Results

Choice of diet for rearing of Spodoptera litura

Spodoptera litura is a seasonal pest and is highly prone to infections. So, the standardization of the rearing of this insect on a suitable diet was essential to ensure the availability of the insect in the laboratory, throughout the experimental period. Diet related fluctuations of some of the characteristics has been observed in various insects like Cnaphalocrocis medinalis (Tojo et al., 1985), S. litura (Itoyama et al., 1999) and B. mori (Batcha, 2002).

The study was performed by comparing some of the variable characteristics of S. litura reared under different dietary conditions, in order to use these as the yardstick for establishing the favorable diet.

As shown in the Table 1, the duration of the life cycle (with reference to the final larval stadium) was normal for the insects reared on castor leaves but comparatively longer for insects reared on artificial diets. This might be an indicator of comparatively compromised diet quality (Itoyama et al., 1999). But, there was no pronounced lengthening of the larval period in the insects reared on the diet containing soybean and red kidney bean. The values for body weight and length varied significantly for the larvae reared on the castor leaves from
those of the semi-synthetic diets. Soybean diet mix, were favorable in terms of increasing the body weight and length, comparatively shorter duration of life cycle and most significant of all the relatively low rate of mortality. The biliverdin content was taken as an indirect reference for the biliverdin-binding protein (BPs) content, along with which storage proteins are taken up by the fatbody (Yoshiga et al., 1998) that are utilized for the adult body formation (Pan and Telfer, 1996). Though in this regard, a diet of castor leaves seems to overrule the rest of the diets, the major drawback was comparatively the higher level of mortality rate.

**TABLE 1. Biology of S. litura reared on different diets**

<table>
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<th>Parameters</th>
<th>Castor fed</th>
<th>Chick pea fed</th>
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<td>Duration of life cycle (day)</td>
<td>23-37</td>
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<td>Weight of larvae*(g)</td>
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<td>Length of larvae #(cm)</td>
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<td>20-30</td>
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<td>Biliverdin content (intensity of the bluish green hue, visually determined)</td>
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* average weight of 10, fifth instar larvae
# average length of 10, fifth instar larvae
Developmental changes in the protein profile of the whole animal, fat body, haemolymph, ovary and egg samples

The whole animal profile of the TCA precipitated samples were carried out for the larval age ranging from day 3 to day 10 (II instar day 1 to V instar day 2) on 7% SDS-PAGE (Fig. 3a). The protein profile indicated that there were at least 15-18 different polypeptides resolved in the molecular weight region, ranging from 205 to 29 kDa that exhibited differential staining intensities. The intensities of silver staining were greater for the fifth instar, second day larvae, than the preceding days. This could be due to the accumulation of various proteins and also due to voracious feeding, which might have caused an overall increase in the protein content of the penultimate day of fifth instar larvae.

The electrophoretogram of the fatbody samples collected from 9 to 15 days old larvae (V instar day 1 to VI instar day 3), revealed the presence of prominently stained bands in the 200, 66, 43 and 30 kDa regions (Fig.3b). A protein band that was localized, approximately in the region of 200 kDa in the whole animal and fatbody samples (Fig.3a and 3b), was initially presumed to be Vg. The intensity of this silver stained band was the highest in the fatbody samples of the fifth instar larval stadium, after which its intensity slightly declined. The larval haemolymph (Fig. 4) too was seen to contain a polypeptide in the 200 kDa region. There was a great fluctuation in its staining intensity in the larval haemolymph too. A slight decline in the staining intensity of this band was seen during the latter days of the fifth instar, followed by a raise in the course of the final instar. There are three types of storage proteins in S. litura. SL-1 is methionine-rich, SL-2 moderately rich in methionine, and SL-3 is arylphorin, rich in aromatic amino acids (Tojo and Yoshiga, 1994;
Zheng et al., 2000). The arylphorin rich storage protein was postulated to be a source of amino acid for Vg synthesis (Chinzei et al., 1991). Thus, the study of storage proteins gains importance in this context. The storage proteins (SL) in *S. litura*, which are hexamers of 70-80 kDa subunits (Yoshiga et al., 1997), were detected in the haemolymph and fatbody of one day old sixth instar larvae. The SLs formed the bulk protein fraction in larval fatbody and haemolymph during final instar, final day. The biliverdin-binding proteins (BP), which are dimers of 165 kDa subunits (Yoshiga and Tojo, 1995) were seen right from the early larval instar in the haemolymph and appeared in the fatbody only on the final day of sixth instar. This is an interesting finding because usually all proteins in the haemolymph are generally synthesized in the fatbody and are thus seen to appear in the fatbody prior to their appearance in the haemolymph. The BP showed maximum staining intensity in the haemolymph during the fifth instar, after which there was a gradual decrease which may be accounted for, by its appearance in the final instar, final day of the larval fatbody.

Results of the Coomassie stained bands of the pupal fatbody profile (Fig. 5), revealed about 10 polypeptides with majority of them resolving in the 200, 80 and 30 kDa regions. The polypeptide in the 200 kDa region, which was presumed to be Vg (as they were generally found to fall approximately in the 200 kDa region for most of the lepidopterans), was not seen in the pupal fatbody. This finding partially ruled out the assumption that the polypeptide localized in the 200 kDa region could be Vg. A polypeptide band was observed in the 205 kDa region which was almost consistent in its staining intensity until the day 5 of pupal age after which it reduced in its concentration and disappeared completely on the day 7 of pupal age (pharate adult day). A novel
band with a molecular weight of 190 kDa appeared in the pharate adult with a simultaneous appearance of such band in the pupal haemolymph (Fig. 6). This protein was presumed to be Vg. The storage proteins in the 70-80 kDa regions were the predominant proteins that showed great variations in their staining intensities (Fig. 5 and 6). There was a slight slump in the intensity of the SL in the fatbody sample of 2 days old pupae, after which, a steady increase was observed till day 5 of pupal age and then a decline in the day 6 of pupal age followed by disappearance in the day 7 of the pupal age (Fig. 5). Thus, the SL that are synthesized and secreted in large amounts by fatbody cells during the final instar stage are selectively sequestered during pupal stage thus highlighting the most important synthetic and storage functions of the fat body tissue. The dormant pupa utilizes the storage proteins as an energy source and the rest goes in for the organogenesis of the adult moth as in the case of most of the holometabolous insects. The BP was found throughout the pupal stage except the pharate adult day.

The pupal haemolymph profile (pupal age of day ‘0’ to day ‘7’) showed the presence of about 14 polypeptides in the Coomassie blue stained gel (Fig. 6). These proteins fall in the range of 205 to 24 kDa. The polypeptides, which fall approximately in the range of 205, 190, 80 and 30 kDa were prominent all along the pupal developmental stages. The polypeptide falling approximately in the 200 kDa region presumed to be Vg disappeared in the haemolymph too, further disproving the assumption. The 205 kDa polypeptide showed higher staining intensity in the earlier stages than the later stages. In the day 5 of pupal age, the 205 kDa diminished and was sparingly seen in the day 6 of pupal age, after which it was completely exhausted in the day 7 of pupal age. A polypeptide
Fig. 3  Electrophoretic profile of the TCA precipitated whole animal proteins and fatbody protein of the larvae of *Spodoptera litura* during the developmental stages, on 7% SDS-PAGE.

(a)  TCA precipitated whole animal protein samples. Lane 1-Marker protein Lane 2- second instar, first day larvae Lane 3- second instar, second day larvae Lane 4- third instar, first day larvae Lane 5- third instar, second day larvae Lane 6- fourth instar, first day larvae Lane 7- fourth instar, second day larvae Lane 8- Fifth instar, first day larvae Lane 9- Fifth instar, second day larvae

(b)  TCA precipitated fatbody protein samples. Lane 1- fifth instar, first day larvae Lane 2- fifth instar, second day larvae Lane 3- fifth instar, third day larvae Lane 4- fifth instar, fourth day larvae Lane 5- Sixth instar, first day larvae Lane 6- Sixth instar, second day larvae Lane 7- Sixth instar, third day larvae. L3 to L15 represent the age of the larvae irrespective of the instars.

Fig. 4  Protein profile of the haemolymph by 7% SDS-PAGE, throughout the developmental period of the larvae of *Spodoptera litura*

Lane 1- fourth instar, second day larvae Lane 2- fifth instar, first day larvae Lane 3- fifth instar, second day larvae Lane 4- Fifth instar, third day larvae Lane 5- Fifth instar, fourth day larvae Lane 6- Sixth instar, first day larvae Lane 7- Sixth instar, second day Lane 8- Sixth instar, third day larvae L8 to L15 represent the age of the larvae irrespective of the instars.

Fig. 5  7% SDS-PAGE profile of the fatbody during the development of the pupae of *Spodoptera litura*

Lane 1- day ‘0’ of pupal age Lane 2- day ‘1’ of pupal age Lane 3- day ‘2’ of pupal age Lane 4- day ‘3’ of pupal age Lane 5- day ‘4’ of pupal age Lane 6- day ‘5’ of pupal age Lane 7- day ‘6’ of pupal age Lane 8- day ‘7’ of pupal age or the pharate adult day.
polypeptide by the ovaries were evident from the absence of this polypeptide in the adult ovaries (Fig. 10). This polypeptide was expected to be taken up and processed to another polypeptide in the developing oocyte. The 200 kDa polypeptide was seen in the adult fatbody and haemolymph, throughout the profile. The staining intensity of the protein diminished gradually towards the day 3 of the adult age while it was seen to sustain in the adult ovary signifying its role in the adult ovarian development. But, the identity of the protein and its site of origin and mode of uptake remains obscure and requires further investigation. The 165 kDa BP was found in the haemolymph but not in the fatbody, which implies that the BP might get deposited in the ovary straight away from the pupal haemolymph. All the proteins were fairly more intense in their staining intensities, in the day 1 adult samples, which then gradually decreased, implicating that the proteins are being utilized by the developing oocytes.

The adult ovary profile (Fig. 10) from day 0 adult to day 4 adult age unravels some interesting findings. The 190 kDa polypeptide in this stage would have been processed to vitellin (Vtn) (Choi et al., 1997). The protein presumed to be Vtn was very prominent in its staining intensity in the day 0 and day 1 of the adult age. Maximum staining intensity was noted on the day 2 of the adult age after which it diminishes through day 3 and day 4 of the adult age. This corresponds to the period when the insect prepares itself for oviposition. It apparently implies that the protein is utilized in the egg maturation. The 200 kDa polypeptide was seen all along the adult ovarian profile too, and was observed to decrease in the day 4 of the adult ovary. This strongly implicates the essential role of this polypeptide in the ovarian development. The BP was
present throughout the developmental stages of the adult ovary for its subsequent incorporation into the eggs.

The egg profile (E₀- day of laying to day of hatching-E₃) represents a prolific increase in the protein, presumed to be Vtn (Fig.11) on the E₀ day, which gradually decreased on the subsequent days and disappeared on E₃ day. The overall protein content on the E₀ day was greater and gradually reduced on the succeeding days. This suggests that the Vtn and other yolk proteins were utilized as a source of nourishment by the growing embryo. The BP were present in the egg giving it a green tinge, which signifies the role of BP in camouflage (Maruta et al., 2002). There were some low molecular weight proteins in the 30 kDa regions similar to that seen in the pupal fatbody and pupal haemolymph, which were almost consistently present throughout the developmental stages of the adult moth and egg. This protein could be microvitellogenin but its identity and function remains to be confirmed.

The apolipoprotein-II with a molecular weight of 79 kDa, was found throughout the developmental stages of S. litura, in all the tissues including ovaries and eggs except for the pharate adult haemolymph and fatbody.

**Western blotting and Molecular weight of vitellogenin**

The preliminary experiments were targeted towards the determination of the identity and molecular weight of Vg. To determine the identity of Vg, the larval haemolymph, larval fatbody, pupal haemolymph, pupal fatbody, pupal ovary, adult haemolymph, adult fatbody, adult ovary and egg samples were subjected to western blot. No signal of cross reactivity was identified in the
larval haemolymph and fatbody suggesting the absence of synthesis of Vg in the larval stages. The Vg antibody showed cross reactivity only with the polypeptide in the 190 / 180 kDa region (in the case of adult ovaries and egg) of the pharate adult haemolymph, pharate adult fatbody, adult female haemolymph, adult female fatbody, adult ovary and egg samples (Fig.12). This suggested the 190 kDa to be Vg and the 180 kDa to be Vtn. Further, from the above result the sub unit molecular weight of Vg and Vtn were established to be 190 and 180 kDa, respectively. It could also be inferred from the result that the Vg synthesis started only in the pharate adult as a immunoreactivity signal with the Vg antibody was seen to start only from the pharate adult stage and not before that. So, unlike in the cases of majority of lepidopterans, where the Vg synthesis started in the V instar larval stage like in the case of B. mori (Izumi et al., 1980 a, b), in this study, the Vg synthesis and the ovarian development was seen to be procrastinated. A relationship can be drawn between the commencement of Vg synthesis and the life span of the adult moth. When the Vg synthesis instigated at an earlier stage like in the case of B. mori, the adult moth was found to have a comparatively shorter life span in contrast to the lepidopterans in which Vg synthesis was delayed and the adult moth were found to have a prolonged life span as observed in the case of S. litura.

**Densitometric Scanning with respect to vitellogenin and vitellin**

The densitometric scan (Fig.12a) pattern of the bands corresponding to Vg/ Vtn of haemolymph, fatbody and ovary protein profile, show the peak areas of the different days of the various developmental stages (pupa, adult moth and egg) of S. litura. The results were coherent with the observations of the developmental changes with respect to Vg/Vtn in the protein profile, as
Fig. 10  7% SDS-PAGE of the ovaries during the developmental stages of the adult moth of *Spodoptera litura*, on 7% SDS-PAGE

Lane 1- Marker Lane 2- day ‘0’ of the adult moth Lane 3- day ‘1’ of the adult moth Lane 4- day ‘2’ of the adult moth Lane 5- day ‘3’ of the adult moth Lane 6- day ‘4’ of the adult moth

Fig. 11  Protein profile of the eggs of *Spodoptera litura*, by 7% SDS-PAGE, all through its developmental days

Lane 1-Marker Lane 2- day ‘0’ of egg Lane 3- day ‘1’ of the egg Lane 4- day ‘2’ of the egg Lane 5- day ‘3’ of the egg

Fig. 12  Immunoblot analysis for the presence of vitellogenin/vitellin during the different stages of development of *Spodoptera litura*, using anti-Vg antibody.

The identity of Vg was probed with Vg antibody after transferring the proteins from 7% SDS-PAGE to the NC membrane.

(12A) - Lane 1- The pharate adult haemolymph protein profile Lane 2- The pharate adult fatbody protein profile Lane 3 and Lane 4- The immunoblot of the pharate adult samples showing cross reactivity with Vg antibody.

(12B) - Lane 5- The day ‘0’ adult haemolymph protein profile Lane 6- The day ‘1’ adult fatbody protein profile Lane 7- The day ‘0’ adult ovary protein profile Lane 8, Lane 9 and Lane 10- The Vg in the pharate adult samples showing cross reactivity with Vg antibody.

(12C) - Lane 11- The day ‘1’ egg sample protein profile Lane 12- Egg sample showing a positive signal for Vtn in response to probing by Vg antibody
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| 205    | 205    |
| 97     | 97     |
| 66     | 66     |
| 43     | 43     |
| 29     | 29     |

- Vtn 180
- BP 165
- ApL 79
- 30

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- Vtn 180
- BP 165
- ApL 79
Fig. 12a  Densitometric scanning of the vitellogenin bands obtained from the 7% SDS-PAGE of the different days in the developmental profile of *Spodoptera litura*.

P7 F-Pharate adult fatbody  P7H- Pharate adult haemolymph  A0F-Day ‘0’ adult fatbody  A1F- Day ‘1’ adult fatbody  A2F- Day ‘2’ adult fatbody  A3F- Day ‘3’ adult fatbody  A0H- Day ‘0’ adult haemolymph  A1H- Day ‘1’ adult haemolymph  A2H- Day ‘2’ adult haemolymph  A0O- Day ‘0’ adult ovary  A1O- Day ‘1’ adult ovary  A2O- Day ‘2’ adult ovary  A3O- Day ‘3’ adult ovary  A4O- Day ‘4’ adult ovary  E0- day ‘0’ of egg  E1- day ‘1’ of the egg  E2- day ‘2’ of the egg  E3- day ‘3’ of the egg
reported above. Maximum peak area of 5874 and 5678 were observed for day ‘0’ and day ‘1’ of egg, respectively. It is also evident that the Vtn is completely consumed on the day ‘3’ of the egg. This emphasizes on the role of Vtn in the maturation of the egg and development of the embryo. The results also support the protein profile studies with respect to the staining intensities of Vg during the developmental stages as comprehended visually because, the trend followed by Vg is comparable to the peak areas obtained by the densitometric scan.

**Nature of vitellogenin**

Though established in general, it was found essential to determine the chemical nature of Vg in *S. litura*. A pink band was obtained in the 190 kDa region on subjecting the pharate adult haemolymph to PAS staining, thus confirming the glycoprotein nature of Vg (Fig.13A). The reddish brown staining of the 190 kDa Vg polypeptide with Oil Red ‘O’ proved the lipoprotein nature of Vg (Fig.13B). The appearance of green hue in the 190 kDa region established the phosphoprotein nature of Vg (Fig.13C). Therefore, the Vg protein was found to be phosphoglycolipoprotein as evident from the results of differential staining. Thus, Vg serves as an ideal nutritional package as it contains all major macromolecules.

**Day and time specific synthesis of vitellogenin**

ELISA was performed to determine the day and time of synthesis of Vg (Fig.14). The pupal samples (fatbody, haemolymph), adult samples (fatbody, haemolymph and ovary) and egg homogenate were subjected to ELISA. The male haemolymph of *S. litura* was used as a negative control as it was proven to be devoid of Vg (Maruta *et al.*, 2002). The female pupal haemolymph of
B. mori was used as a positive control, as it consists Vg and since vitellogenesis in B. mori had been proven to start in the fifth instar larval stage itself (Vanisree, 1998). A positive signal was obtained in the day ‘0’ of the pharate adult fatbody and haemolymph, further vouching for the earlier findings from the immunoblot, regarding the day of appearance of the Vg. No positive signals were seen in any of the samples collected from the days preceding the pharate adult stage i.e the pupal samples did not exhibit immuno reactivity with the Vg antibody suggesting the absence of synthesis of Vg in the days and time prior to the pharate adult stage. Therefore, it was confirmed that Vg is synthesized in the pharate adult day at ‘0’ hour. Thereon a positive signal for the presence of Vg was seen in the adult female fatbody, haemolymph, ovaries and egg. Though the adult ovaries and eggs contain Vtn, which is the processed form of Vg, a cross reactivity with the antibody was seen. The titre of the Vg protein in the different samples were visually recorded based on the intensity of the colour developed. The maximum titre of Vg were seen in the pharate adult fatbody at 22 hour, 24 hour, pharate adult haemolymph at 24 hour marking the active synthetic phase of the pharate adult fatbody. Similarly, maximum titre was recorded for day ‘0’ adult moth fat body, day ‘1’ adult moth fatbody, day ‘0’ adult moth ovary, day ‘1’ adult moth ovary, day ‘2’ adult moth ovary, day ‘0’ adult moth haemolymph and day ‘1’ and day ‘2’ of the egg samples while no signal was found on the day ‘3’ (Table 3), suggesting the accumulation of Vg in the early stages of adult and egg, and release or utilization of the protein in the later stages. This also implies that Vg after synthesis in the fatbody is released into the haemolymph followed by sequestration by the adult moth ovary and complete utilization by the developing egg.
Purification of vitellogenin

In order to perform oocyte membrane binding assay for Vg and to examine the specificity and binding activity, it was imperative to have pure Vg. For separation of Vg from the major haemolymph lipoprotein, lipophorin and other proteins, the female adult haemolymph was subjected to preparative ultracentrifugation in a KBr density gradient. The lipoprotein fraction floated in the upper part of the tube while other haemolymph proteins remained in the subphase. The fraction was collected with a Pasteur pipette and dialysed. The dialyzed fraction was electrophoresed on native gel. This procedure gave partially pure Vg, as it was contaminated with two unknown proteins or subunits (Fig.15A, L1). So, gel filtration chromatography (Sephadex G-200) was carried out. Elution profile obtained by gel filtration chromatogram of the lipoprotein portion obtained from the preparative ultracentrifugation showed the presence of pure Vg in the fractions from 50 to 55. The presence of other contaminating proteins was also evident from the presence of other peaks (Fig.16). The fraction numbers ranging from 50 to 55 were clubbed and concentrated. The concentrate was applied to Native PAGE and SDS-PAGE (Fig.15A, L2 and 15B, L2) to confirm the purity of Vg. Vg was established to be completely pure as only a single band was obtained in the Vg molecular weight region in both SDS and native PAGE. The native molecular weight of Vg was found to be 490 kDa and subunit molecular weight as 190 kDa. Further, these results proved Vg to be a homodimer though the possibility of an oligomer could not be ruled out.
Fig.13 Characterization of vitellogenin in *Spodoptera litura* by differential staining of the electrophoretogram of the samples subjected to 7% SDS-PAGE

(A)- Periodic Acid Schiff staining (PAS) showing the glycoprotein nature of Vg.
(B)- Oil Red ‘O’ staining to showing the lipoproteic nature of Vg
(C)- Methyl green staining showing the phosphoproteic nature of Vg

Fig.14 Direct Antigen Coating ELISA to determine the day of synthesis and approximate titre of vitellogenin, in *Spodoptera litura*

**TABLE 2** giving the details of the sample

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NC-*Spodoptera litura* male haemolymph as the Negative control BC-Buffer control PC- Silk worm, *Bombyx mori*, female pupal haemolymph as the positive control
Day ‘2’ adult moth haemolymph 39- Day ‘3’ adult moth haemolymph 40- Day ‘4’ adult moth haemolymph 41- Day ‘5’ adult moth haemolymph

TABLE 3 representing the titre of the immunoreactivity based on colour intensity recorded visually

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Approximate titre of vitellogenin based on colour intensity
+ : low intensity colour indicating a low titre of vitellogenin
++ : medium intensity colour indicating a medium titre of vitellogenin
+++ : high intensity colour indicating a high titre of vitellogenin

Fig. 15 Electrophoretic analysis of the fractions containing vitellogenin obtained during the purification procedure involving density gradient ultracentrifugation and gel filtration chromatography.

(A) 7% Native PAGE of the adult haemolymph protein of *Spodoptera litura* obtained by purification procedure
M-Marker L1- Partially purified Vg in the fraction obtained by density gradient ultracentrifugation L2- Purified Vg in the fraction obtained from gel filtration chromatography.

(B) 7% SDS-PAGE of the purified protein
M-Marker L1-day ‘1’ adult haemolymph L2- Purified Vg in the fraction obtained from gel filtration chromatography.
Fig. 16 Elution profile obtained by gel filtration (Sephadex G-200) chromatogram of the subphase acquired from density gradient ultracentrifugation. Fractions 50-55 were found to contain vitellogenin in pure form.
**Isolation of ovarian membrane protein**

To determine the ability of binding of Vg to its native oocytes receptor present in the oocytes membrane extract, the oocytes membrane proteins were isolated and subjected to Native PAGE and SDS-PAGE (Fig. 17). Three bands appeared in the Native PAGE (Fig.17A). Two of them were in the region of high molecular weight proteins (545 and 410 kDa) and one of them in the low molecular weight protein region (66 kDa). It was interesting to note that there are limited numbers of bands, which denote the fewer ovarian membrane proteins. The identity of the VgR on the SDS-PAGE and Native gel was confirmed by western blot (Fig.17B). The molecular weight of the subunits of VgR were found to be 205 kDa and that of the native VgR was found to be 410 kDa as inferred by the immunopositive signal obtained in these positions. From the molecular weights and the blot results, it can be confirmed that the VgR is a homodimer as no other band of cross reactivity with the VgR antibody in the SDS and native PAGE were observed, but the presence of oligomer cannot be dismissed.

**Receptor binding studies**

To determine the suitable conditions for binding of Vg to ovarian membrane, the variations of binding activities induced from the change in Ca$^{2+}$ concentration, Vg concentration, inhibitor concentration (Suramin) and ovarian membrane extract concentration were examined by a modification of Dot blot (Fig. 18). As the pH 7 of the binding buffer was found to trigger good binding activity, this was not varied. In these experiments only total binding was determined.
A Ca\textsuperscript{2+} ion concentration of 2 mM in the binding buffer was found to be optimum for the binding activity of Vg to the ovarian membrane extract as evident from the result obtained by plotting the densitometric scan intensity of the signal obtained on dot blot (Fig.18A and 19A). For the various amounts of ovarian membrane proteins incubated with the constant amounts of Vg, the saturation took place at 8\textmu g ovarian membrane protein. This result showed the presence of high affinity, specific binding site on the ovarian membrane for the Vg as obvious from the saturation in the Vg binding by the increasing concentration of VgR (Fig.18B and 19B). Various concentrations of Vg were used, while keeping the concentration of the VgR constant, to determine the Vg binding concentrations and to confirm specific binding activity of Vg to the VgR. A concentration of 3\mu g/\mu l was found to be the saturation point beyond which binding of Vg was not seen, further vouching for the specificity and concentration dependent binding of Vg to VgR (Fig.18C and 19C).

Since suramin, a polysulfated polycyclic hydrocarbon had been shown to be very effective in blocking the binding of lipoproteins to their receptors (Gondim and Wells, 2000) and cause their disassociation from the receptors, its effect was tested in the binding process. Suramin was found to completely abolish the binding of Vg to the ovarian membrane Vg receptor at a concentration of 5 mM as evident from the absence of the immunoreactivity signal of Vg binding to VgR, at a higher concentration of suramin (Fig.18D and 19D).
Fig. 17  Electrophoretic profile of the ovarian membrane extract of *Spodoptera litura* and western blot analysis for identification of the vitellogenin receptor

(A) 7% Native PAGE of the solubilized oocyte membrane extract
M-Marker L1- Protein profile of the oocyte membrane extract
L 2- Immunoblot showing positive signal for the presence of VgR.

(B) 7% SDS-PAGE of the solubilized oocyte membrane extract
M-Marker L1- Protein profile of the oocyte membrane extract
L 2- VgR showing positive signal for VgR antibody.

Fig 18. Receptor binding studies by modified Dot blot. Analysis of the parameters affecting binding of Vg to its receptor.

(A) Determination of the effect of Ca$^{2+}$ concentration on binding of VgR (6μg/ml) to Vg (3μg/ml) using binding buffer containing a) 1.5 mM Ca$^{2+}$ b) 2mM Ca$^{2+}$ c) 2.5 mM Ca$^{2+}$

(B) Analysis of binding activity at constant Vg concentration (3μg/ml) but membrane concentration of c) 4μg/ml d) 6μg/ml e) 8μg/ml and f) 10μg/ml of binding buffer, with a) male haemolymph as negative control b) 5μg/ml purified Vg as positive control

(C) Concentration dependent binding of Vg to its receptor at a constant membrane concentration (6μg/ml) and increasing Vg concentration of b) 2μg/ml c) 2.5μg/ml

(d) 3μg/ml (e) 3.5μg/ml and (a) 5μg/ml purified Vg as positive control

(D) Detection of the binding activity of VgR (6μg/ml) to Vg (3μg/ml) in the presence of varying concentration of suramin a) 4 mM b) 4.5mM c) 5mM and d) 5.5mM in the binding buffer.
Fig. 19 Quantitation of the dot blot result of receptor binding studies in relation to Ca$^{2+}$ concentration, vitellogenin concentration, inhibitor concentration (Suramin) and ovarian membrane extract concentration, by densitometric scanning

(A)- Plot of the effect of Ca$^{2+}$ concentration on the binding of Vg to the ovarian membrane proteins

(B)- Saturation plot of the vitellogenin binding to the oocyte membrane preparation in relation to membrane concentration.
(C) Analysis of the vitellogenin affinity for ovarian membrane preparation in the presence of increasing concentrations of \( \text{Vg} \)

![Graph showing the effect of Vg concentrations on bindability](image1.png)

(D) Plot of the effect of suramin on the binding of \( \text{Vg} \) to the ovarian membrane extract

![Graph showing the effect of suramin concentration on binding affinity](image2.png)
Organization of the ovary and distribution of vitellogenin

Fluorescent microscopy was employed to study the organization of the ovary of *S. litura* and to determine the cell-specific uptake and site-specific distribution of Vg in the ovarian tissues. From this study, sufficient information on the morphological aspects of the developing ovary of *S. litura* could be obtained which facilitated the interpretation of the results obtained for the process of receptor-mediated endocytosis. Apart from this, the present work was expected to highlight some unique aspects of embryological development of the ovaries, though the general pattern was found to be similar in the holometabolous insects (Buning, 1994). For instance, though most of the ovarian differentiation into its constituent ovarioles, takes place during late larval phase and pupation in most of the insects, in *Spodoptera*, such prominent differentiation occurred in the pharate adult and imago stage.

The fluorescein isothiocyanate tagged antibody and counter stain, Propidium iodide provided the labeling pattern for the immunofluorescence detection. The immunofluorescence signal for the presence of Vg were localized exclusively in the oocyte while the staining for PI (Propidium iodide) was entirely limited to the follicle cells and the nurse cells (Fig. 20 and 21) in the early and mid stages of ovarian development. The ovary consisted of numerous ovarioles (Fig. 30e), each containing one maturing oocyte with its associated nurse cells and follicle cells. The Fig. 22 clearly showed the presence of the follicle cells with distinct nucleus arranged in monolayer around the oocyte. It was evident from the above findings that the ovarioles were meroistic polytrophic in nature. The ovarioles were differentiated into an anterior elongated germarium, containing the youngest egg chambers, and a posterior
vitellarium containing more developed egg chambers and oocytes (Fig. 23 and 24). The distal end of each ovariole is called the terminal filament. The incomplete cytokinesis of the terminal oogonial divisions resulted in a cluster of cells called cystocytes (Fig. 23 and 24), which were seen connected by cytoplasmic bridges. One of these cells was found to be differentiated into the oocyte, and the rest became the nurse cells. The nurse cells, which become highly polyploid were noted to provide macromolecules to the developing oocytes via the intercellular bridges. The presence of Propidium iodide staining in the oocyte, vouch for this (Fig. 21). Each ovariole contained a linear series of eggs in progressive stages of maturation, giving the appearance of a "chain of beads" (Fig. 25). As the developing eggs move down the ovariole, they grow in size by absorbing yolk. The yolk proteins specifically Vg, are synthesized in the fatbody and are taken up by the oocyte as evident from fluorescein stained yolk body or endosomes (Fig. 25). The small bud like structures prominently observed in the terminal end could be the extension by undifferentiated cells. The terminal filaments suffer a retraction to the distal end due to moving forward the portion of the ovariole containing germinative cells (Fig. 25). The oocytes receive the nurse cell products and yolk proteins to become mature oocyte. The Vg accumulated together to ultimately form a large transitional yolk body (Fig. 23, 24, 26 and 27). The basal portion of the ovariole was seen to have a large globule, interpreted to be the germinative cells (Patricio and Cruz-Landim, 2002) or the oviduct (Fig. 26 and 27). The cytoplasm of the poliendric cells was clear and their nuclei presented three nucleoli (Fig. 26 and 27). A remarkable partitioning of the yolk material and the dense dot-like stained pigments were detected in the oocyte (Fig. 26 and 27). A pore sort of intracellular bridge for the transfer of nucleic acid was obvious. The oocyte
Fig. 20  Immunofluorescent localization of Vg in sections of the ovaries of *Spodoptera litura*. The FITC signal for the presence of Vg were localized exclusively in the oocyte while the staining for PI (Propidium iodide) was entirely limited to the follicle cells (F). The nuclei of the follicle (Fn), the vitelline membrane (Vm), yolk body (Yb) in the and the cytoplasm (C) are prominently seen. Scale bar-10 μm.

Fig. 21  Immunofluorescent image of the section of the ovaries of *Spodoptera litura*. The FITC signal for the presence of Vg were solely seen in the oocytes, while the staining for PI (Propidium iodide) was entirely limited to the follicle cells and nurse cells. The Vg were localized in the endocytic vesicles (E) and the transitional yolk body (TYB) in the oocyte (O), surrounded by the follicle cells (F) containing the nucleus (Fn). The presence of the outer peritoneal membrane (Pm) and the inner non-cellular tunica propria (tp) are visible. The nurse cells (Nc), compound nurse cell nucleolus (NI), intercellular bridge connecting a nurse cell to the oocyte, transfer of nucleic acid (NA) are apparent. Scale bar-10 μm.

Fig. 22  Immunofluorescent micrograph of the ovarioles that are differentiated into an anterior germarium (G), containing the youngest egg chambers with cystocytes (Cy) or nurse cells (Nc), and a posterior vitellarium (V) containing more developed egg chambers with the oocyte (O) with the transitional yolk body (TYB), surrounded by nurse cells (Nc) and follicular epithelium (F). A portion of tunica propria (tp) is seen. Scale bar-10 μm.

Fig. 23  Immunofluorescent image of the longitudinal section of a follicle showing the vitellogenic growing oocyte (O) containing the endocytic vesicle (Ev) with vitellogenin (Vg), enclosed by the vitelline membrane (Vm) surrounded by the follicle cells (F) with distinct nucleus (Fn) and nurse cells (Nc) with nucleolus (NI) in the vitellarium (V). Scale bar-10 μm.
assumed a longitudinal structure with a concave shape on one end and tapering edge on the other. A plane of bilateral symmetry was apparent along the main axis of the oocyte. May be this occurred when the oocyte was transforming into an egg or was undergoing its first meiotic division. This uneven distribution of the cytoplasm and the pigments might be considered as an indicator of qualitatively different areas in the cytoplasm of the egg. The mid axial region of the oocyte, vitally stained with Propidium iodide reflected some peculiarities in cytoplasm, which were related to the distribution of the nucleic acid. Similar observations had been made in the eggs of some amphibians (Bilinski et al., 1998).

**Receptor-mediated endocytosis**

To expound the process of receptor-mediated internalization of Vg and to substantiate the cellular organelles involved in the event, Immunogold electron-microscopic study was carried out using gold labeled anti Vg antibody and gold labeled anti VgR antibody as primary antibody and gold labeled anti rabbit secondary antibodies. Though isolated works on receptor-mediated endocytosis had been carried out in a few insects, it remains to be elaborated in many others, particularly in lepidopterans for facilitating comparison and exploitation for some beneficial purpose. Immunogold electron-microscopic analysis of the oocyte using VgR antibody is very scarce in insects and so this study was performed to determine cell-specificity of the VgR in the ovarian tissues. The complete process of receptor-mediated endocytosis has been elucidated. The invagination of the plasma membrane of the oocytes forming the early endosome, coated vesicles, late endosomes and transitional yolk body containing gold particles were evident (Fig. 28a). The presence of microvilli and
Fig. 24 Immunofluorescent image of the ovarian follicles (OF) differentiated into an anterior germarium (G), containing the youngest egg chambers with cystocytes (CY), and vitellarium (V) containing more developed egg chambers with the oocyte harboring the endosomes (E) and transitional yolk body (TYB) containing FITC labeled vitellogenin, nurse cells (NC), and the oocyte surrounded by vitelline membrane (VM). Scale bar-10 μm.

Fig. 25 Immunofluorescent micrograph showing segmentally arranged ovarioles differentiated into the terminal filaments (TF) containing the undifferentiated cells, oocyte (OC) containing numerous endocytic vesicles (E), Nurse cells (Nc) and portions of nucleolus (N11, N12 and N13), tunica propria (tp) and yolk body (YB). Scale bar-10 μm.

Fig. 26 Flourescent microscopic images of the sections of the ovaries stained with fluorescein thiocyanate and propidium iodide showing the pattern of distribution of the pigments (Pg), the pore (P) forming the intercellular bridges for the passage of Nucleic acid (NA) to the oocytes (which receive these components and grow eventually becoming mature oocytes), Nucleoli of the nurse cells (No), the presence of the Germinative cell (Gc) or the oviduct Ov) and the transitional yolk body (TYB). The uneven distribution of the cytoplasm and the pigments and the presence of nucleic acid in both the oocytes are seen. Scale bar-10 μm.

Fig. 27 Immunofluorescent micrograph of the ovarioles showing the presence of pore (P) forming the intracellular bridge connecting a nurse cell to the oocyte, a portion of the compound nuncleolus (No) of the nurse cells, transitional yolk body (TYB) and the pigments (Pg). Scale bar-10 μm.
the tubular vesicle without Vg, emanating from the transitional yolk body were discernible (Fig.28a and 28f). The early endosomes were observed to fuse with a mature granular endosome, in which Vg particles congregated to form the transitional yolk body (Fig.28b). Coated vesicles containing Vg were observed to amalgamate (Fig.28d) forming mature yolk body (Fig.28c). The distribution and utilization of vitellin in the yolk body of the egg were evident from the electron micrographs (Fig.29a to 29f). The peculiar distribution of yolk (Fig.28e), the existence of cortical granule and pigments in the yolk body were conspicuous (Fig.29c and 29d). This might in some way contribute to the uneven distribution of yolk in the egg where more yolk was observed in the animal hemisphere than in the vegetal hemisphere. These unique finding were concurrent with the findings of the Immunofluorescent studies of ovaries, though further confirmation is required (Fig.26 and 27). Spherical yolk platelets showing the concentration of Vtn at specific site in dimorphic electron dense and electron lucent regions were detectable (Fig.29a and 29b). The electron dense regions were predicted to be the pigments. Vitellophages were also prominently visible (Fig.29a and 29c). It was interesting to observe the occurrence of specialized structures derived from endocytic vesicles called the multivesicular bodies (Fig.29a). Fig.29e and 29f showed the pigment distribution and cortical granules respectively, at a higher magnification. The existence of these structures had not been described in insects. The gold labeled Vg receptors were located on the ooplasm and oocyte’s cortex indicating the mobilization of the VgR (Fig. 30a and Fig. 30c). The occurrence of large vesicles containing signals for the presence of VgR indicated that these vesicles could be transitional yolk body (Fig. 30d). The presence of the tubular vesicles (Fig. 30c), which exclusively contain VgR, were seen near a large vesicle. The
Fig. 28 Immunogold labeling of vitellogenin in the ultrathin sections of oocyte of *Spodoptera litura*, representing the process of receptor mediated endocytosis. Scale bar-1 μm.

a- The process of receptor mediated endocytosis is seen. The process starts with the invagination of the oocytes membrane (MV). Once the Vg binds to VgR, early endosomes (EE) are formed. The clathrin-coated vesicles (CV) contain the Vg bound to VgR. They dispense the Vg in the transitional yolk body (TYB) and the empty vesicles called the tubular vesicle (TV) containing the VgR alone are recycled back to the oocytes membrane, microvilli are also seen. X 15,000

b- Fusion of the early endosomes (EE) with the transitional yolk body. The labeled Vg is accumulated in the transitional yolk body (TYB). Numerous endosomes carrying Vg are seen near the TYB. X 11,000

c- An electron dense mature yolk body (MYB) containing vitellin is seen. X 12,500

d- Fusion of labeled Vg containing early endosomes to form late endosome. EF- endosome fusion. X 8, 200

e- Unique notched yolk body (YB) containing gold labeled Vg concentrated towards the upper region. X 10,000

f- The presence of follicular epithelium (F), oocyte membrane (OM), transitional yolk body (TYB), tubular vesicles (TV) and late endosome (LE) are prominently seen.
Fig. 29  Immunogold labeling of vitellin in the ultrathin sections of the egg of *Spodoptera litura*. Scale bar-1 μm.

a - Yolk platelets (Yp) showing dimorphism in the distribution of the gold labeled vitellin (Vtn). The electron dense region represents pigments (Pg). Vitellophages (Vp) containing yolk granules (Vp) and multivesicular bodies (MVB) containing specialized structures derived from endocytic vesicles are obvious. X 9500

b - Yolk platelets (YP) showing dimorphic regions and unique distribution of vitellin (Vtn) and pigments. X 8,000

c - Micrograph of egg showing layer of cortical granules (Cg), pigment (Pg), vitellophages (Vp) with vitellin, scantily distributed vitellin (Vtn) in the yolk (Y) and the presence of plasmalemma (Pl). X15,000

d - Egg in the later stages, showing the presence of sparsely distributed yolk (Y), vitellin and abundant cortical granules (Cg). X 13,000

e - Magnification of the distribution of yolk (Y) containing vitellin (Vtn) and distribution of pigment (Pg). X 9000

f - Magnification of the cortical granule (Cg) and the distribution of vitellin (Vtn) in the yolk (Y). X 11,000
large vesicle was noticed to be completely devoid of VgR signals, suggesting it to be the mature yolk body (Fig. 30c). These findings endorse the concept of recycling of the VgR after the release of Vg into the transitional yolk body and also the transition of the transitional yolk body to mature yolk body. No VgR were observed in the follicular epithelium or surrounding tissues (Fig. 30a). Fig.30f represents SEM electronmicrograph of the external view of the ovary of S. litura. This micrograph was taken out of curiosity and has not been discussed in the present work.
Fig. 30 Immunogold localization of VgR in the ultrathin sections of *Spodoptera litura* oocyte for visualization of vitellogenin receptor

a - VgR is localized on the oocyte membrane (OM) of the oocyte, in the coated vesicles and coated pits. The follicular epithelium (F) is clearly visible. Scale bar -1 μm. X 10,000

b - VgR is localized in the coated vesicles (CV). X 8,400

c - The tubular vesicle (TV) containing VgR and the mature yolk body (MYB) without the signal for VgR are visible. X 9,000

d - Transitional yolk body with VgR is apparent. X 8,000

e - Image of the ovariole (OV) in the dissected adult moth of *Spodoptera litura*. The differentiation of the ovary into its constituent ovarioles takes place during the pharate adult day. Scale bar -1 μm.

f - SEM view of a ovarian follicle dissected from adult moth O- ovarian membrane. Scale bar -1 μm. X 5,000