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REVIEW ARTICLE

Mechanisms and control of vitellogenesis in crustaceans

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Abstract Crustaceans produce complex yolk proteins to meet the substrate and energy requirements of embryonic development. Early electron microscopic investigations point to a biphasic yolk synthesis, first within the ovary, followed by heterosynthesis at extra-ovarian sites. Recent advances in molecular techniques have enhanced our understanding of the genetic control of yolk synthesis in crustaceans. Amino acid sequencing of crustacean vitellogenin (Vg) has enabled the elucidation of the cDNA sequence associated with it, the identification of genes, and the examination of their expression patterns in different tissues. Yolk processing in crustaeans involves cleavage of the pro-Vg at consensus sites by subtilisin-like endoproteases within the hepatopancreas, hemolymph and oocytes. The structural elucidation of crustacean yolk proteins, as well as the comparison of amino acid sequences of vitellogenins from various crustacean species, has revealed molecular phylogenetic relationships not only among them but also with other large lipid transfer lipoproteins of disparate function. The combinatorial effects of eyestalk neuropeptides and a variety of trophic hormones achieve the hormonal coordination of molting and reproduction. Biogenic amines secreted by the central nervous system may also play an integrative role by stimulating neuropeptide secretion.

Keywords Vitellogenesis · Vitellogenin receptor · Yolk processing · Neuropeptides · Methyl farnesoate · Ecdysteroids · 17β-Estradiol

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Introduction

Many malacostracan crustaceans produce large numbers of yolk-laden eggs and brood them externally for extended periods. Hence, vitellogenesis, the process of yolk formation, is central to oogenesis. In Crustacea, vitellogenesis is a biphasic event consisting of autosynthesis and heterosynthesis [1]. This contention is supported by recent molecular studies demonstrating yolk protein gene expression both in ovary and hepatopancreas. Receptor-mediated endocytosis of the yolk precursor molecule, vitellogenin (Vg), into growing oocytes has been established in crustaceans [2]. The molecular transformation of Vg into final yolk products for deposition in the mature oocyte is another crucial event in vitellogenesis.

A defining feature in the endocrine regulation of vitellogenesis in Crustacea is the occurrence of inhibitory hormones in the neurosecretory cells of the X-organ/sinus gland complex within the eystalk. Conversely, many hormonal factors as diverse in nature as methyl farnesoate (MF) and vertebrate steroidal hormones have been implicated in the stimulation of vitellogenesis. However, we are far from having achieved a clear understanding of the exact regulatory mechanisms relating to the vitellogenic processes in Crustacea, mainly because of the species-specific nature of the effector molecules. Yet, recent molecular studies on the primary structure of the major vitellin molecules, as well as the deciphering of their gene sequences and the elucidation of their synthetic sites, are paving the way to an understanding of the transcriptional control of the vitellogenin gene in light of what is already known about insect and vertebrate vitellogenesis. Homology searches and molecular phylogenetic analysis of various crustacean Vgs have revealed unexpected results on their closer relationship with several members of the large lipid transfer lipoprotein

superfamily as compared to their own orthologous Vg molecules. This review undertakes a critical analysis of the various mechanisms involved in the vitellogenic process and their hormonal control.

Vitellogenesis

Molecular composition of crustacean yolk proteins

Crustacean yolk proteins, referred to as lipovitellin, are complex molecules comprising a high-density lipoprotein (HDL) conjugated to carbohydrates and carotenoid pigments [3]. Crustacean lipovitellin differs from that of vertebrates in that it lacks protein phosphates and has high lipid content. In the mole crab *Emerita asiatica*, purified lipovitellin contains neutral lipids, glycolipids and phospholipids, among which phospholipids are the dominant lipid class, with phosphatidyl choline and phosphatidyl serine being the major species [4, 5]. However, the proportion of lipid to protein seems to be higher in the precursor protein, Vg. Crustacean lipovitellin characteristically contains a variety of carotenoid pigments. They include beta-carotene, astaxanthin, canthaxanthin and *cis*-canthaxanthin, among other minor intermediary metabolites [1]. Crustacean lipovitellin also possesses a higher carbohydrate content than vertebrate vitellins. In *E. asiatica*, most protein-bound carbohydrates found in lipovitellin are hexosamines and hexoses [5]. *Emerita* lipovitellin also contains galactosamine as well as *O*-linked oligosaccharides with *N*-acetyl hexosamine as the terminal residue, whereas sialic acid is specifically absent. Khalaila et al. [6] have identified the glycosylation sites in the vitellogenin of the crayfish *Cherax quadricarinatus* and characterized the glycan moieties. Besides providing an important source of carbohydrates for the developing embryos, the glycosylation of Vg has an important role in the folding and subunit assembly of these molecules. The glycan moieties may also play an equally important role in the recognition of the Vg membrane receptor during yolk accumulation. After uptake into the oocytes, they may also be involved in packaging and compressing the yolk precursor proteins into the yolk bodies [5].

Biogenesis of yolk

In Crustacea, vitellogenesis occurs in two stages: a primary vitellogenesis or previtellogenic phase characterized by the differentiation of endoplasmic reticulum and the formation of endogenous yolk stored in vesicles; and a secondary vitellogenesis corresponding to an intensive phase of uptake and storage of exogenous yolk precursor molecules, which accumulate into large yolk globules [7]. Early electron microscopic investigations point to this biphasic yolk synthesis, first within the ovary, followed by a heterosynthetic yolk formation in somatic tissues such as the hepatopancreas or subepidermal fat body [1].

Support for autosynthetic yolk formation came from in vitro incubation studies using ovaries of crayfish *Procambarus* sp. and of the crab *Pachygrapsus crassipes* [8]. In the shrimp, the yolk content of the egg is meager, and hence oocytes may be in a position to synthesize most of them, with only a very limited contribution deriving from extra-ovarian sites. In the kuruma prawn *Marsupenaeus japonicus*, under in vitro conditions, only the ovary incorporated radioactive amino acids into a protein immunologically identical to lipovitellin [9]. Similar in vitro studies on the vitellogenic ovaries of another shrimp *Penaeus semisulcatus* also revealed that the ovary is the primary organ of vitellin synthesis [10].

Egg maturation in penaeid shrimp is characterized by vitellogenesis and cortical rod protein (CRP) formation. In *M. japonicus*, Kim et al. [11] showed that CRP mRNA is highly expressed before the onset of vitellogenesis and that Vg mRNA exhibited high expression during intense vitellogenesis, suggesting that different genes are involved in the ovarian synthesis of CRP and Vg proteins. Okumura et al. [12] provided further evidence that eyestalk ablation induced both Vg and CRP synthesis within the ovary. Khayat et al. [13] demonstrated high levels of Vg mRNA in the vitellogenic ovary of *P. semisulcatus*, as evidenced by its ability to direct the cell-free synthesis of large amounts of Vg. However, unlike the other decapods, where autosynthesis of yolk has been shown to occur within the oocytes [8], in penaeid shrimp, the ovarian synthesis of yolk probably takes place in the follicle cells. Thus, in *M. japonicus*, an immunofluorescence study with anti-vitellin IgG was suggestive of yolk protein synthesis by the follicular epithelium rather than by the oocytes. Northern blot analysis and in situ hybridization have revealed that mRNA encoding vitellogenin was expressed in the follicle cells of the vitellogenic females [14]. Tsang et al. [15] also showed the expression of the vitellogenin gene, *MeVg1*, in the ovary and hepatopancreas of *Metapenaeus ensis*, suggesting equal contributions from both tissues.

In recent years, several gene expression studies, using quantitative real-time PCR techniques, have demonstrated that the ovary remains the principal organ that synthesizes yolk proteins in several penaeid shrimp species. Interestingly, in species such as *M. japonicus* and *P. semisulcatus*, one and the same Vg is expressed in the ovary and hepatopancreas [16, 17]. However, in other species, such as *Litopenaeus merguiensis*, *M. ensis* and *P. monodon*, more than one Vg may be involved in the tissue-specific expression of the gene in both the ovary and hepatopancreas [18–20]. Especially in *L. merguiensis*, the patterns of Vg mRNA expression between the hepatopancreas and ovary

differ in that the expression level in the hepatopancreas is much lower than that in the ovary at all stages of ovarian development [18]. Evidently, the relative contributions of the ovary and hepatopancreas to overall yolk production may differ among various shrimp species.

Vitellogenin

In addition to being the precursor of ovarian lipovitellin, crustacean vitellogenin is considered to be an important transporter of lipids to the ovary from the hemolymph during vitellogenesis. In general, lipid transport through the hemolymph is accomplished by two HDLs and a very high-density lipoprotein (VHDL) [21, 22]. Female-specific vitellogenin is one of the HDLs, with its production being correlated with ovarian development in female crustaceans, whereas the other HDL as well as VHDL are found in both males and females. In the penaeid shrimp, *P. semisulcatus*, the non-sex-specific hemolymph lipoprotein, LP I, consists of one 110-kDa peptide unit, whereas the sex-specific LP II consists of 3 subunits of 200, 120, and 80 kDa [23]. Interestingly, the same subunits were also present in the lipovitellin of this shrimp. Furthermore, the lipid compositions of these two HDLs in *P. semisulcatus* also differ: LP II (Vg) has a lower lipid content than does LP I, in addition to differences found in lipid classes linked to the apolipoprotein. Apparently, vitellogenin and lipovitellin have similar protein structures, but show differences in their lipid contents, with the lipovitellin having more percentage lipid acquired through adsorption within the oocytes. LP I is also different from LP II in its protein composition, as the former does not cross-react with anti-vitellin antiserum. In the crayfish *Cherax quadricarinatus*, Yehezkel et al. [24] observed that the hemolymph lipoprotein II, equivalent to Vg, appears only at the onset of secondary vitellogenesis. In the mole crab *E. asiatica*, Subramoniam and Gunamalai [25] have described three hemolymph lipoproteins: LP1, LP2, and LP3. LP1 is non-sex-specific, but is accumulated into the oocytes along with LP2, which is the female-specific Vg. LP3, which appears only during the premolt of male and female crabs, plays a role in the transport of lipids to the epidermis for the purposes of cuticle formation. In addition to transporting a variety of lipophilic compounds such as triglycerides and phospholipids, crustacean Vgs are known to transport steroidal hormones like ecdysteroids and vertebrate steroids, including estradiol 17β and progesterone [26, 27]. These hormones are stored within the oocytes as conjugates of yolk proteins and serve regulatory functions during embryogenesis.

Site of vitellogenin synthesis

Initial electrophoretic and isotope tracer studies have implicated several organs such as the hemocytes in crabs [28, 29], the fat body in isopods and the amphipods [30, 31], and the subepidermal adipose tissue in *Palaemon serratus* [32], and *Scylla serrata* [33] as the synthetic sites of Vg. However, the hepatopancreas has proven to be the most important organ synthesizing Vg outside of the ovary in the majority of crustacean species analyzed. The crustacean hepatopancreas is the functional homolog to the fat body in insects and the liver in vertebrates. Subsequent investigations employing molecular techniques have revealed that the hepatopancreas is the sole site of Vg synthesis in the giant freshwater prawn, *Macrobrachium rosenbergii*. Chen et al. [34] cloned a cDNA fragment encoding Vg in this species and found its expression in the hepatopancreas of the vitellogenic female. In addition, Yang et al. [35] obtained cDNA fragments for four vitellins; using these cDNA fragments as probes, they found the exclusive expression of Vg mRNAs for the four vitellins in the hepatopancreas of vitellogenic female *M. rosenbergii*. Using quantitative real-time PCR techniques, Jayasankar et al. [36] measured the expression levels of mRNA in the hepatopancreas of this species and also determined Vg levels using enzyme immunoassay. Vg mRNA expression in the hepatopancreas and hemolymph Vg levels showed a gradual increase concomitant with increasing gonadosomatic index. Vg mRNA expression was, however, negligible in the ovary, confirming that the hepatopancreas is the principal site of Vg synthesis in *M. rosenbergii*. In general, Vg expression may occur at multiple sites, but expression patterns nevertheless vary according to species. That one and the same gene for vitellin and Vg can be simultaneously expressed both in the ovary and hepatopancreas was shown in *P. semisulcatus* [37]. Multiple genes may also show tissue-specific expression of Vg in the ovary and hepatopancreas, as demonstrated in another penaeid shrimp, *Metapenaeus ensis*, where two Vgs (MeVg1 and MeVg2) have been identified [15]. The *MeVg1* gene is expressed equally in the ovary and hepatopancreas, whereas *MeVg2* is expressed only in the hepatopancreas. Furthermore, the *MeVg2* gene gives rise to smaller transcripts, resulting in the production of many smaller MeVg2 subunits destained for ovarian uptake [19].

Evidently, the ovary is the primary site of yolk synthesis in penaeid shrimp, as indicated by gene expression studies enumerated above; on the contrary, large-bodied decapods such as crabs and lobsters seem to rely largely on extraovarian organs such as the hepatopancreas for the synthesis of Vg. Using molecular techniques, Li et al. [38] have demonstrated that in the Chinese crab *Eriocheir sinensis*, the hepatopancreas is the main site of Vg synthesis, although immunocytochemical studies have suggested a parallel role for ovary. However, in the red crab *Charybdis feriatus*, northern blot analysis revealed that the crab expresses the Vg precursor only in the hepatopancreas.

In addition to the major 8.0-kb transcript, a large proportion of smaller *C. feriatus* Vg-specific transcripts are also detected in the hepatopancreas. These transcripts most likely result from the alternative splicing and alternative use of promoter and/or termination signals [39]. The occurrence of many Vg subunits in the crab hemolymph may also result from autoproteolysis due to intrinsic protease activity in Vg itself [40]. In a recent study using quantitative real-time PCR techniques, Zmora et al. [41] found evidence that Vg is primarily expressed in the hepatopancreas of the vitellogenic females, with only minor expression in the ovary of the blue crab *C. sapidus*. Furthermore, Vg expression in the hepatopancreas of this brachyuran anecdysic crab is correlated with ovarian maturation, with a remarkable 8000-fold increase in expression from stage 3 to 4 of ovarian development. Recent cloning and expression studies on the Vg in the lobster *Homarus americanus* also adduced further evidence that the hepatopancreas is the primary organ for yolk precursor synthesis in lobsters [42]. The lobster *HaVg1*, expressed mainly in the hepatopancreas, comprises 14 introns and 15 exons. This study also revealed that the sizes and locations of the exons and introns of *Vg* are conserved among crustaceans. The HaVg1 precursor contained the lipoprotein domain at the N-terminus, followed by a domain of unknown function in the middle. The von Willebrand factor type-D domain is located at the C-terminus of the precursor. A unique feature of crustacean Vg is that it contains several cleavage sites, resulting in increased subunit composition. More numbers of Vg subunits may also arise from smaller transcripts, as reported for the crab *C. feriatus* [43].

All these studies lead to the compelling conclusion that the hepatopancreas is the principal site of Vg synthesis in brachyuran crabs, lobsters, and probably other representative species under the suborder Pleocyamata. Conversely, in Dendrobranchiata, including mainly the penaeid shrimp, both the hepatopancreas and ovary provide equal contributions towards Vg synthesis. The Vg synthesized at extraovarian sites such as the hepatopancreas undergoes several modifications, such as glycosylation and lipid addition, bringing about changes in molecular weight when compared with the final yolk products accumulated within the ovary [41]. To sum up, besides being the precursor protein molecule that supplies the amino acid pool for the developing embryo, vitellogenin can also serve other subfunctions, such as the transport of a variety of organic and inorganic molecules required for embryonic development.

Phylogenetic analysis of crustacean vitellogenin

Crustacean Vg is a multidomain apolipoprotein that is cleaved into distinct yolk proteins. Multiple alignments of all known crustacean Vg sequences have revealed almost similar cleavage sites. ClustalW alignment of *M. rosenbergii* Vg with that of 17 other crustacean species has shown that the first common cleavage site RXRR occurs at amino acid residues 707–710, and the homology for the first segment is high when compared with the rest of the module. The results from BLAST searches indicate that the N-terminal region of crustacean Vgs is conserved, as in the apolipoproteins that are involved in lipid transport. This property is in accord with the fact that Vg, insect apolipophorin II/I, apoB, and MTP are members of the same multigene superfamily of large lipid transfer proteins (LLTP) [44]. Next to the N-terminal segment, the middle segment is comparable to a lipovitellin domain of unknown function called DUF1943. The C-terminal domain of *M. rosenbergii* Vg harbored a von Willebrand-factor type D domain (YGP4) found in mammals. Similarity in amino acid sequence of the von Willebrand factor at the C-terminal region has also been reported for another LLTP protein, the insect apolipophorin [45].

A phylogenetic tree constructed based on the alignment of amino acid sequences of 18 crustacean Vgs using the ClustalW programme shows six distinct lineage groups: Penaeidea (A), Brachyura (B), Astacidea (C), Caridea (D), Copepoda, and Brachiopoda (E), and Thalassinidea (F) (Fig. 1; Table 1). The Vgs of the penaeidian species seem to be highly homogeneous, with >92% identity in amino acid sequence, except in the case of *M. ensis*. In *M. ensis*, the two Vgs (MeVg1 and MeVg2), identified by Tsang et al. [15] and Kung et al. [19], are expressed independently in the ovary and hepatopancreas, with only a sequence identity of 56% between them. These MeVgs also showed less homology with Vgs of other penaeid shrimp, revealing a greater evolutionary distance from other penaeid species [46]. As seen from Fig. 1, *Upogebia major*, representing Thalassinidea, has taken a separate lineage near the Brachyura. In addition to all the above decapods, the two copepods and a branchiopod, *Daphnia*, formed a separate clad in-between Caridia and Thalassinidea in the radial tree.

The structural elucidation of Vg from different crustacean species has also been helpful in solving phylogenetic relationships with other arthropod groups. In a primitive brachiopod, *Daphnia magna*, two Vgs, *DmagVg1* and *DmagVg2*, have been isolated. Interestingly, the lipid transport module in the N-terminal region of DmagVg1 is more closely related to those of insect Vgs than to those of decapod crustacean Vgs [47]. Yet again, the intergenic region of the two genes contains sequences resembling juvenile hormone-responsive and ecdysone-responsive elements, typical of insect Vgs [48]. The close homology found between *Daphnia* and insect LLT Vg modules may be due to either divergence or convergence.

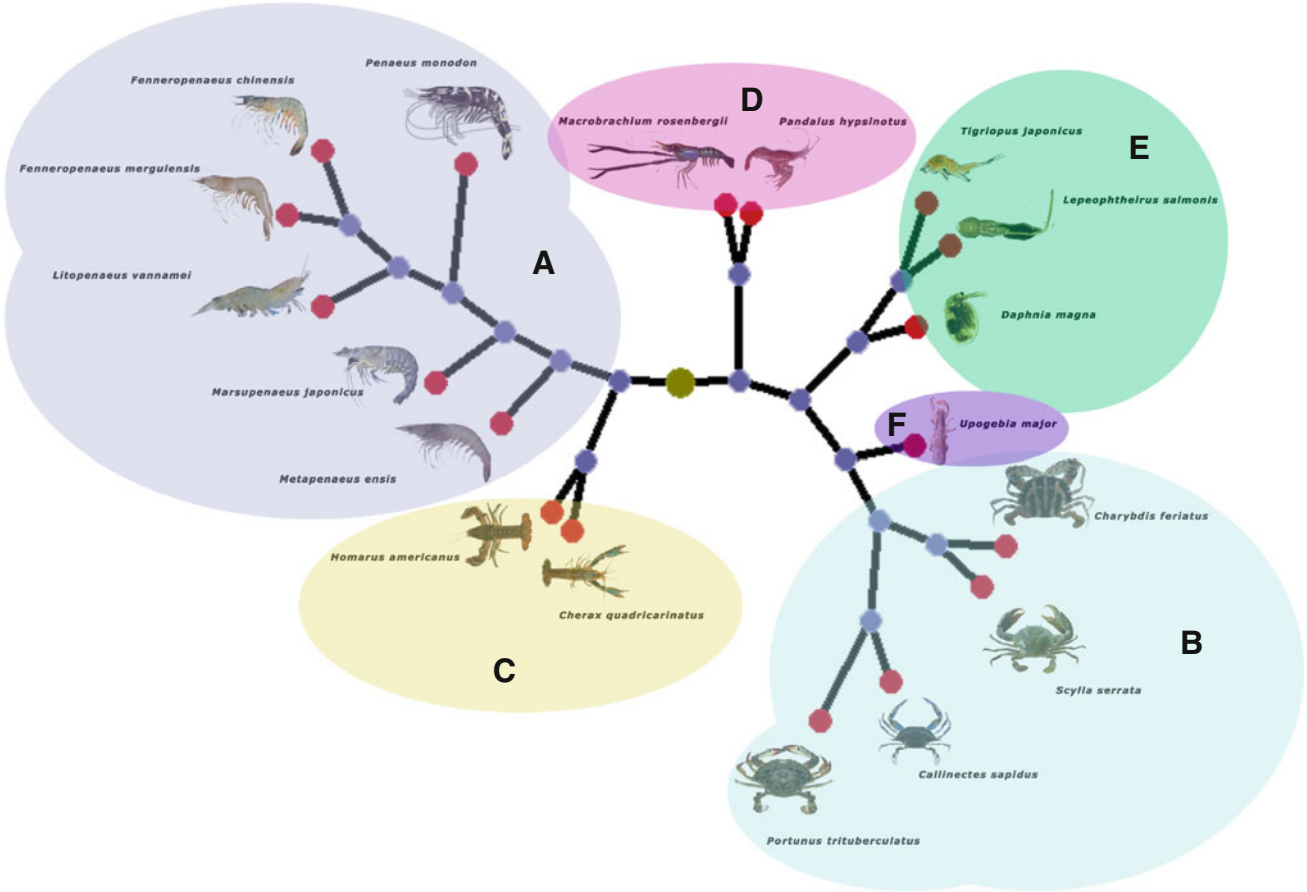

Fig. 1 Phylogenetics of eighteen crustacean Vgs with two dominant domains, (*A*) penaeoidean and (*B*) brachyuran, at both ends of the radial tree. Other major domains such as Astacidea (*C*) and Caridea (*D*) are found on either side of the tree. Copepods and brachiopods form a common grouping (*E*) along the brachyuran side. Thalassinidea (*F*) also has a separate lineage along the brachyuran crabs. The protein sequence accession numbers for all the Vgs are given in Table 1

In relation to other invertebrates, crustacean Vg has shown homology in amino acid sequence with molluscan and coral Vgs [49, 50], although homology among the coral Vg, GfVg, and the shrimp Vg (*L. vannamei*) is much closer. These homology studies purportedly point to the emergence of Vg as an egg protein precursor before the cnidarian–bilaterian divergence. The origin and evolutionary progression of Vgs from a common ancestral molecule at the cnidarian–bilatrian divergence denotes a landmark interception in crustacean arthropods, giving rise to other lipid-carrying apolipoproteins that perform disparate physiological functions. Even among crustaceans, we find a number of lipoproteins, such as crustacean clotting proteins and hemocyanin, that show limited amino-acid sequence homology with vitellogenin [2].

The inclusion of crustacean Vg among other LLTP proteins is also justified by the immunological relatedness found between Vg of the crab *S. serrata* and apoB, the major protein component of LDL and VLDL [40]. Warrier and Subramoniam [40] demonstrated the recognition of Vg by antibodies to apoB-containing mammalian lipoproteins LDL and VLDL, and not to HDL (Fig. 2). Furthermore, the apoB antibodies reacted with greater efficacy to *S. serrata* Vg, thereby providing corroborative evidence for the structural identity of apoB with Vg.

Avarre et al. [51] conducted a homology study between crustacean Vgs and other members of the LDL superfamily of lipoproteins, and arrived at the conclusion that crustacean Vgs are closer to mammalian LDL and insectan apolipophorins. However, the vertebrate apo-lipo B line of proteins is thought to have diverged from the vertebrate Vg line, which, in turn, arose from the ancient egg yolk storage proteins of invertebrates [52]. The closer relationship between apoB and crustacean Vg discussed above not only indicates the high conservancy in the lipid-binding domains of both these proteins, but may also point to the evolutionary derivation of vertebrate apo-lipo B proteins at the crustacean Vg level.

Vitellogenin receptors and yolk protein uptake

In crustaceans, only a few studies have been carried out with reference to Vg receptors. In the giant freshwater

Table 1 Vg accession numbers of 18 crustaceans, along with their taxonomic classifications

Species	Accession no.	Systematics
Penaeus monodon	ABB89953.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Penaeus
Fenneropenaeus chinensis	ABC86571.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Fenneropenaeus
Fenneropenaeus merguiensis	AAR88442.2	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Fenneropenaeus
Litopenaeus vannamei	AAP76571.2	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Litopenaeus
Marsupenaeus japonicus	BAD98732.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Marsupenaeus
Metapenaeus ensis	AAT01139.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Metapenaeus
Homarus americanus	ABO09863.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Astacidea; Nephropoidea; Nephropidae; Homarus
Cherax quadricarinatus	AAG17936.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Astacidea; Parastacoidea; Parastacidae; Cherax
Portunus trituberculatus	AAX94762.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Brachyura; Eubrachyura; Portunoidea; Portunidae; Portunus
Callinectes sapidus	ABC41925.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Brachyura; Eubrachyura; Portunoidea; Portunidae; Callinectes
Charybdis feriatus	AAU93694.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Brachyura; Eubrachyura; Portunoidea; Portunidae; Charybdis
Scylla serrata	ACO36035.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Brachyura; Eubrachyura; Portunoidea; Portunidae; Scylla serrata
Macrobrachium rosenbergii	BAB69831.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Caridea; Palaemonoidea; Palaemonidae; Macrobrachium
Pandalus hypsinotus	BAD11098.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Caridea; Pandaloidea; Pandalidae; Pandalus
Upogebia major	BAF91417.1	Malacostraca; Eumalacostraca; Eucarida; Decapoda; Pleocyemata; Thalassinidea; Callianassoidea; Upogebiidae; Upogebia
Daphnia magna	BAE94324.1	Branchiopoda; Diplostraca; Cladocera; Anomopoda; Daphniidae; Daphnia
Lepeophtheirus salmonis	ABU41135.1	Maxillopoda; Copepoda;Siphonostomatoida; Caligidae; Lepeophtheirus
Tigriopus japonicus	ABZ91537.1	Maxillopoda; Copepoda; Neocopepoda; Podoplea; Harpacticoida; Harpacticidae; Tigriopus; Tigriopus japonicus

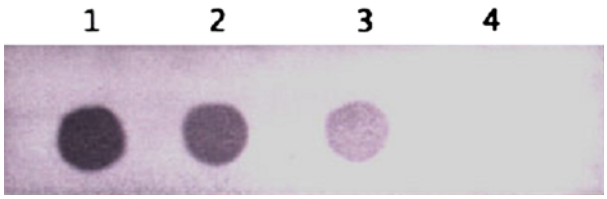

Fig. 2 Dot blot analysis of crab Vg (*1*), rat LDL (*2*), VLDL (*3*), and HDL (*4*) using anti-crab Vg antibodies (dilution 1:2000). Anti-Lv antibodies are seen to react well with Vg, LDL, and VLDL, but there is no reaction with HDL (from Warrier and Subramoniam [40])

prawn *M. rosenbergii*, Jugan and Soyez [53] demonstrated Vg uptake by the oocytes by employing colloidal gold-conjugated vitellin. The labeling was visualized in the microvilli, coated pits, and intraooplasmic vesicles. These authors further observed that a sinus gland neuropeptide inhibited vitellogenin endocytosis, possibly by blocking the membrane receptors. Laverdure and Soyez [54] solubilized the vitellogenin receptor from the oocyte membrane of *H. americanus*, and characterized it using an enzyme-linked immunosorbent assay. Binding of Vg with the solubilized receptors increased at the onset of vitellogenesis, but decreased in older oocytes of the freshwater crayfish *Orconectus limosus* [55]. The solubilized oocyte membrane receptor with a molecular weight of 28–30 kDa binds specifically to Vg of *O. limosus*. Warrier and Subramoniam [56] purified the vitellogenin receptor in the mud crab *Scylla serrata* using HPLC and found a still higher molecular weight of 230 kDa. In direct binding studies using ^{125}I-labeled Vg, crab VgR was observed to have increased affinity to its ligand in the presence of Ca^{2+} and was inhibited by suramin, a polysulfated polycyclic

hydrocarbon. These authors also showed an immunological relatedness between VgR of *S. serrata* and LDLR by virtue of the ability of VgR to bind rat LDL and VLDL.

In a recent study, the cloning and characterization of a cDNA encoding a putative Vg receptor from the tiger prawn *P. monodon* (PmVgR) has been reported [57]. PmVgR has a molecular weight of 211 kDa, and is ovary specific. It consists of conserved cysteine-rich domains, EGF-like domains and YWTD motifs, similar to the mammalian LDL receptor as well as to the Vg receptors of insects and vertebrates. PmVgR expression in the ovary coincides with the rapid pace of Vg production by the hepatopancreas. Immunological detection of PmVgR in the oocyte membrane during intense vitellogenesis has also been done in this prawn. Further, PmVgR expression was knocked down in animals after they were injected with PmVgR dsRNA, leading to a decrease in vitellin content in the ovary, and at the same time elevating the levels of hemolymph Vg. A similar molecular characterization of VgR has also been reported for the kuruma prawn *M. japonicus* [58]. The expression dynamics of MjVgR during vitellogenesis have been found to be similar to those of *P. monodon*. Furthermore, structural analysis of the VgR of this shrimp also reconfirmed its inclusion in the LDLR superfamily. The results of these studies are comparable with those of *S. serrata* with respect to molecular weight and functional characteristics [56]. It would be of interest to know whether crustacean VgR also facilitates the endocytosis of other hemolymph lipoproteins into the ovary, similar to avian VgRs [59] and insectan lipophorin receptor [60].

Receptor-mediated internalization of Vg into the oocytes has been demonstrated by an immunogold electron microscopic study using anti-Vg as the primary antibody in *S. serrata* [56]. Immunogold labeling against Vg antibody was first visualized in the coated pits found on the plasma membrane of the vitellogenic oocytes. This is followed by their appearance in the pinched-off coated vesicles as well as in early endosomes, which fuse together to form the mature electron-dense late endosomes (Figs. 3, 4). Such an endocytotic entry of Vg into the oocytes to form the yolk body is similar to that described for insects [61]. In *P. monodon*, after the binding of Vg with VgR, the complex moves into the oocyte cytoplasm, aided by internalization signals present in VgR [57]. Interestingly, the VgR of *P. monodon* has two putative internalization signals (i.e., FANPGFG and FENPFF) found in vertebrate VgRs as well as several IL and LI sites characterizing the insect VgR and *Drosophila* yolk peptide receptors [57]. This redundancy with the internalization signals present in the shrimp oocytes could increase the efficiency of receptor-ligand binding during crustacean vitellogenesis.

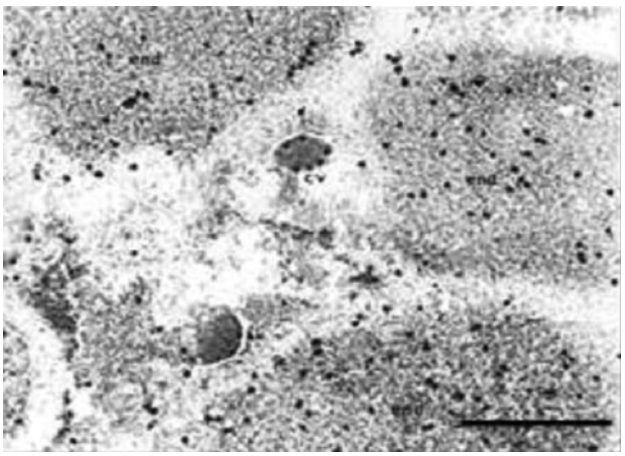

Fig. 3 Immunogold labeling of Vg in ultrathin sections of the ovary of *Scylla serrata*, examined with a Philips CM10 transmission electron microscope to demonstrate the endocytosis of Vg. Vg labeling is seen along the luminal surface of the coated vesicle (*cv*), which fuses into a mature endosome. Electron-dense particles representing Vg molecules are densely packed within the endosomes. *Scale bar* 0.5 μm (from Warrier and Subramoniam [56])

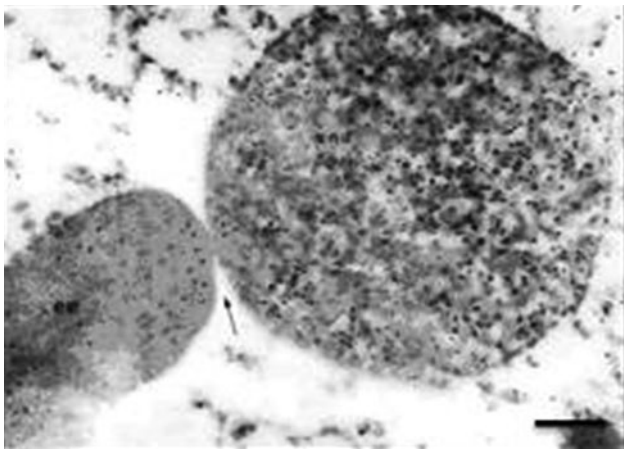

Fig. 4 Immunogold labeling of Vg in ultrathin sections of the ovary of *Scylla serrata*. In this micrograph, fusion of an early endosome (*ee*) with a mature endosome is observed (indicated by an *arrow*). Electron-dense particles of Vg are extensively labeled in the mature endosome compared to the early endosome. The mature endosomes finally form the yolk bodies. *Scale bar* 0.5 μm (from Warrier and Subramoniam [56])

Yolk processing

In general, Vg undergoes post-translational proteolytic cleavage at the site of synthesis (e.g., insects [61]) or after sequestration into the ovary (e.g., amphibians [62]). In crustaceans, SDS-PAGE analysis of hemolymph and ovary yolk proteins has indicated the occurrence of varying numbers of Vg and vitellin (Vn) fractions, suggesting that Vgs are already fragmented at the time of endocytotic uptake into the ovary. In the isopod *Armadillidium vulgare*, four female-specific glycoprotein bands in hemolymph, detected on SDS-PAGE,

were found to be the same in the ovarian extract [63]. In this isopod, an anion-exchange HPLC separation has yielded 6 vitellins from the ovary, ranging in molecular weight from 112 to 205 kDa [64]. The N-terminal sequencing of these proteins showed identical amino acids except for the 112 and 59 kDa proteins. PCR-assisted cloning of the 5′ region of a cDNA encoding Vg revealed the presence of an amino-terminal sequence identical to those of the 112 and 122 kDa yolk proteins, suggesting that the Vg gives rise to the Vn fractions by cleavage either in the hemolymph or ovary.

In *M. rosenbergii*, Vg, after being synthesized as a single precursor protein, undergoes initial cleavage at amino acids 707–710 by a subtilisin-like endoprotease to give rise to two subunits, A and pro-B, within the hepatopancreas [65]. After secretion into the hemolymph, subunit A is sequestered as is into the ovary, whereas pro-B is cleaved by another processing enzyme to give rise to subunits B and C/D (Fig. 5). The ovary subsequently takes them up to give rise to the yolk proteins, VnA, VnB, and VnC/D. Examination of subunit composition of Vg in hemolymph and Vn into the ovary by SDS-PAGE and

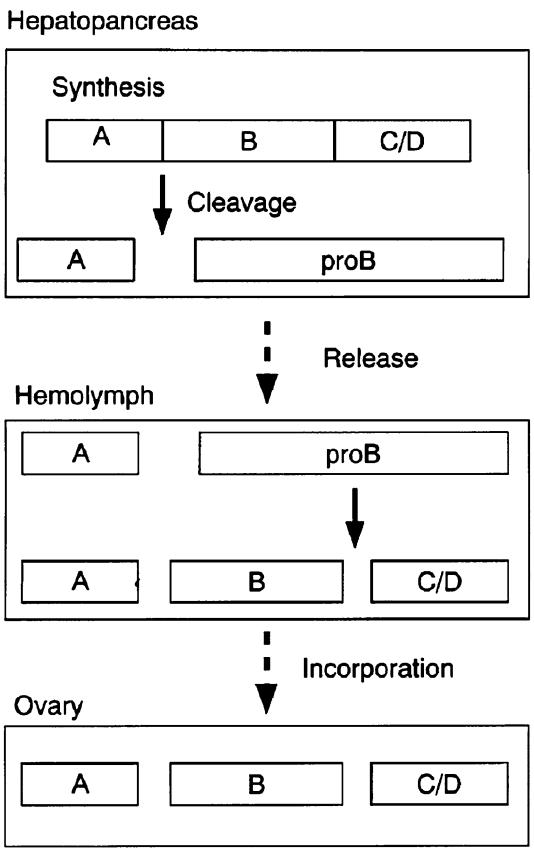

Fig. 5 Schematic representation of synthesis and processing of vitellogenin in *Macrobrachium rosenbergii*. Vg is synthesized as a single precursor molecule, A–B–C/D, in hepatopancreas, which is then cleaved into two subunits, A and proB. Subunits A and proB are released into the hemolymph, and proB is cleaved to form two subunits B and C/D. The three processed subunits A, B, and C/D are incorporated into the ovary. (From Okuno et al. [65])

western blotting has also supported the above sequence of Vg conversion to Vn fractions. Furthermore, identity in the N-terminal amino acid sequences of these Vg and vitellin fractions that appear in hemolymph and ovary has also provided final support to the scheme of Vg processing in this freshwater prawn [65]. Further studies on the processing of other decapod crustacean vitellogenins have revealed conservancy in the first cleavage site at amino acids 707–710, although the subsequent cleavage sites may differ among many species. In *Litopenaeus vannamei*, Raviv et al. [66] predicted an N-terminal sequence of 78 kDa, with the first cleavage site occurring at an RTRR consensus cleavage for subtilisin-like endoprotease. These authors isolated five HDL polypeptides of masses 179,113, 78, 61, and 42 kDa from the ovary and found that all of these polypeptides are derived from the 179 kDa second fraction of the premature Vg of *L. vannamei*. These results are in accord with those described for *M. rosenbergii* yolk protein processing. In a recent study on the mud shrimp, *Upogebia major*, belonging to the infraorder Thalassinidea of Decapoda, Kang et al. [67] found three polypeptides in the oocytes. These subunits were found to be derived from a single long polypeptide translated from the *Vg* transcript in the hepatopancreas. This precursor polypeptide of 289 kDa is cleaved to produce two Vg subunits at the consensus cleavage site, RLRR, which is recognized by subtilisin-like endoproteases. These two subunits are also suggested to undergo further processing upon or immediately after incorporation into oocytes.

Evidence for the secondary cleavage of vitellogenin after its uptake into the ovary is given in other decapods such as the freshwater crayfish, *Ibacus ciliates* [68]. A low-density lipoprotein isolated from the ovary of this crayfish degraded Vg into apolipoprotein fragments, which are similar to the lipovitellin subunits of the egg. Furthermore, the Vg digested by LDL exhibited proteinase activity whereas the native Vg did not have it. The instability of Vg and its susceptibility to undergo proteolytic cleavage may be a general feature, but in a brachyuran crab *Scylla serrata*, Vg itself possesses proteinase activity [40]. Warrier and Subramoniam [40] demonstrated that conformational changes in the native Vg could bring about such proteolytic cleavage, as indicated in a study using urea as a destabilizer. Whereas Vg showed a spectral change with 8 M-urea treatment due to exposure of the hydrophobic core containing aromatic residues (absorption at 274 nm), lipovitellin did not show such a spectral shift. Clearly, Vg is a relatively unstable lipoprotein, but the ovarian lipovitellin is more stable.

Yolk utilization

Yolk proteins primarily evolved to supply both energy as well as organic building blocks to support embryonic

growth in oviparous animals. Understandably, yolk utilization is the central event of embryogenesis, and is accomplished by a host of hydrolytic enzymes acting on the complex yolk molecules. Subramoniam [69] has reviewed the existing information on crustacean embryonic nutrition from the perspective of yolk utilization. During yolk utilization, the complex lipovitellins are dismantled by esterases, proteases and glycosidases, resulting in the release of conjugated steroidal hormones [70]. The regulated release of active ecdysteroids from their conjugates by nonspecific esterases at specific times in embryogenesis may not only trigger embryonic cuticle formation but may also accomplish larval molting and egg hatching [26, 71]. Direct utilization of lipovitellins in the egg by way of proteolytic cleavage in the developing embryos has also been documented in the blue crab *C. sapidus* [72].

As much as the yolk proteins meet the metabolic demands of embryonic development, they are also used in early larval development. In an extreme case of cirripede development, a new protein is expressed during the non-feeding cypris stage of the barnacles. This protein, called cypris major protein, is interestingly related to the heavy chain of barnacle yolk protein both structurally and immunologically [73]. Another glycoprotein, called settlement-inducing protein complex (SIPC), which is found in juvenile and cyprid larvae of the barnacle *Balanus amphitrite*, also showed immunological and peptide sequence similarity with cirripede yolk proteins [74]. Evidently, cirripede larval storage protein and the SIPC may share a common ancestor with yolk protein. Alternatively, crustacean yolk protein genes would have undergone duplication to give rise to different proteins necessary for larval metamorphosis and gregarious larval settlement in these sessile barnacles.

Endocrine regulation of vitellogenesis

In most malacostracan crustaceans, except the diecdysic crabs, vitellogenic activities are sandwiched between two molt cycle stages. Such an inextricable linkage between molting and vitellogenesis is accomplished by a delicate multihormonal interaction unique to crustaceans. Egg brooding within the pleopods of several malacostracans provides another intervention in the coordinated control of molting and reproductive cycles. Essentially, the hormonal controlling mechanisms enabling the temporal separation of these two processes involve principally the inhibitory neuropeptides—vitellogenesis-inhibiting hormone (VIH) and molt-inhibiting hormone (MIH)—originating from the X-organ/sinus gland complex in the optic ganglia. Thus, the hormonal coordination of both molting and vitellogenesis becomes vital to accomplishing continued body growth and increased fecundity [75]. The endocrine factors that control vitellogenesis can be considered under two categories: gonad-inhibiting and gonad-stimulating hormones.

Gonad-inhibiting hormones

Gonad-inhibiting hormones of Crustacea mainly reside in the eyestalk X-organ/sinus gland complex. The crustacean hyperglycemic hormone (CHH) superfamily of neuropeptides that mainly originate from this neuronal complex include important regulatory molecules to control somatic growth and reproduction. CHHs themselves play a pivotal role in the regulation of glucose metabolism. However, they also exhibit considerable cross-functional activities with other peptides such as MIH, VIH, and mandibular organ inhibitory hormone (MOIH). The application of peptide sequencing as well as PCR-based cloning techniques has resulted in the isolation of many cDNA sequences of CHH family members involved in diverse regulatory functions. In addition, these investigations have facilitated sequence homology studies to establish structural relationships among them. Their neuronal distribution outside eyestalk ganglia implicates other parts of CNS such as supraesophageal ganglia, thoracic ganglia and ventral nerve cord in the regulatory roles of molting and reproduction.

Vitellogenesis-inhibiting hormone

Vitellogenesis-inhibiting hormone belongs to the CHH family of neuropeptides, and shows inhibitory effects on ovarian growth and vitellogenesis. Our present understanding of endocrine regulation of crustacean vitellogenesis per se is mainly based on experimental studies involving the removal of VIH by way of eyestalk extirpation. VIH was first characterized in the American lobster *H. americanus* as a 78-residue peptide that exists as two enantiometric isoforms, both of which have a molecular mass of 9135 Da, an amidated C-terminus and a free N-terminus [76, 77]. However, the vitellogenesis-inhibiting effect was found in only one isoform when tested with an in vivo heterologous assay developed in the grass shrimp *Palaemonetes varians*. VIH has been subsequently isolated and characterized from many malacostracans, and has been shown to play a prominent role in the regulation of reproduction, especially vitellogenesis [78]. Amino acid sequence homology studies on the VIH of several crustacean species have uncovered considerable similarities with other CHH family peptides such as MIH and MOIH, claiming a separate subgroup (Type II) from the CHH molecules [79].

Bioassay studies to test VIH activity have been carried out either using an ovarian growth index [80, 81] or by in vitro culturing of ovarian tissue and monitoring the inhibition of protein synthesis [82, 83]. Inhibition of

gold-labeled vitellin binding to oocyte microvilli in incubation medium containing sinus gland extract is another bioassay method that was followed by Jugan and Soyez [53]. Another in vivo bioassay system involving the measurement of vitellogenin levels in the hemolymph by a highly sensitive sandwich enzyme-linked immunosorbent assay was employed to quantify Vg in eyestalk-ablated *P. monodon* [84]. In this species, only two of the HPLC-purified eyestalk peptide fractions were found to reduce hemolymph Vg concentrations in a time-dependent manner, suggesting their direct inhibitory effect on Vg synthetic sites. In *H. americanus* females, the highest hemolymph levels of VIH were observed during the immature and previtellogenic stages [85]. Edomi et al. [79] isolated two VIH sequences from the eyestalk of the Norway lobster *Nephrops norvegicus*. Interestingly, mRNA expression of VIH in this lobster was detected not only in the eyestalks but also in the supraoesophageal ganglia. In a recent study using double-stranded RNA (GIH-dsRNA), Treerattrakool et al. [86] knocked down GIH expression both in the eyestalk ganglia and abdominal nerve cord in *P. monodon*. This resulted in a conspicuous increase in *Vg* transcript level in the ovary of GIH-knockdown shrimp, although Vg expression in the hepatopancreas was less significant.

The inhibitory effects of eyestalk hormones with particular reference to VIH were further investigated by Tsutsui et al. [16] in another penaeid shrimp species, *M. japonicus*. Using a quantitative real-time PCR system, Vg mRNA expression levels were measured both in the hepatopancreas and the ovary in normal and eyestalk-ablated adult shrimp. Their study indicated a significant increment in mRNA levels in the ovary but not hepatopancreas, suggesting that VIH exerts its effects primarily through vitellogenin gene expression in the ovary. Hepatopancreatic gene expression may not be significantly affected by VIH, although in this shrimp, vitellogenin cDNA from the hepatopancreas is identical to that isolated from the ovary [14]. In a more recent study, Tsutsui et al. [87] isolated as many as six sinus gland peptides with vitellogenesis-inhibiting activities in the whiteleg shrimp *L. vannamei*. These VIHs caused varying degrees of inhibition in Vg mRNA expression in ovarian fragments of *M. japonicus* incubated in vitro. Marco et al. [88] have predicted that the presence of a C-terminal amide in two CHHs of *J. lalandii* could be responsible for VIH activity, based on tests done using a *P. semisulcatus* ovarian incubation system. Recently, Ohira et al. [89] produced a recombinant VIH from the American lobster *H. americanus* and tested its inhibitory activity on ovarian fragments of *M. japonicus* in a culture system. The amidated C-terminus of this recombinant neuropeptide has also been shown to be responsible for its vitellogenesis-inhibiting activity. In *M. japonicus*, cyclic nucleotides such as cAMP and cGMP, Ca^{2+}, and protein kinase C appear to serve as second messengers in mediating Vg mRNA synthesis in the ovary [90]. Cyclic AMP and cGMP probably mediate the action of VIH on Vg synthesis in the follicle cells of the ovary. In this shrimp, the responsiveness of the ovary to VIH is high during previtellogenesis, compared to the vitellogenic ovary [91].

Eyestalk ablation affecting Vg synthesis has also been demonstrated in the hepatopancreas of the giant freshwater prawn *M. rosenbergii*. In the adult female, Vg mRNA expression increases significantly in the hepatopancreas, with concurrent elevations in hemolymph Vg levels as well as gonadosomatic index [36]. More significantly, such increases in mRNA levels in the hepatopancreas, increased levels of Vg in the hemolymph, and elevated gonadosomatic index have been shown in eyestalk-ablated juvenile female prawns [92]. In the eyestalk-less isopod *Armadillidium vulgare*, VIH suppressed Vg synthesis in incubated fat body tissues [93]. Taken together, the above results are indicative of the fact that VIH acts on the target tissues such as ovary, hepatopancreas and fat body that are involved in Vg synthesis in all species investigated thus far.

Androgenic gland hormone

Vitellogenin is a female-specific hemolymph protein, and in a sense, can be said to be a secondary sexual characteristic in the reproducing female. In many submammalian vertebrates such as amphibians and fishes, Vg expression can be induced in the male liver by exogenous estrogen [94]. In crustaceans too, Vg induction has been shown in the fat body of androectomized male isopods [30, 95]. These studies have demonstrated yet another Vg-inhibiting factor that resides in the androgenic gland of male crustaceans. The VIH-like effects of androgenic gland hormone are well known in the protandric hermaphrodites. In the hermaphroditic caridean prawn *Pandalus hypsinotus*, Vg is not expressed in males and immature females, but becomes detectable from the late male phase associated with the degeneration of the androgenic glands and the appearance of vitellogenic oocytes in testicular tissues [96]. In another sexually plastic crustacean, the freshwater crayfish *C. quadricarinatus*, Vg is not expressed in intersex individuals, while transcription of the gene is induced in the hepatopancreas when the androgenic glands are removed [97, 98]. Evidently, the androgenic gland plays an essential role in negatively regulating the expression of the female-specific Vg gene in intersex individuals.

Mandibular organ inhibitory hormone

Just as the glandular Y-organ is controlled by MIH from the X-organ/sinus gland, and with the analogy of allatostatins

controlling juvenile hormone synthesis by the corpora allata in insects, the synthesis of crustacean juvenoid, MF, in the mandibular organs (MOs) is inhibited by an eyestalk neuropeptide named MOIH [99]. First described in the spider crab *Libinia emarginata*, MOIH exists in three forms, all of which repress MF synthesis to a degree of 70–80% [100]. However, the crude extract of the sinus gland showed more than 90% inhibition, suggesting that there is a combined effect of a group of neuropeptides from the eyestalk that controls MF synthesis. MOIH isoforms of *L. emarginata* have a molecular weight of 8,400 Da, while sharing other features of CHH family peptides, including N-terminal blockade by pyroglutamic acid [100]. MOIH was also characterized biochemically in the shore crab *Cancer pagurus* by Wainwrite et al. [101], who identified two isoforms (MOIH I and MOIH II) with almost identical amino acid sequences, except for the replacement of Lys in MOIH I with Glu at position 33 of MOIH II. In this crab, the sensitivity of MO to MOIH I is high at the beginning of vitellogenesis and declines drastically during peak vitellogenesis, indicating a stage-specific role for MF on Vg synthesis [102]. The mechanism of MOIH action on MF synthesis involves the inhibition of farnesoic acid *O*-methyltransferase (FAOHeT), the enzyme that catalyzes the final step of MF biosynthesis in the MOs, by affecting the methylation of FA to produce MF. In *C. pagurus*, high MF titers occur before or during early vitellogenesis, and coincide with or are preceded by elevated levels of putative FAOMeT mRNA in the MOs [103]. Sequence studies on MOIH I and II peptides of *C. pagurus* have revealed their close identity with MIH and VIH, although none of the CHH peptides exhibited MOIH activity in this crab [103]. On the other hand, in the spider crab *L. emarginata*, all the three isoforms of MOIH exhibited CHH activity when injected into the eyestalk-ablated fiddlercrab *Uca pugilator* [100]. Inhibition of MF synthesis by MOIH and other CHHs assumes greater physiological significance in view of the dual role that the MOs play in the regulation of both reproduction and molting in decapod crustaceans.

Vitellogenesis-stimulating hormones

It is possible that crustaceans employ multiple hormonal factors to positively control vitellogenesis. They may be species specific or combinatorial in action, and they are varied in chemical nature. They include (1) the neurosecretory hormones from the brain/thoracic ganglia, (2) methyl farnesoate, a structural homolog of insect juvenile hormone III, and farnesoic acid (FA), secreted by the mandibular organs, (3) ecdysteroids, and (4) a variety of steroidal hormones, including estrogen and progesterone of uncertain origin. Biogenic amines secreted from the central nervous system also seem to play a pivotal role in the control of female reproduction by influencing the secretion of both gonad-stimulatory and -inhibitory neuropeptides. Although much experimental evidence exists to implicate these hormonal factors in vitellogenesis, the action of these hormones at the level of gene transcription in Crustacea is only beginning to be understood.

Gonad-stimulating hormones

The first evidence for a gonad/vitellogenesis-stimulating principle in the central nervous system of Crustacea was obtained by Otsu [104], who noticed precocious ovarian development in the crab *Potamon dehaani* after the implantation of thoracic ganglia. Following this discovery, several attempts have been made to implant brain and thoracic ganglia or to inject their extracts to stimulate vitellogenesis in different crustacean species [105–107]. In this context, the role of differing biogenic amines in influencing the release of neurosecretary peptides from different neurosecretory neurons is relevant to understanding their integrative role in crustacean vitellogenesis. That the administration of serotonin (5-hydroxytryptamine; 5-HT) is effective at stimulating ovarian maturation was indicated first in the fiddler crab, *Uca pugilator* [108]. Subsequently, Sarojini et al. [109, 110] demonstrated in the freshwater crayfish *Procambarus clarkii* that dopamine (DA) inhibits 5-HT-stimulated ovarian maturation by inhibiting the release of gonad-stimulating hormone (GSH) from the brain or thoracic ganglia, or enhancing the release of VIH from the eyestalk neurosecretory centers. The opposing effects of 5-HT and DA on vitellogenesis were demonstrated in several other crustacean species, including the Indian spiny lobster *Panulirus homarus* [111]; but in the giant freshwater prawn *M. rosenbergii*, Chen et al. [112] provided experimental evidence that the site of action of DA is at the thoracic ganglia through the inhibition of the release of GSH, and not by the enhancement of VIH secretion.

Crustacean hyperglycemic hormones secreted from the X organ/sinus gland complex of the American lobster *H. americanus* are shown to have multiple functions, including molt inhibition and gonad stimulation [113]. In this lobster, CHH exists as two isoforms, CHH-A and CHH-B. Interestingly, both GIH and CHHs are produced in the same neuroendocrine cells. mRNA levels as well as CHH titers in the hemolymph indicate that CHH-B expression in particular peaks during intense vitellogenesis [113]. Furthermore, the hemolymph levels of GIH are high when CHH is low, and vice versa. CHH-A and CHH-B are also present in parts of the nervous system other than the optic ganglia, raising the question of whether they are the same substances as the so-called gonad-stimulating hormones of the brain and thoracic ganglia.

Molt-inhibiting hormone

The cross-functional role of the CHH family of peptides is also found with MIH of several decapod crustaceans [114]. MIH exists in two isoforms, MIH-A and MIH-B, in the penaeid shrimp *M. ensis*. Interestingly, MIH-B is expressed not only in the X-organ/sinus gland, but also in the ventral nerve cord, thoracic ganglia, and brain during vitellogenesis. The levels of MIH-B mRNA transcript in the eyestalk decrease in the initial phase of gonad maturation and increase towards the end of maturation, suggesting a stimulatory role for this neuropeptide in the initiation of vitellogenesis. Further, the injection of rMIH-B delayed the molting cycle of the maturing female [114] and increased levels of Vg mRNA expression and Vg synthesis in the ovary and hepatopancreas of this shrimp [115]. Injection of MeMIH-B dsRNA into female shrimp also caused a decrease in MeMIH-B transcript levels in the thoracic ganglia and eyestalks. Similarly, in *C. sapidus*, Zmora et al. [116] recently found that MIH titers are significantly higher in the mid-vitellogenic stages rather than in the early vitellogenic stages. While high MIH levels during intermolt suppress molt hormone synthesis by the Y-organs in the anecdysic blue crab *C. sapidus* [117], the specific elevation of MIH coincides with mid-vitellogenesis, when Vg transcription and translation is intense, which is noteworthy [117]. The stimulatory role of MIH in Vg synthesis is further substantiated by the specific binding to its receptors in the hepatopancreas followed by the modulation of a cAMP pathway involved in the Vg synthesis of *C. sapidus*. In contrast, the mode of action of MIH on the Y-organs occurs via binding to high-affinity receptors and increasing the levels of *c*GMP in *C. maenas* [118]. Actinomycin D blocks the stimulatory effects of MIH on Vg mRNA and Vg synthesis, while cycloheximide lowers only Vg levels, confirming the role of MIH in Vg transcription and translation [117]. In this way, MIH and GIH have important roles in the integrative control and coordination of molting and reproduction in decapod crustaceans. In the anecdysic crab *C. sapidus*, as well as the penaeid shrimp *M. ensis*, MIH achieves this coordination by stimulating Vg synthesis and, at the same time, extending the intermolt conditions, making it favorable for vitellogenic activities.

Gonadotropin-releasing hormone

The recent discovery of the neuropeptide gonadotropin-releasing hormone (GnRH) in the central nervous system (CNS) of *M. rosenbergii* gives further evidence that vertebrate-like steroidal control of vitellogenesis is possible in crustaceans [119]. GnRH is a well-known decapeptide initiating hormonal induction in the brain–pituitary–gonadal axis in vertebrates [120]. Several studies have reported on the occurrence of GnRH or GnRH-like peptides in diversified invertebrate phyla ranging from corals to prochordates. In many invertebrates, especially the molluscs, the synthesis of GnRH is related to reproductive activities [119]. GnRH peptides have been demonstrated by immunocytochemistry in the CNS of the giant freshwater prawn *M. rosenbergii*; however, interestingly enough, they are also found in the previtellogenic as well as early vitellogenic oocytes, suggestive of a specific role in ovarian maturation in this prawn [119]. Likewise, in the tiger prawn *P. monodon*, GnRH-I immunoreactivity was also localized to the follicular cells of proliferative, vitellogenic, and mature ovaries [121]. As expected, ir-GnRH in shrimp was more closely related to octGnRH and lGnRH-III than to other forms. Hepatopancreatic extract from *P. monodon* could induce luteinizing hormone (LH) release from rat anterior pituitary glands in vitro, demonstrating the potential role of LH in crustacean reproductive function [122]. In *M. rosenbergii* and *P. monodon*, immunoreactivity for GnRH has also been found in the neurons as well as the nerve fibres innervating such neurons in thoracic ganglia, suggesting that they regulate the synthesis and release of serotonin, as well as of GSH neuropeptides that are involved in the stimulation of oocyte maturation. Moreover, their occurrence in late previtellogenic and early vitellogenic oocytes could imply a stimulatory role for GnRH in the synthesis and release of sex steroids in the ovaries of these decapods, as reported in the protochordate *Ciona intestinalis* [123]. Alternatively, they could be involved in the control of ovarian maturation and ovulation, as in mollusks [124]. With this preliminary data, it is not possible to postulate a mechanism for GnRH action in gonadal control in Crustacea, especially in the absence of a verterbrate-like pituitary in these invertebrates. However, an early report is suggestive of the stimulation of oogenesis in the sand shrimp *Crangon crangon* by a human gonadotropin [80].

Methyl farnesoate and farnesoic acid

Hinsch [125] first revealed a functional role for MF from her observations that the active MO implants stimulated ovarian growth in the immature female spider crab *Libinia emarginata*. Subsequent measurement of MF levels in the hemolymph and MOs demonstrated increased synthesis and secretion of MF during vitellogenesis in this crab, suggesting a role in crustacean reproduction [126]. In the blue crab *Cancer pagurus*, MF concentrations in the hemolymph varied throughout ovarian development, exhibiting a peak at the beginning of secondary vitellogenesis (140 μg/ml) and falling to basal levels thereafter [102]. Rodriguez et al. [127] showed the positive effects of MF on oocyte growth when MF was injected alone or in combination with 17β-estradiol in the crayfish *Procambarus clarkii*. In addition, a higher

level of incorporation of labeled leucine was also induced by MF in isolated pieces of ovary. Obviously, MF has a positive influence on vitellogenic activity within the ovary of this crayfish. Interestingly, crayfish ovary has been repeatedly shown to engage in the autosynthesis of yolk proteins, unlike many other decapod crustaceans [1]. That MF has an influence on Vg uptake into the ovary was also revealed in the finding that MF injection activated protein kinase *C*, an isoenzyme involved in Vg uptake by oocytes and follicle cells of the crayfish *Cherax quadricarinatus* [128].

However, recent studies have indicated that MF has no effects on Vg gene expression in the hepatopancreas of shrimp (*M. ensis*), lobster (*H. americanus*), and crab (*Charybdis feriatus*) [19, 42, 43]. Using in vitro explant culture, the above studies showed that the treatment of hepatopancreas fragments with FA, a precursor to MF, resulted in the enhanced expression of the Vg gene. In a more recent study on *H. americanus*, Tiu et al. [129] revealed that the combined use of FA and 20E increased *HaVg1* gene expression synergistically in the hepatopancreas.

Previous studies indicated three major physiological effects of MF: regulation of reproduction, molting [130–132], and juvenile development [133]. Hence, in many of the reported results on reproduction, MF's effect may result from its action as a juvenilizing or molting hormone. Hence, the physiological status of the experimental animal is a significant factor in deciding its effect. However, in a recent report on the shrimp *Penaeus monodon*, Marsden et al. [134] indicated an inhibitory role for MF in the late stage of ovary development, which is unlikely to be due to its molting or juvenilizing effect. MF also has a purported role in male reproduction and sexual behavior [135–137]. Nevertheless, recent gene expression studies (reviewed above) may indicate a combinatorial effect of FA with 20E on Vg synthesis in the hepatopancreas of several decapods.

Crustaceans do not produce juvenile hormone (JH), but several workers have used exogenous JHs to induce vitellogenesis in decapod crustaceans. The effects of JH on crustacean vitellogenesis vary greatly among species. For example, methoprene inhibited vitellogenesis in the xanthid crab *Rhithropanopeus harrisii* [138], but seemed to promote vitellogenesis in the spider crab *Libinia emarginata* [127]. In the field crab *Paratelphusa hydrodromous*, Sasikala and Subramoniam [139] found a stimulatory effect of injected JH on vitellogenesis. Despite the non-occurrence of JH in crustaceans, a JH-responsive element has been recently reported in the promoter region of the Vg gene of a cladoceran, *Daphnia magna* [48]. These authors showed the occurrence of a nucleotide sequence, in-between two Vg genes, that resembled juvenile hormone-responsive and ecdysone-responsive elements in *D. magna*. When JH agonists, such as pyriproxyfen and fenoxycarb were injected, there was a strong repression of the Vg gene expression in *D. magna.* The occurrence of JH-responsive elements in *Daphnia* Vg may be only a remnant of the progressive evolution to other hormonal effector molecules that developed later in crustaceans.

Steroidal control of vitellogenesis

Ecdysteroids

Ecdysteroids are the principal hormonal factors in the inducement of molting in all arthropods. They also play a definitive role in the transcriptional activation of the Vg gene in dipteran insects and certain ticks and mites [140]. Although a similar role for ecdysteroids in crustacean vitellogenesis is inconclusive, several reports implicate its role in female reproductive activity. For example, Arvy et al. [141] found evidence that there is a rise in hemolymph ecdysteroids coincident with the initial stages of gametogenesis, e.g., oogonial and spermatogonial mitoses in the shore crab *Carcinus maenas*. In amphipods and isopods, hemolymph Vg levels parallel ecdysteroid titers during the vitellogenic cycle, suggesting a role in Vg synthesis [142]. In the shrimp *Lysmata seticaudate*, vitellogenin synthesis occurs under a high titer of ecdysone [143]. Similarly, in the freshwater prawn *M. nipponense*, Okumura et al. [144] found a close correlation between hemolymph ecdysteroid titer and the corresponding ovarian maturation stages during the reproductive molt cycle. Similarly, in the crab *E. asiatica*, the 20-hydroxy ecdysone (20E) titer in the hemolymph showed a gradual rise in the intermolt stage corresponding to ovarian maturation, whereas there was a rapid increase in 20E levels during the premolt stage [71]. In the lobster *H. americanus*, 20E could also stimulate *HaVg1* gene expression in the ovary alone or in combination with FA [42]. Notwithstanding these positive effects of ecdysteroids on vitellogenesis, Demeusy [145] showed the total noninvolvement of ecdysteroids in vitellogenesis of the shore crab *Carcinus maenas*, as Y-organ removal did not halt this process. In the anecdysic oxyrhynchan crab *Acanthonyx lunulatus*, the Y-organ degenerates at the pubertal molt, and there are then two more vitellogenic cycles that are completed in the absence of ecdysteroids [146]. In *M. rosenbergii* and *P. monodon*, both hemolymph and ovarian ecdysteroid levels declined from the immature to the late vitellogenic ovarian stages [147, 148].

The question of ecdysteroid control of vitellogenin synthesis in Crustacea can be resolved only by molecular studies pertaining to their receptor activities. In crustaceans, ecdysteroid receptor (EcR) has been identified in the blastimal tissues of regenerating limbs of *Uca pugilator*, but it dimerizes with retinoid X receptor (RXR) [149]. In another

study, Durica et al. [150] found the coexpression of these two receptors (UpEcR and UpRXR) in the ovary of *U. pugilator* during the ovarian cycle, suggesting that the ovary is a potential target tissue for ecdysteroid action. Further work is necessary to ascertain whether this receptor activity is related to the ovarian synthesis of yolk protein. Interestingly, Tokishita et al. [48] have recently described the occurrence of an ecdysone-responsive element in the upstream of the vitellogenin gene in a cladoceran, *Daphnia magna*. This, along with other binding sites for E74, E75, and those for GATA factors in the *D. magna* genome, suggest that ecdysteroids activate the transcription of *D. magna* Vg genes, as in insects [151], although this activation is antagonized by JH agonists [152]. It should be noted here that there is significant sequence homology between *D. magna* and insect Vgs (see above).

Vertebrate steroids

Vertebrate steroids such as estradiol and progesterone, together with their metabolic products, have been identified in the ovary and hepatopancreas of several decapod crustacean species [78]. These steroid hormones exhibit characteristic fluctuations during gonadal maturation, indicating a role in the control of reproduction. In the tiger prawn *Penaeus monodon*, both 17β-estradiol- and progesterone in free and conjugated forms increase in the ovary during vitellogenesis, but fall in the post-vitellogenic stages [153]. Two of their metabolic precursors, pregnenolone and dehydroepiandrosterone, also show a peak during the major vitellogenic stages within the ovary, suggesting that a biosynthetic pathway is operational in the crustacean ovary, in a similar manner to that of vertebrates. In *P. monodon*, 17β-estradiol and progesterone levels in the hemolymph, hepatopancreas and ovary were also shown to fluctuate closely with those of serum vitellogenin levels during ovarian maturation [154]. Similar fluctuations of these hormones during the ovarian cycle have also been reported in several decapods such as the brachyuran crab *S. serrata* [28], the spiny lobster *P homarus* [155], the anomuran crab *E. asiatica* [156], and the giant freshweater prawn *M. rosenbergii* [156]. In *M. rosenbergii*, such hormonal fluctuations were found only during the reproductive molt cycle, whereas during the nonreproductive molt characterized by a nondeveloping inactive ovary, the level of estradiol in the hemolymph was not detectable at any molt stage. The immature ovary and hepatopancreas showed only basal levels of estrogen during the nonreproductive molt cycle, and progesterone levels were totally undetectable. These studies suggest that estradiol potentially plays a role in crustacean vitellogenesis, either by upregulating Vg synthesis as in vertebrates, or by stimulating certain metabolic pathways initiated during vitellogenesis, such as lipogenesis and/or ion transport.

Further evidence of the influence of vertebrate steroids on vitellogenesis has been adduced from the injection of exogenous hormones. In *Penaeus japonicus*, injection of progesterone and 17α-hydroxyprogesterone induced ovarian maturation in *M. ensis* [157] and stimulated Vg secretion in *P. japonicus* [158]. In vitro culture of previtellogenic ovary of immature *M. japonicus* with 17β-estradiol resulted in the inducement of Vg synthesis into the medium, as well as the appearance of primary vitellogenic oocytes [159]. Similarly, explants of hepatopancreas of *M. ensis*, incubated in vitro with 17β-estradiol and progesterone stimulated Vg mRNA synthesis in the early vitellogenic ovary of the crayfish *Cherax albidus* [160]. Thus, 17β-estradiol and progesterone have positive effecte on Vg synthesis both in the ovary and hepatopancreas in these species. In the amphipods, a vitellogenesis-stimulating ovarian hormone (VSOH) has been proposed to induce Vg synthesis in the fat body [161]. With the knowledge that the ovary is the site of synthesis of the vertebrate sex steroids that stimulate Vg synthesis, could these steroids then be the VSOH suggested for amphipods?

Injection of exogenous estradiol resulted in the stimulation of vitellogenesis in the ovary of premature female sand crab *E. asiatica*, in addition to eliciting a new Vg fraction in the hemolymph. Significantly, *M. rosenbergii* in the nonreproductive molt cycle also initiated vitellogenesis following estradiol injection (unpublished observation). In oviparous vertebrates, the synthesis of vitellogenin is tighhtly controlled by an estrogen hormone signal transduction pathway, which is mediated by estrogen receptor and heat shock protein 90 (Hsp90) [162]. A recent report by Wu and Chu [163] on the Hsp90 activity during vitellogenesis in *M. ensis* has indicated a strong correlation between estrogen hormones and Hsp90 expression, suggesting that the expression of Vg may be under the regulation of estrogen through a mechanism similar to that in vertebrates.

The occurrence of nuclear receptors for both progesterone (PR) and estrogen (ER) has recently been reported in the freshwater crayfish *Austropotamobius pallipes* [164]. By using immunohistochemistry and western blotting approaches, these authors showed the presence of PR in the ovary and hepatopancreas and ER only in the hepatopancreas of this crayfish. Whereas ER has a direct role in the transcriptional control of Vg gene in hepatopancreas, the presence of PR in the hepatopancreas of *A. pallipes* suggests that progesterone plays a genomic role mediated by its receptor, as opposed to the hypothesized function of progesterone as a precursor of estradiol necessary for vitellogenesis [165]. EST analysis of the cDNA library established from vitellogenic ovary of *P. mondon* revealed the expression of a progesterone receptor-releated protein P23 (Pm-p23) during vitellogenesis. In situ hybridization

also indicated that Pm-p23 was localized in the ooplasm of previtellogenic oocytes [166].

Future perspectives

A proper understanding of all aspects of vitellogenesis is necessary to formulate control measures to increase egg production in commercially important decapod crustaceans. Recent molecular and immunological approaches to the study of crustacean vitellogenesis have appreciably improved our understanding of the mechanism and control of yolk formation. Gene expression studies relating to vitellogenin synthesis in different organs including the ovary have shed new light on resolving the problem of yolk precursor synthetic sites. A general consensus that has emerged from gene expression studies is that penaeid shrimp produce yolk both in ovary and hepatopancreas, whereas in many other decapods such as crabs and lobsters, vitellogenin synthesis is restricted to the hepatopancreas. Yolk synthesis within the ovary may be considered an earlier evolutionary feature in marine shrimp in view of scanty yolk in the egg combined with the reproductive behavior of free spawning and the occurrence of nauplius larvae. Conversely, crabs and other species belonging to the suborder Pleocyemata lay numerous yolk-laden eggs and incubate them in a brood until the eggs hatch into advanced zoea larvae. These forms naturally rely on the hepatopancreas for increased yolk precursor production.

Our knowledge of the hormonal control of vitellogenesis is mainly based on experimental studies involving the extirpation or implantation of endocrine organs of decapod crustaceans. With the advent of gene expression studies on Vg synthesis using in vitro culture systems, verification of hormonal influence at the transcriptional control level becomes possible. In this respect, the cross-functional activities of the CHH family of peptides have revealed their multiple controlling effects on both vitellogenesis and molting. Furthermore, hormone receptor expression studies during vitellogenesis have also adduced evidence on

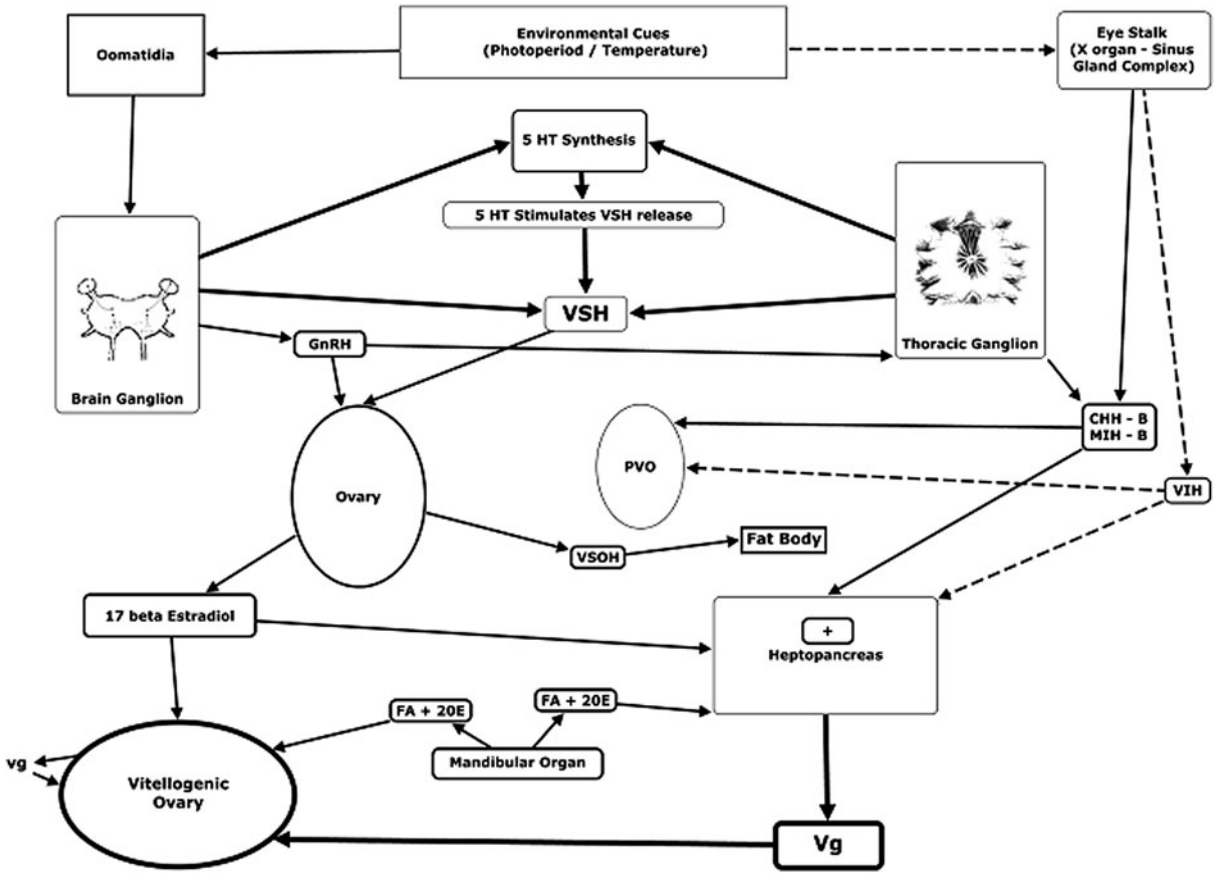

Fig. 6 A hypothetical model of the neuroendocrine control of vitellogenesis in decapod crustaceans. Different endocrine pathways included in this diagram reveal the involvement of various hormonal factors operating in different crustacean species (see main text for details). The existence of inhibitory peptides may be unique to crustaceans, but their cross-functional activity likely fine-tunes the regulation of vitellogenic activities. *PVO* previtellogenic ovary, *Vg* vitellogenin, *MF* methyl farnesoate, *FA* farnesoic acid, *20E* 20-hydroxyecdysone, *VSOH* vitellogenesis-stimulating ovarian hormone, *GnRH* gonadotropin-releasing hormone. *Solid line* stimulatory, *broken line* inhibitory

proximate endocrine factors controlling yolk precursor synthesis in several crustacean species. Vitellogenin gene expression occurring under the specific influence of FA secreted by the MOs and 20E in the hepatopancreas as well as the ovary provides direct evidence that these two organs are involved in yolk synthesis in shrimp species and to a certain extent lobsters. Analysis and identification of the hormone-responsive elements occuring in the Vg gene regulatory regions are expected to throw more light on this aspect. The identification of DNA sequences comparable to these insect hormone-responsive elements in the brachiopod *Daphnia* not only points to this possibility, but also indicates the relatedness of the hormonal regulation of vitellogenesis between insects and crustaceans. In addition, vertebrate sex steroids have been thought to have a role in the control of vitellogenesis in as much as the same hormones control egg maturation in vertebrates. Expression profiles of estrogen receptor and progestrone receptor in reproductive tissues during vitellogenesis in the freshwater crayfish lend support to the transcriptional control of Vg synthesis by estrogen in these crustaceans. Evidently, crustaceans employ multiple hormonal factors often synergistically in the control of vitellogenesis and related reproductive phenomena in order to successfully accomplish egg production without affecting somatic growth. Figure 6 depicts the hypothetical hormonal controlling mechanisms operating in typical decapod crustaceans. The occurrence of multiple hormonal factors in crustaceans to control reproduction obviously arises from the condition that both molting and reproduction occur either sequentially or in an overlapping manner in various species. Nonetheless, the evolution of endocrine regulatory mechanisms to control Vg gene expression appears to occur in the insectan line. It seems promising to imagine that the common ancestor of insects and crustaceans possessed all these hormonal factors. Whereas insects went on to use JH and 20E as the chief gonadotropic hormones in egg production, crustaceans experimented with a variety of hormonal effector molecules, probably in an attempt to augment yolk production to meet the enormous demands of embryonic nutrition. Incidentally, the use of steroidal hormones in Vg gene regulation by vertebrates would have originated from invertebrates. With all the recent advancements in understanding the endocrine control of crustacean reproduction, it is now time for the reproductive endocrinologists to use such information in their efforts to control the reproduction of commercially significant crustaceans.

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