General Discussion and Conclusion
RECOGNITION OF LDL AND VLDL BY CRAB VITELLOGENIN RECEPTOR

In the present study a membrane protein which binds Vg with high affinity was identified in the oocyte membrane extracts of *S. serrata*. In addition, by employing ligand blotting analysis and solid phase filtration assay, Vg receptor was also observed to bind LDL and VLDL of rats. All molecularly characterised Vg receptors belong to the low density lipoprotein receptor supergene family (Schonbaum *et al.*, 1995) which includes the LDL receptors, VLDL receptor and LDLR related protein (LRP). The LDLR is the patriarch of a family of cell-surface receptors that transport macromolecules into cells by receptor-mediated endocytosis in clathrin-coated pits. Chicken oocyte receptor for Vg has been demonstrated in earlier reports to bind apolipoprotein-B lipoproteins such as LDL and VLDL (George *et al.*, 1987). Subsequently, it was observed that chicken VgR also recognizes mammalian apolipoprotein E (Steyrer *et al.*, 1990). Analyses of the chicken 95 kDa Vg receptor protein revealed that this receptor mediates the endocytosis of both Vg and VLDL into growing chicken oocytes (Barber *et al.*, 1991) and that both Vg and VLDL bound at identical sites on VgR. The present investigation demonstrated the binding affinity of crab VgR to mammalian LDL and VLDL which is consistent with earlier studies elucidating the heterogeneity of ligands binding to VgR.
In addition, it was observed that this VgR was inhibited from ligand recognition by a negatively charged compound, suramin, which is a known inhibitor of the LDL receptor family of proteins. It has been shown previously that suramin, a polysulfated polycyclic hydrocarbon inhibited the recognition of Vg by its receptor in birds, amphibians (Stifani et al., 1990a), insects (Röehrkasten and Ferenz 1986) and fish (Núñez Rodriguez et al., 1996). Furthermore, in the present study it was also seen that the binding process of VgR to Vg was enhanced in the presence of excess Ca\(^{2+}\). The role of Ca\(^{2+}\) in stabilizing LDLR has been reported to be by the formation of Ca\(^{2+}\) cages (Brown et al., 1997). The evidence thus adduced from these experimental approaches suggests that the crab VgR is similar to the LDL receptor family of proteins.

The LDL receptor binds two structurally unrelated ligands, apoB and apoE. ApoB binding requires a combination of repeats 3 to 7, whereas apoE binding primarily requires repeat 5 of the LDLR. Therefore, VgR which is also a member of LDLR superfamily, may also possess binding sites for apoB and E. As previously stated, in this study it was observed that crab Vg revealed a kinship to mammalian apoB. It is thus possible that the VgR recognizes the putative apoB-like moiety of Vg which is present in LDL and VLDL. Further work on different receptor binding domains of crab VgR will elucidate the exact nature of binding of multiple ligands and their specificity to Vg receptor.
DETERMINATION OF THE MOLECULAR WEIGHT OF CRAB VgR

The oocyte membrane protein fractions were solubilized by triton and VgR was purified from the extracts by preparative electrophoresis followed by gel filtration HPLC. The molecular weight of VgR of S. serrata was thus estimated to be 230 kDa in this study. Vg receptor characterised so far in vertebrates and invertebrates have molecular weights ranging from 96 kDa to 205 kDa. In birds, VgR of chicken was estimated to be 96 kDa (Stifani et al., 1988). Recently, it was seen that among reptiles, the lizard VgR had an M, of 115 kDa (Romano and Limatola, 2000). Stifani et al. (1990a) characterised the Xenopus VgR to be a 115 kDa protein. Among fishes the M, of VgR was found to be 113 kDa in the trout, Oncorhynchus mykiss (Núñez Rodriguez et al., 1996), 100 kDa in the Mediterranean sea bass Dicentrarchus labrax (Mananos et al., 1997).

Among invertebrates, in Caenorhabditis elegans, an LDLR supergene family member was isolated from a genomic library (Yochem and Greenwald 1993). This gene which had a similarity to the mammalian LDL related protein (LRP) gene had a predicted protein of 4753 residues long. The resulting protein whose expression, however, has not yet been proven, would have a molecular weight of more than 300 kDa. In insects the VgR has been identified in a variety of species. The receptor from Aedes egyptii was thus identified to be 205 kDa glycoprotein (Dhadialla et al., 1992) whereas the VgR of the cockroach Nauphoeta cinerea was a 200 kDa protein (Indrasith et al., 1990). In Drosophila, the ovary-specific yolk protein receptor was identified to be a
protein of 210 kDa having sequence similarity to LDLR superfamily protein (Schonbaum et al., 1995). The locust receptor is apparently somewhat smaller, in the range of 156-190 kDa (Röehrkasten et al., 1989; Hafer and Ferenz 1994). In crustaceans a preliminary study on oocyte membrane protein in crayfish, Orconectus limosus reported the protein to have a low molecular weight of 30 kDa (Jugan & Van Herp, 1989). However, following this study on this crayfish, no further studies on it have been reported. In the present investigation, VgR of S. serrata was estimated, by employing gel filtration HPLC, to be a high molecular weight protein of 230 kDa. This is consistent with high molecular weight range of most invertebrate VgRs characterised thus far. These results further strengthen the hypothesis that VgR of lower forms such as arthropods is about twice the molecular mass of the VgR of oviparous vertebrates. As the Vg molecules are also larger and more complex in arthropods than in vertebrates, VgR probably requires to be large to facilitate easy accession and binding of the Vg molecules at the oocyte membrane.

ENDOCYTOTIC PATHWAY OF VITELLOGENIN IN S. SERRATA

The current study has elucidated the model of the endocytotic pathway of Vg in the oocyte by transmission electron microscopy following immunolocalization of Vg particles by using rabbit anti Lv antibodies and colloidal gold labeled anti rabbit antibodies. It was observed that in the vitellogenic ovary of S. serrata, that electron-dense endosomes had an intense packing of Vg molecules. Vg was also observed within the internal lamina of coated vesicles. This phenomenon has been well
characterised in several insects (Lauverjat et al., 1984; Telfer et al., 1982) and is best studied in *Rhodnius prolixus* (Davey, 1981) and *Manduca sexta* (Van Antwerpen et al., 1993). In this insect, dramatic enlargement of the interfollicular channels, a condition known as patency, occurs at the onset of vitellogenesis and directly correlates with the increase in Vg uptake by the ovary (Oliviera et al., 1986). Follicle cells lose patency at the time of cessation of vitellogenesis.

When compared to other serum proteins, Vg is taken up by growing oocytes 20-25 times more rapidly (Opresko and Wiley, 1987a, 1987b) during vitellogenesis. Chicken oocytes have been shown to incorporate upto 1.5 g of protein per day immediately before oviposition (Johnson, 1986). The vitellogenic crab oocytes, as observed in this study also displayed an enormous load of Vg within the oocytes containing several Vg laden endosomes.

Immunohistochemistry has been employed in several crustaceans to assess the presence of vitellin immunoreactivity in ovary and hepatopancreas (Meusy et al., 1983; Tom et al., 1987; Chen et al., 1999). By immunofluorescence microscopy and phase contrast microscopic analysis, it was observed in the tiger prawn, *Penaeus monodon*, that vitellin commences to accumulate in the secondary vitellogenic (yolk globular stage) oocytes. Vitellin was not present in the primary vitellogenic oocytes which is the pre yolk stage (Chen and Chen, 1994). It was also demonstrated that the vitellin of degenerating oocytes is transferred to the newly matured oocytes.
A number of researchers have observed rough endoplasmic reticulum in developing oocytes of crabs and other decapods (Duronslet et al., 1975; Goudeau, 1984; Rankin and Davis, 1990). Lee and Walker (1995) suggested that the developing oocytes within the ovaries are responsible for lipovitellin synthesis by ELISA and immuno-histochemistry. They demonstrated in the blue crab, *Callinectes sapidus*, the small developing oocytes reacted to anti lipovitellin antibodies localized in the perinuclear yolk-nuclear complexes. In the freshwater prawn, *Macrobrachium rosenbergii*, immunoprecipitation reactions and immunohistochemical studies revealed that hepatopancreas is the site of Vg synthesis (Chen et al., 1999). This observation was further corroborated recently by Soroka et al. (2000) by labeling the Vg particles in the hepatopancreas using antiVg antibodies and fluorescein labeled secondary antibody. The accumulation of Vg in the electron dense endosomes and the transition of endosomes from the electron lucent to dense stage as reported in the current study, has been observed in *Xenopus* oocyte development by Yoshizaki (1992), who noted that Vg gradually fills the lumen of early endosomes which is ultimately transformed to multivesicular endosome. Confinement and accumulation of Vg in the yolk granules was also demonstrated by electron microscopy in the stick insect, *Carausius morosus* (Giorgi et al., 1998).

Receptor-mediated endocytosis is now recognized as a ubiquitous mechanism for internalizing functionally important macromolecules in animal cells (Goldstein et al., 1985). Following binding, receptor complexes concentrate
inside coated pits that invaginate and pinch off to form intracellular coated vesicles. Coated vesicles carry their load into the next cellular compartment, the endosome, which plays a key role in directing subsequent intracellular routes for both the ligand and receptor (Mellman et al., 1986). The uncoupling of receptor and ligands is promoted by acidification of the endosomes followed by the recycling of receptors back to the cell surface. The internalized Vg is, however, not immediately recycled or degraded but instead in the mosquito it was observed that the Vg-laden endosomes coalesce into a transitional yolk body in which Vg undergoes condensation and begin to crystallize (Raikhel 1984). In the present study, an intense accumulation of Vg was observed in the electron dense organelle, the endosome, confirming the earlier observations that endosome is the storage organelle of endocytosed Vg.

Investigations of endocytotic organelles by previous workers have led to the conclusion that the coated vesicle is a universal organelle for macromolecular transport in eukaryotic cells (Keen, 1990; Pearse and Robinson, 1990). The coated vesicles carry the ligand/receptor complexes into the cell, become uncoated, allowing the coat component to recycle back to the plasma membrane. The uncoated vesicles fuse with one another to form early endosomes. In the current investigation Vg was observed to be localized in coated pits on the plasma membrane, the coated vesicles and the early and late endosomes. These results, clearly elucidate the route of Vg into the oocyte to be, initially, docking in the coated pits from where
it is taken to the ooplasm by coated vesicle. These vesicles then fuse to form the early endosome which on further packing of Vg becomes the electron dense mature endosome. It is, thus, apparent that the sequence of events taking place in the crab oocyte when Vg is endocytosed is identical to that observed in the other oviparous species, so far studied. Therefore, these studies will facilitate a better insight into the multistep pathway for yolk formation in the crab oocyte.

INSTABILITY AND PROTEINASE ACTIVITY OF VITELLOGENIN

It is now well established that Vg is initially synthesized as a yolk protein precursor at various candidate tissues and is thereafter secreted into the hemolymph. In crustaceans, the immunological identity of Vg and Lv have been demonstrated several times over (Chang et al., 1993; Vafopoulou and Steel, 1995). Lipovitellin is considered to be derived from Vg by subunit rearrangement and reshuffling of polypeptides caused by the proteolytic action in the oocytes. In fact, in several crustaceans a majority of the Lv polypeptides can be traced back to the precursor Vg (Wilder et al., 1994, Lee et al., 1997; Okuno et al., 2000; Pateraki and Stratakis 2000). In the present study, it was observed that Vg from the hemolymph was much more unstable than its counterpart in the ovary, lipovitellin. The sensitivity of Vg degradation and its relative instability has been reported in teleosts (Tyler and Sumpter, 1990, Goodwin et al., 1992). Furthermore, Silversand et al. (1993) observed that purified vitellogenin partly dissociated into smaller polypeptides which were immunoreactive to Vg antisera. In the current
investigation also, it was seen that the crab Vg purified by gel filtration chromatography and preparative electrophoresis degraded rapidly from a single band to three electrophoretic bands. Purified Lv, however, remained stable and resistant to degradation.

It has been difficult to obtain purified vitellogenin in fishes as a single homogenous species because on purification the polypeptide becomes partially degraded or aggregated (Kanungo et al., 1990; Tao et al., 1993; Silversand et al., 1993; Specker and Sullivan, 1994). Vitellogenin, isolated from four teleost species were found to be unstable on isolation with the degree of susceptibility varying among different species (Silversand et al., 1993). It was hypothesized that Vgs which were overtly vulnerable to degradation existed in more than one form. As vitellogenin is a lipoprotein, oxidation of acyl moieties in the lipid gives rise to carbonyls. Protein carbonyls are generated from Vg as a result of Vg being extremely susceptible to oxidative stress (Nakumura et al., 1999). Vitellogenin shares a common ancestry with enzymes and factors involved in the clotting mechanism such as the von Willebrand factor (Baker, 1988b), crayfish plasma clotting protein (Hall et al., 1999) and also proteases such as fibrinogen (Doolittle and Riley, 1990). It was observed that in crayfish, a novel LDL had proteinase activity (Komatsu and Ando, 1992) with which it was able to cleave Vg. The LDL-proteolysed Vg had a molecular weight of about 100 kDa which showed definite proteinase activity with which it could cleave native Vg. Furthermore, it was demonstrated that native Vg had no proteinase activity suggesting that the proteolytic activity of Vg was in a latent
form which was generated after digestion by ovarian LDL. Komatsu and co-workers (1996), observed that in the eel, *Anguilla japonica*, Vg displayed a protease activity only after tryptic treatment. It has been suggested that the subunit form of Vg acquires a proteinase activity because of the exposure of the cutting site caused by unmasking from the other subunits (Lee et al., 1997). Interestingly, it was observed that the apolipoprotein profile of proteolyzed Vg was similar to that of ovarian Lv, although no proteolytic activity was exhibited by Lv (Komatsu and Hayashi 1994). These results are consistent with the observations of the present study in which only Vg and not Lv exhibited a proteinase activity. Furthermore, the observations in the present study indicated that the Vg was proteolytically active at alkaline pH and behaved similar to a serine protease. These findings corroborate the previous studies on Vg from crayfish (Komatsu and Hayashi, 1994) which had a pH of 7.0 as optimum for protease activity and cleaved at the tryptic cleavage sites. Based on these reports and those observed in the present study, it can be hypothesized that Vg circulating in the hemolymph has probably a protective factor, adhering to it, and is possibly lost during the purification process. As a result, isolation and purification renders Vg to be extremely labile. It is also possible that upon internalization into the oocyte, Vg loses its natural stability in the circulating medium and becomes susceptible to degradation either by itself or other oocyte proteases to be finally processed into the stable, storage protein, the lipovitellin.
In this investigation, the relative instability of Vg was observed in the presence of a denaturing agent such as urea. It was seen that whereas Lv did not undergo any major structural change the Vg molecule was labile and readily underwent conformational changes in the presence of urea. Previous experiments have suggested that upon extensive lipid binding, a protein becomes more resistant to disrupting agents and thereby more stable (Wilson et al., 1991). The extent of lipidation of Lv is much greater than that of Vg as the internal milieu within oocyte in crustaceans contains very high reserves of lipids (Holland, 1978). Thus a lipid cage formed around Lv possibly resists the action of denaturants and proteases.

From these collective results, it is possible to conclude that the precursor vitellogenin is a relatively unstable protein when compared to its ovarian product lipovitellin. Possibly, the conformational balance achieved by Lv within the oocyte is a stable one whereas Vg probably has a more labile structure to facilitate easy degradation in the oocytes.

RELATIONSHIP OF VITELLOGENIN TO MAMMALIAN APOLIPOPROTEIN B

In this investigation, Vg and Lv purified from S. serrata were analysed for immunological identity to apolipoprotein B of mammals. Based on immunoturbidimetric assay and Western blotting employing an antiserum against apo B100, Vg and Lv were confirmed to have immunological reactivity to anti apoB antibodies. Furthermore similarity of rat LDL and VLDL, possibly by virtue
of their apoB moiety, to Vg of *S. serrata* was established. This was done by dot blot analysis of LDL and VLDL using antiserum raised against purified Vg.

The extracellular transport of water insoluble lipids is mediated by macromolecular protein-containing complexes, the lipoproteins. The assembly, secretion and metabolism of the different lipoprotein classes involve several exchangeable and nonexchangeable apolipoprotein components. In vertebrates some of these apolipoproteins are apolipoprotein B (apoB), apoA, apoC and apoE. Apo B-100 having *M*ₚ ~ 510 kDa, is part of VLDL, intermediate density lipoprotein (IDL) and LDL (Brown and Goldstein, 1986) and functions as a transporter of cholesterol and triacylglycerols to cells throughout the body. The first 2150 amino acids of apoB-100 constitute apoB-48 which is found in chylomicrons and chylomicron remnants. The biological similarities of Vg and apoB-100 strongly suggest that both these lipoproteins arose from a common ancestor. Both bind hydrophobic molecules such as phospholipids, triacylglycerols and cholesterol to transport these to target cells. Also apoB-100 and Vg bind to specific membrane receptors belonging to the same super family and enters the cell by endocytosis. Baker (1988a) suggested that the transfer of cholesterol and other lipophilic compounds to cells by apoB-100 descended from the similar actions of Vg. The degree of homology between Vg and apoB as deciphered by sequence alignment was found to be high with 12 of 16 strictly conserved residues in Vg being also conserved in human apoB. By the construction of phylogenetic trees it was shown that apoB line of proteins diverged from the vertebrate Vg line suggesting that the apoB line arose from
a Vg ancestor (Chen et al., 1997). This data was further extended by Babin et al. (1999) by showing that Vg, insect apolipoporhin II/I, apoB and microsomal triglyceride transfer protein (MTP) are members of the same multigene superfamily comprising of large lipid transfer proteins (LTP). Molecular modelling of the amino termini of apoB between aminoacids 1-587 and of MTP (amino acid residues 22-603) on the structure of crystalline lamprey Lv revealed unexpected structural and functional relationship between Vg, apoB and MTP (Mann et al., 1999). Presumably the significant structural differences between these proteins would play a role in their different lipid binding and lipid transfer properties.

The regions of similarity between vertebrate Vg and human apoB lie within the first 17 and 16 exons of Vg and apoB, respectively. The three main regions of sequence similarity are region I covering exons 3,4,5 and 6 of apoB which corresponds to exons 3,4 and 5 of chicken VgII; region II covering exons 11,12 and 13 of apoB which corresponds to part of exon 10 and exon 11 and 12 of chicken Vg II and region III covering exon 15 and part of exon 16 of apoB which corresponds to exons 15, 16 and part of exon 17 of chicken VgII. Therefore, the whole of apoB upto exon 16 share a common ancestor with the Vg gene. These observations strongly suggest the possibility that the common ancestor of Vg and apoB had more introns (Byrne et al., 1989). It was also observed that the sequences conserved between apoB and vertebrate (chicken and Xenopus) Vg are conserved in nematode Vg also. Many leucine and isoleucine residues are conserved in apoB and the Vgs of
different species. The highest degree of similarity is found in region II and the derived amino acids sequence of exon 12 is the most highly conserved between vertebrate and nematode Vg. This region, which is present conserved in apoB, probably encodes an important structural or functional domain involved in sequestering lipids. Very recently, it was reported that apoB-MTP interaction, required for the assembly of VLDL utilizes the homodimerization surfaces of Lv conserved in apoB and MTP (Shellness and Seller, 2001).

Another interesting observation regarding the conservation of apoB and Vg is a pattern in the induction of these two proteins by estrogen. It was observed that although apoB is synthesized in the small intestine, liver and kidneys in chicken, only apoB synthesized by liver is increased by estrogen (Kirchgressner et al., 1987). These findings support the theory that not only Vg coding sequence but also the estrogen response elements found upstream of Vg gene are conserved in apoB. All these findings provide an overwhelming evidence pointing to the sequence similarity and conservation among Vgs and human apoB. However, the actual functional similarity of these proteins by studying the epitope recognition of the antibodies has yet to be investigated. In crustaceans, studies on Vg in relation to its homology to lipid transporting proteins of higher order has, so far, not been performed. This study is thus one of the first investigations establishing an immunological relationship between crustacean Vg and mammalian apoB. The first requirement to support such relationship would be to demonstrate the recognition of Vg with antibodies to
human LDL, VLDL. It was seen that Vg reacted with both antiLDL and antiVLDL although reactivity with latter was less, suggesting that the common sequences of similarity between Vg and VLDL were present in lower proportions in VLDL. These considerations are compatible with fact that the proportions of apoB in VLDL is lesser than LDL. Therefore, to demonstrate that the actual link of similarity connecting these mammalian serum lipoproteins to Vg of crab is the apoB part, Vg was tested for recognition to anti apoB antibody. It was observed that apoB antibodies reacted with greater efficacy to Vg thereby providing corroborative evidence for the structural identity of apoB with Vg. Additionally, to test the converse of the recognition of rat LDL and VLDL by anti crab Vg antibodies, a dot blot analysis was done. Rat high density lipoprotein (HDL) was also included in this analysis. It was demonstrated that LDL and VLDL reacted efficiently with anti crab Vg antibodies, which was in agreement with the preposition of the existence of an immunological compatibility of mammalian LDL, VLDL with crab Vg. Contrastingly, the non-recognition of HDL by anti-Vg antibodies as revealed in dot blot analysis strongly suggests that the major protein component of HDL, the apoAI, does not share homology with Vg. Interestingly, it may be noted that although Vg is a high density lipoprotein in terms of its density in crustaceans (Lee and Puppione, 1988; Komatsu et al., 1993), the present work suggests that the crab Vg is immunologically more identical with rat very-low-density and low-density lipoproteins.
It is, therefore, apparent from these data that Vg can be considered as
the ancestor of the mammalian lipoprotein, apoB, which arose after the
divergence of chordate and invertebrate lineages (Chen et al., 1997). The
structural and functional evolution of Vg will thus provide a unifying pattern
for the invertebrate origins of the vertebrate lipid transporting system.

INFLUENCE OF STEROID HORMONES ON CRUSTACEAN
VITELLOGENESIS

The crustacean endocrine system consists of classical epithelial-type
endocrine glands and endocrine structures of neural origin (Fingerman, 1987).
Growth and reproduction, being the major energy demanding physiological
processes in Crustacea have been investigated in great detail with regard to
their endocrine regulations. The hormonal control of vitellogenesis in
crustaceans appears to be multipronged with several factors, peptides and
hormones playing a role. The classical vertebrate steroids controlling
vitellogenesis and egg maturation, estradiol and progesterone have been
reported in the recent years to influence crustacean vitellogenesis. In the
present study, the fluctuations of the levels of estradiol 17β(E2) and
progesterone (PG) were studied in the different vitellogenic stages of the crab,
Scylla serrata. It was observed by performing radioimmunoassay (RIA) that
the levels of both these hormones increased sharply as the animals entered the
vitellogenic stage. The identity of E2 and PG was further confirmed by HPLC
separation of these two hormones from the hepatopancreas. The profiles of
estradiol and progesterone were observed to follow a different pattern in the hepatopancreas, ovary and hemolymph.

In vertebrates, although the chief hormone initiating Vg synthesis is estradiol, several other hormones have been demonstrated to influence Vg expression either directly or indirectly. In fish, elevated cortisol levels associated with stressful condition was shown to decrease plasma Vg levels by reducing estrogen receptor (ER) number in the liver (Lethimonier et al., 2000). The thyroid hormone, triiodothyronine (T₃) potentiated the E2 induced activation of Vg genes and also autoinduced ER in *Xenopus* hepatocytes (Rabelo and Tata, 1997). However, the pituitary prolactin was observed to abolish this effect by blocking the thyroid hormone action. It was demonstrated that in the anuran, *Rana esculenta*, the pituitary hormones, prolactin and growth hormone (GH) have a role in the Vg induction by E2 (Carnevali and Mosconi, 1992). Very recently, it was shown that in this frog, insulin-like growth factor 1 (IGF-1) induced Vg synthesis at a faster rate than GH by following a different pathway of activation (Carnevali et al., 2000). It was observed that IGF-1 affects Vg synthesis by inducing ER, whereas GH mediated Vg synthesis relied on tyrosine kinase activation. In invertebrates, among crustaceans as in vertebrates a multihormonal and multifactorial mechanism is operational in controlling vitellogenesis (Subramoniam 2000). The occurrence of the steroids estradiol and progesterone in *S. serrata* in peak vitellogenesis as observed in this study concur with the previous reports.
displaying a correlation between E2 and PG levels and Vg synthesis (Quinitio et al., 1991; 1994; Fairs et al., 1990).

In oviparous vertebrates the synthesis of Vg in the liver is chiefly under the control of estradiol (Tata, 1978; Tata and Smith, 1979). The control of the Vg genes occurs in a time-, sex-, tissue- and hormone-specific pattern, largely as a result of interactions between cis- and trans- acting elements of the gene expression machinery (Wahlì and Ruffel, 1985). In Xenopus, Skipper and Hamilton (1977) demonstrated that pituitary gonadotropins stimulated follicle cells to secrete estrogen which reaches the liver and exerted its positive action on Vg synthesis. These workers also revealed that upon estrogen injection the hepatocytes show enormous expansion of the rough endoplasmic reticulum and Golgi apparatus needed for production and secretion of vitellogenin. In the present investigation, it was observed that the vitellogenic hepatopancreas had the highest concentration of estradiol. This suggests that similar to vertebrates, hepatopancreas of this crab is possibly the site of action of E2 in influencing vitellogenesis. In decapod crustaceans, the ovary (Browdy et al., 1990; Khayat et al., 1994), hepatopancreas (Fainzilber et al., 1992; Yang et al., 2000) and subepidermal adipose tissue (Suzuki et al., 1989; Rani and Subramoniam, 1997) have been suggested to be the Vg synthetic site. The accumulation of E2, the chief vertebrate hormone inducing Vg gene, in the hepatopancreas as revealed in the current investigation, strongly suggest that the site of Vg synthesis to be hepatopancreas.
The concentration of progesterone was observed to be highest in the vitellogenic ovary. The occurrence of progesterone in crustaceans (Yano et al., 1987; Quinitio et al., 1991; 1994) has been attributed to ovarian maturation and to the release of Vg to the hemolymph. In Xenopus, progesterone secreted by the follicle cells stimulate the breakdown of the germinal vesicle leading to ovulation (Wallace, 1984). In this study it was also observed that the circulating level of PG was high in the hemolymph, suggesting a possibility that the site of PG synthesis could be hepatopancreas wherefrom it is transported to the ovary through the hemolymph. In the ovary, PG in this crab possibly acts by stimulating oocyte maturation by initiating the germinal vesicle breakdown.

In the current investigation, the occurrence and variations in the levels of E2 and PG in the developing embryos of the mole crab, Emerita asiatica and mud crab Scylla serrata were studied. Maternal hormones are present in embryos of several vertebrates (Wilson and McNabb, 1997). Thyroid hormones are transferred into fish eggs (Brown et al., 1987; Kobuke et al., 1987) and cross the placenta in rats (Morreale de Escobar et al., 1985) to accelerate embryonic development. In the invertebrates the molt hormone, ecdysteroid has been detected in the eggs of insects (Bownes et al., 1988) and in crustaceans (Subramoniam et al., 1999). The presence of ecdysteroid in the egg has been correlated with a definite morphogenetic role in embryonic development (Bownes et al., 1988 and Espig et al., 1989). Occurrence and the role of E2 and PG in the eggs and embryo of vertebrates have been well
documented (Albrecht and Pepe, 1990). The present study demonstrates not only the presence of E2 and PG in the embryos of the crab but also a stage-specific upsurge in their levels. The concentrations of these two hormones were highest in the stage V of embryonic development of *Emerita*. In mammals, 17β estradiol in the uterus creates an overall mitogenic response by the stimulation of several pathways. It upregulates the expression of growth factors such as, PDGF (Gray *et al*., 1995), IGF-1 (Hana and Murphy, 1994) and also growth factor receptors (Gray *et al*., 1995). It also stimulates connective tissue (Lye *et al*., 1993), muscle protein actin (Hsu and Frankel, 1987) and other cytoskeletal elements. Estrogen also facilitates sexual differentiation of the brain (Olmos *et al*., 1987), differentiation of the bone (Komm *et al*., 1988) and also plays a role in cardiovascular physiology (Orimo *et al*., 1993). Progesterone promotes the growth of blood vessels in the endometrium. It also regulates LDL receptor mRNA in human placenta (Shetty, 1992) by which it regulates steroidogenesis. The function of E2 and PG in the crustacean embryo could also be to promote and sustain embryogenesis by stimulating the hormone regulated mRNA synthesis. The steep increase of the two hormones in the stage V of *Emerita* embryos is suggestive of a possible steroidogenic capacity of the embryo. This stage of embryogenesis marks the development of the eye and the appendages. Furthermore, biochemical studies demonstrated that maximum protease and esterase activity is observed in stage V (Subramoniam, 1991, 2000) implying that the sudden upheaval of the hormones at this stage
could be by the release of protein bound and conjugated steroids into the already existing general hormone pool.

An interesting aspect of the hormonal role in vitellogenesis is the ability of vitellogenin itself to bind hormones. In salmonoids, high density lipoproteins and Vg have been identified as the major carriers of thyroxine (T₄) and triiodothyronine (T₃) (Cyr and Eales, 1992 and Babin, 1992). Very recently, Monteverdi and Diguilio (2000) showed that when the estuarine killifish, Fundulus heteroclitus when exposed in vivo to radiolabeled T₄, the radioactivity was found associated with Vg in the sera. The hormones were accumulated in the oocyte subsequent to Vg uptake. In some insects the molt hormone, ecdysteroid, bind vitellin which are released temporally during embryonic development (Lagueux et al., 1981; Hoffman et al., 1986; Bownes et al., 1988). In crabs, conjugated ecdysteroids were detected in lipovitellin in the eggs of E. asiatica (Subramoniam et al., 1999). In the present study, Vg purified from the hemolymph and lipovitellin purified from the ovary and the eggs showed the presence of both 17β estradiol and progesterone. Vitellogenin, being the ancestral molecule, of the atherogenic proteins involved in lipid transport, contains hydrophobic clusters in the α-helical domain (Babin et al., 1999).

Homology of Vg to apolipoprotein B (Baker, 1988a) and microsomal triglyceride transfer protein (Mann et al., 1999) triacylglycerol lipase (Persson et al., 1989) which are involved in mammalian lipoprotein assembly has been
established. Therefore, binding of the cholesterol derivatives, progesterone and estradiol, to Vg as noted in this study possibly occurs at the lipid binding sites conserved in the vitellogenin molecule. These steroids are probably carried from the hemolymph to the oocytes and finally to the embryos. Together, these data indicate an additional function of Vg as a vector of maternal transfer of steroid hormones. The controlled release of these hormones at an appropriate time during embryogenesis imply a role in morphogenesis.