mechanisms is of course, highly interesting and necessitated. It is certain that many similarities, yet many more differences, will be found in comparison to what is known in other arthropods, for example, insects. There also exists much potential for this knowledge to be applied to further aquacultural development—to control reproduction and stabilize seed production operations in the hatchery, as put forth in the Introduction. Japan as a country consumes 300,000 tons per year of shrimp; yet its production based on aquaculture (of the kuruma prawn, *M. japonicus*) is under 2,000 tons, while about 25,000 tons is provided by conventional fisheries activity, mainly of cold-water species (MAFF 2008).

The use of re-circulating aquaculture systems as a means of promoting sustainable shrimp culture without impacting the environment has received a great deal of attention in recent years. In Japan, the authors of this monograph have been involved in the experimental phase of setting up re-circulating culture systems for *L. vannamei*; the system now operates on a commercial basis (owned by Myoko Yuki-Guni Suisan Co. Ltd.) in Myoko City, in a mountainous area. The system has proved to be stable for the low-salinity culture of *L. vannamei* starting from PL15 (Jayasankar et al. 2009), and the resultant product, Myoko Yuki Ebi® (“Myoko Snow Shrimp”), has been shown to be of high quality in terms of the content of flavor-producing amino acids (Okutsu et al. 2010). However, it remains necessary to import seed from Thailand or Hawaii, and the authors are now aiming to develop improved seed production methodology making use of knowledge of the reproductive mechanisms of this species.

Recently, Treerattrakool et al. (2008, 2010) have conducted studies using double-stranded RNA in *P. monodon* to investigate VIH (named gonad-inhibiting hormone; GIH in these reports) function, and as a means of inducing ovarian maturation and spawning on-site in Thailand, where this species is an important target of culture. The results of Treerattrakool et al. (2010) show that the use of GIH-dsRNA to create GIH-knockdown shrimp, is as effective as unilateral eyestalk ablation in promoting ovarian maturation and spawning. The results are extremely promising, and appear to be almost at the level of being able to be commercially utilized in the field. However, there is still little information on how VIH itself fluctuates in the hemolymph, although the work of de Kleijn et al. (1999) has demonstrated the dynamics of CHH and GIH storage in the sinus glands and their release into the hemolymph in the lobster *H. americanus*. In relation to reproduction. More information of this nature in other commercially important species may lead to even more effective means of exploiting, for example, the use of dsRNA methodology to artificially control maturation. Of course, if a stimulatory factor influencing vitellogenesis can be conclusively demonstrated, hormonal treatment could also be used in combination with the environmental control of light, salinity and water temperature in the hatchery.

In conclusion, further research on the hormonal control of vitellogenesis in Crustacea is expected to provide greater insight into reproductive mechanisms in general, and have many new, exciting applications in aquaculture, helping to thus provide greater sustainability to this important industry.
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References


Chan S-M, Mak AS, Choi C-L, Ma THT, Hui JH, Tu SHK. Vitellogenesis in the red crab, Charybdis feriatus: contributions from small vitellogenin transcripts (CiVg) and farnesoid acid stimulation of CiVg expression. Ann. NY Acad. Sci. 2005; 1040: 74–79.


De Chaffoy de Courcelles D, Kondo M. Lipovitellin from Cherax quadricarinatus (Crustacea, Decapoda). Contribution from small vitellogenin transcripts (CfVg) and farnesoid acid stimulation of CfVg expression. Comp. Biochem. Physiol. 2005; 141: 5175–5180.

Chevalier J-P, Jansen KPC, Waddy SL, Martin GM, Herp F. Expression of the crustacean


Jugan P, Van Herp F. Introductory study of an oocyte membrane protein that specially binds vitellogenin in the cray-


Lui CW, O’Connor JD. Biosynthesis of crustacea lipovitellin. III. The incorporation of labeled amino acids into the purified subunits. J. Mol. Biol. 1977; 110: 266.


Meeratana P, Wisbyachumnakul B, Damrongphol P, Wongprasert K, Suseangtham A, Sobhon P. Serotonin-induced ovarian maturation in the giant freshwater prawn


Qiu YW, Ng TB, Chau KH. Purification and characterization of vitellin from the ovaries of the shrimp Metapenaeus salicker.


