

## LIPID ACCUMULATION IN THE OVARIES OF A MARINE SHRIMP *PENAEUS SEMISULCATUS* (DE HAAN)

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### Summary

By the end of oocyte development, the ovaries of *Penaeus semisulcatus* have accumulated almost equal amounts (approximately 16 mg lipid g<sup>-1</sup> protein) of phospholipids and triacylglycerols. The phospholipids consist mainly of phosphatidylcholine (75–80%) and phosphatidylethanolamine (20–25%). Approximately 30% of the total fatty acid content of both phospholipids and triacylglycerols is made up of polyunsaturated fatty acids. In fractions obtained by centrifugation of ovarian homogenates, most of the increase in levels of ovarian lipids during ovarian maturation was associated with an increase in triacylglycerol levels in the floating fat fraction and of phospholipids in the infranatant fraction. The presence of polyunsaturated fatty acids in the ovaries indicates the occurrence of lipid transport to the ovary during oocyte maturation. The gradual decrease in the relative abundance of polyunsaturated fatty acids as the ovaries matured supports previously published results suggesting intra-ovarian synthesis of saturated and mono-unsaturated fatty acids.

Most of the lipids found in the female haemolymph (64.8%) were recovered in the high-density lipoprotein fraction after density ultracentrifugation. The

haemocyanin fraction recovered from this stage of fractionation contained substantial amounts of lipid (16.8%) that could be removed by further sequential centrifugation at a higher NaBr density, leaving less than 0.9% of the total haemolymph lipids associated with this fraction. While 16.2% of the lipids were recovered from the very high-density lipoprotein fractions, these lipoproteins carried only 64–89 µg lipid mg<sup>-1</sup> protein compared with 538.9 µg lipid mg<sup>-1</sup> protein in the high-density lipoprotein fraction, indicating that the high-density lipoproteins are more likely to be the main transporters of lipids to the ovary. However, the contribution of very high-density lipoproteins to lipid transport cannot be ruled out at this stage.

In this study, we present two models for lipid transport to the ovary based on the abundance of phospholipids and triacylglycerols in the haemolymph and on the amounts of polyunsaturated fatty acids accumulated within the ovary during vitellogenesis.

Key words: lipid, ovary, haemolymph, shrimp, *Penaeus semisulcatus*, high-density lipoprotein, very high-density lipoprotein.

### Introduction

Phospholipids (PLs), sterols, triacylglycerols (TAGs) and diacylglycerols (DAGs) are the main lipid classes found in marine shrimps and are associated with the maturation of oocytes and the survival of the initial larval stages (Teshima and Kanazawa, 1980a,b, 1983; Teshima et al., 1982, 1989; Kanazawa et al., 1985; Chen and Jenn, 1991; Alava et al., 1993; Chen, 1993; Xu et al., 1993). Free sterols are commonly found in developing ovaries, where they contribute to membrane structure and form precursors to hormones and steroids. Cholesterol is an essential nutrient for crustaceans since they are incapable of *de novo* synthesis of the steroid ring (Van der Oord, 1966; Zandee, 1967; Teshima and Kanazawa, 1971; Teshima et al., 1982; Kean et al., 1985). In contrast to cholesterol, PLs, TAGs and DAGs are synthesized by

crustacean tissues, including the ovary, provided that fatty acids (including polyunsaturated fatty acids; PUFAs) are supplied to these tissues (for a review, see Harrison, 1990; Shenker et al., 1993; Khayat et al., 1994b).

A dramatic increase in ovarian lipid accumulation occurs during vitellogenesis, with lipids accounting for 18–41% (in various species) of the total ovarian dry mass at the end of ovarian maturation (Teshima and Kanazawa, 1983; Castille and Lawrence, 1989; Lautier and Lagarrigue, 1988; Harrison, 1990; Lee and Walker, 1995). In most species, PLs, TAGs and sterols are the most abundant ovarian lipid classes. In the blue crab (*Callinectes sapidus*), lipid droplet, which form a minor component in immature ovaries, constitute 27% of the total lipids in mature ovaries (Lee and Walker, 1995). The fatty acid

compositions of PLs and TAGs show an abundance of PUFAs, including eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), which are considered important for the future survival of embryos (Middleditch et al., 1980; Cahu et al., 1986, 1994; Lytle et al., 1990; Teshima et al., 1992). Studies using [ $^{14}\text{C}$ ]acetate and [ $^{14}\text{C}$ ]palmitic acid have demonstrated the absence of *de novo* synthesis of PUFAs in penaeid shrimps (Kanazawa et al., 1977, 1979a,b; Castell, 1982; Shenker et al., 1993). Recent results (Mourete, 1996) indicate that the midgut gland (the hepatopancreas) and ovarian cells have a limited capacity to convert linoleic and linolenic acids to C<sub>20</sub>- and C<sub>22</sub>-PUFAs. This indicates that most of the EPA and DHA found in penaeid tissues, including the ovary, must originate from the diet.

The origin of lipids reaching the ovary is not fully understood. Lipids stored in the hepatopancreas have been shown to be transported to the ovary during vitellogenesis (Castille and Lawrence, 1989; Teshima et al., 1988; for a review, see Harrison, 1990). However, the amount of lipids accumulated within the ovaries is greater than that stored in the hepatopancreas (Castille and Lawrence, 1989). Teshima et al. (1986a,b) showed that female shrimps double their food consumption, indicating that lipids accumulating in the ovaries must originate from the food. It is not known whether these lipids pass *via* the metabolic junction in the hepatopancreas or are taken up directly from the gut.

Being insoluble in water, lipids are transported as lipoproteins in the haemolymph. So far, three lipoproteins have been identified and isolated from crustacean haemolymph, all belonging to the high-density lipoprotein (HDL) and very high-density lipoprotein (VHDL) classes (Lee and Puppione, 1978, 1988; Teshima and Kanazawa, 1980a,b; Puppione et al., 1986; Spaziani et al., 1986; Lee, 1990; Komatsu and Ando, 1992; Komatsu et al., 1993; Chen and Chen, 1993, 1994; Khayat et al., 1994b; Hall et al., 1995; Lubzens et al., 1995, 1997). The HDL fraction in haemolymph isolated from crustacean males contains lipoprotein 1 (LP1), while that from vitellogenic females contains LP1 and vitellogenin (Vg), the female-specific protein that shows immunoidentity with vitellin (Vt). Vitellin is the main lipoprotein found in mature ovaries. The LP1 from males was found to be identical with the  $\beta$ -1,3-glucan-binding protein (BGBP) associated with the immune response in crayfish (Soderhall et al., 1994). The N-terminal sequence of LP1 from male *Penaeus vannamei* was found to show high homology with the crayfish BGBP (Ruiz-Verdugo et al., 1997). The VHDL fraction was found to contain the clotting protein (Hall et al., 1995).

In the present paper, we have measured the lipid content and fatty acid distribution in the ovaries of *P. semisulcatus* throughout the course of oocyte maturation. We compare the lipids occurring in lipid droplets, infranatant and membrane fractions of immature and mature ovaries. We show that ovarian lipids contain EPA and DHA, indicating their uptake into the ovary. To provide a dynamic model explaining the mode of transport of lipids into ovaries, the relative abundance

of lipids found in the high-density lipoproteins, very high-density lipoproteins and haemocyanin was measured in the haemolymph of females. Our model is also based on previously published results describing the lipid and protein profiles of haemolymph lipoproteins (Lubzens et al., 1997), the abundance of vitellogenin and the volume of haemolymph (Shafir et al., 1992).

## Materials and methods

### Animals

Marine shrimp, *Penaeus semisulcatus* (de Haan), caught in Haifa Bay, Israel, were kept in circular tanks (3 m $\times$ 1 m, diameter  $\times$  height) in running sea water (salinity 40‰; three exchanges per day) and fed with pelleted food (prepared by Dr George Kissil, National Mariculture Center, Israel Oceanographic and Limnological Research, Eilat, Israel), bait shrimp and squid. Water temperatures ranged between 16 and 20 °C in the winter months (December–April) and between 24 and 26 °C in the summer (May–November). Under these conditions, females produced vitellogenic ovaries all year round.

### Determination of lipid content and fatty acid composition of ovaries

Ovaries were collected from intermoult females and from females immediately after moulting. They were weighed, and a small piece of tissue was removed and fixed in 4% formalin in sea water for determination of the mean oocyte diameter (AOD) as described by Shlagman et al. (1986). Ovaries removed from females immediately after moulting had an AOD close to 0  $\mu\text{m}$  ( $N=3$ ). Previtellogenic ovaries were those with an AOD within the range 46–59  $\mu\text{m}$  ( $N=4$ ), and vitellogenic ovaries had oocytes ranging in diameter from 179  $\mu\text{m}$  to 245  $\mu\text{m}$  ( $N=3$ ) in one group of samples and from 265  $\mu\text{m}$  to 398  $\mu\text{m}$  ( $N=5$ ) in a second group of samples. Late vitellogenic ovaries in which oocytes had an AOD of more than 400  $\mu\text{m}$  usually showed the presence of cortical rods (Browdy et al., 1990). The ovaries were homogenised in phosphate-buffered saline solution (0.01 mol l<sup>-1</sup>; pH 7.4) containing protease inhibitors (final concentrations of phenylsulphonyl fluoride, PMSF, 1 mmol l<sup>-1</sup>; aprotinin and leupeptin at 1  $\mu\text{g ml}^{-1}$ ). Lipids were extracted from these ovarian homogenates and the amounts of PLs, TAGs and cholesterol accumulating during ovarian development were determined (Lubzens et al., 1997). These results are presented in Figs 2–4. In additional experiments (results shown in Fig. 5), immature (early vitellogenic stage; AOD ranging between 135 and 160  $\mu\text{m}$ ) and mature (late vitellogenic stage; AOD between 450 and 630  $\mu\text{m}$ ) ovaries were fractionated after homogenization (as described above) by centrifugation (10 000 g; 4 °C for 30 min). Three fractions were collected: the top layer (or cap), containing the floating fat and lipid droplets, and the infranatant and pellet, containing the membranes. Total lipids, lipid classes (TAGs, PLs and cholesterol) and fatty acid composition were determined as described previously

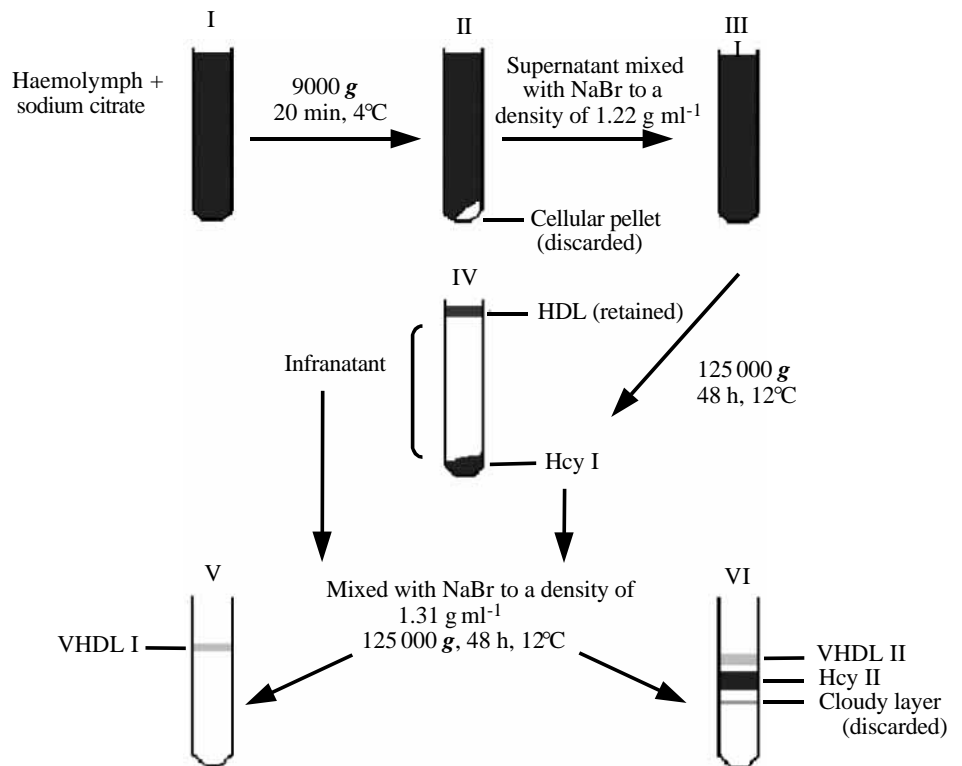
(Lubzens et al., 1997) for all homogenates and for the three fractions collected after centrifugation. Since the cap fraction contained both floating fat and infranatant, the lipid composition of the floating fat was calculated by subtracting the amounts of PLs, TAGs and cholesterol contained in the infranatant fraction. All results were corrected to give milligrams lipid per gram ovarian tissue. The lipid composition of the food provided to the shrimps was also determined.

*Fractionation of haemolymph into high-density lipoproteins (HDLs), very high-density lipoproteins (VHDLs) and haemocyanin*

Haemolymph was obtained by making a cut in the anterior rostrum and collecting haemolymph into a 10% (w/v) sodium citrate solution to avoid clotting. Haemolymph from three females was pooled to give a total volume of approximately 19 ml, and the final concentration of sodium citrate was adjusted to 1% by adding a calculated volume of 10% sodium citrate solution. The haemolymph was centrifuged at 9000g for 20 min at 4°C to remove cells (Fig. 1; step II). After removal of a 0.3 ml sample for total lipid and total protein determinations, the supernatant (17.5 ml) was subjected to NaBr gradient ultracentrifugation by raising the density of the sample to 1.22 g ml<sup>-1</sup> with a saturated NaBr solution (Fig. 1; step III) and centrifugation at 125 000g for 40–48 h at 12°C (Fig. 1; step IV) in a T-875 fixed-angle rotor of a Sorvall (Dupont, USA) ultracentrifuge (Lubzens et al., 1997). Three fractions were collected: (1) the upper orange-coloured layer

containing the HDL, (2) the bottom fluffy layer containing the haemocyanin (Hcy I fraction) and (3) the infranatant between the HDL and haemocyanin. After removal of small samples for protein (from all three fractions) and lipid determinations (from HDL and haemocyanin fractions), the infranatant and Hcy I fractions were separately subjected to further ultracentrifugation. The density of each fraction was raised to 1.31 g ml<sup>-1</sup> with a saturated NaBr solution, and ultracentrifugation was carried out as before (Fig. 1; steps V and VI). A pale orange layer designated VHDL I was obtained from the top of the infranatant fraction and was subjected to lipid extraction and analysis and to protein content determinations. The fraction containing the Hcy I (step VI) showed a pale orange upper band, a lower dark haemocyanin fluffy layer and below this a white cloudy layer that was discarded. The upper and lower fractions were collected separately into two tubes and subjected to additional ultracentrifugation at 1.31 g ml<sup>-1</sup> for 48 h as before (125 000g, 12°C; not shown in Fig. 1). Following this last centrifugation step, weak orange bands from both tubes were combined to give the VHDL II fraction. The lower layers from the two tubes were combined to give the Hcy II fraction. Both VHDL II and Hcy II fractions were subjected to lipid extraction and analysis and total protein content determinations. Lipid analysis included the determination of total lipids, the lipid class distributions (PLs, DAGs, TAGs and cholesterol) and the fatty acid distribution of glycerolipids. Total proteins were determined for each of the fractions collected using a

Fig. 1. Fractionation procedure for haemolymph samples subjected to NaBr gradient ultracentrifugation. Haemolymph samples were collected (step I), and sodium citrate (10% solution w/v) was added to a final concentration of approximately 1%. All samples contained PMSF (0.1 mmol l<sup>-1</sup> final concentration). Following a brief centrifugation to remove cells (step II), the density of the supernatant (step III) was raised to 1.21–1.22 g ml<sup>-1</sup> with NaBr. Samples were subjected to ultracentrifugation (step IV) and, after 48 h, three layers were collected: high-density lipoprotein (HDL), infranatant and haemocyanin (Hcy I). The density of the infranatant and Hcy I fractions was raised to 1.31 g ml<sup>-1</sup> with NaBr, and each fraction was subjected to ultracentrifugation for 48 h (step V for the infranatant fraction; step VI for the Hcy I fraction). The very high-density lipoprotein (VHDL I) fraction was collected (step V) from the infranatant fraction. The upper (VHDL II) and lower (haemocyanin) fractions (in step VI) were collected separately and subjected to additional ultracentrifugation at 1.31 g ml<sup>-1</sup> NaBr. The upper layers of VHDL II and of the haemocyanin-containing (Hcy II) fractions were collected and pooled.



modification of the Lowry procedure (Markwell et al., 1978) or by the method of Bradford (1976).

#### *Polyacrylamide gel electrophoresis (PAGE)*

Native, non-denatured 5% polyacrylamide gels were prepared, and samples from each of the fractions (high-density lipoproteins, very high-density lipoproteins and haemocyanin) were loaded and run as described by Khayat et al. (1994a) and Lubzens et al. (1997). Proteins were visualized by staining with Coomassie Blue.

#### *Statistical analyses*

Analysis of variance (ANOVA), Duncan's test for variability (SAS Institute, Inc., NC) and Student's *t*-tests were used to evaluate the differences in accumulation of PLs, TAGs and cholesterol and the differences in the distribution of fatty acids in PLs and TAGs during oocyte development, in ovarian homogenates and in the floating fat, infranatant and pellet fractions obtained after centrifugation. Regression analyses (see Fig. 2) were performed using SAS/STAT, and lines were plotted using SAS/Graph software (version 6, volume 2, SAS Institute Inc., NC, USA).

## Results

### *Lipid content of ovaries*

Phospholipids, TAGs and cholesterol were identified as the main lipid components of the ovaries during the process of vitellogenesis (Fig. 2). The total fresh mass of the ovaries increased from 638±187 mg in non-vitellogenic ovaries to 2618±259 mg (mean ± S.D.) at the termination of vitellogenesis (Table 1). The amounts of PLs and TAGs increased with oocyte diameter in ovaries throughout the progression of vitellogenesis. Almost equal amounts of PLs and TAGs (approximately 16 mg g<sup>-1</sup> fresh mass) were found in fully mature ovaries (AOD>250 µm; Fig. 2). There was a rapid accumulation of lipid as oocytes increased in diameter from approximately 100 µm to 300 µm. The amount of PLs in previtellogenic ovaries (oocyte diameter <100 µm) exceeds that of TAGs, which were almost completely absent from the ovaries of young females. The amount of phospholipids present increased sharply with the growth of oocytes from 100

to 300 µm in diameter and then reached a plateau. The relative amount of TAGs increased throughout vitellogenesis, almost up to the time of egg extrusion, while the relative amount of cholesterol (approximately 1 mg g<sup>-1</sup> fresh mass) did not change significantly during this period. Comparisons (Duncan's multiple-range test for variables, *P*=0.05) were made after arranging the results into four oocyte size groups on the basis of AOD (Fig. 3). The relative amounts of PLs and TAGs increased significantly at each stage of oocyte development.

The main fatty acids found in ovarian PLs and TAGs were 16:0, 18:1, 20:5 n-3 and 22:6 n-3 (Fig. 4). The percentage of 16:0 in TAGs was significantly higher than that in PLs at all stages of oocyte development (*P* values ranging from 0.03 to 0.006; *t*-test). The percentages of 16:1 n-7, 18:1 and 22:6 n-3 were significantly higher in TAGs than in PLs (*P* values ranging from 0.01 to 0.04) in non-vitellogenic (AOD 46–59 µm; Fig. 4A) and mid-vitellogenic (AOD 179–245 µm; Fig. 4B) ovaries, but these differences were not statistically different in late vitellogenic ovaries (AOD 265–398 µm; Fig. 4C). There were no differences in the abundance of 18:2 n-6 and 20:1 n-9 in PLs and TAGs at the stages tested. Phospholipids contained significantly more 18:0 than TAGs in mid-early and late vitellogenic ovaries (*P* ranged from 0.0016 to 0.02). Phospholipids also contained larger amounts of 20:5 n-3 than of TAGs, but these differences were statistically significant only for the two earlier stages of ovarian development (*P* ranged from 0.0004 to 0.01). The greater abundance of 20:4 n-6 in PLs was statistically significant only in non-vitellogenic ovaries (Fig. 4A; *P*=0.01). Statistical analyses were performed (general linear model procedure) to detect changes in the abundance of each fatty acid in PLs and TAGs at different stages of ovarian development. The analysis revealed that, for TAGs, no significant differences were found in the percentage distribution of all fatty acids, except for 22:6 n-3. The abundance of 22:6 n-3 was significantly lower in vitellogenic ovaries (Fig. 4C) than in non-vitellogenic ones (Fig. 4A; *P*=0.0507). For PLs, significant differences were found in 18:1, 20:4 n-6 and 20:5 n-3 between the early (Fig. 4A) and late (Fig. 4C) stages of oocyte development (*P* ranging from 0.0001 to 0.05).

In phospholipids, there was a 53% increase in mono-

Table 1. *The total lipid, lipid classes, polyunsaturated fatty acid and cholesterol contents of ovaries during vitellogenesis in Penaeus semisulcatus*

Mean oocyte diameter (µm)	Mass of ovaries (mg)	Lipid content (mg)				PUFAs (mg)	
		PL	TAG	Ch	Total	PL	TAG
46–59 (N=4)	638±187	3.55±1.21	1.09±0.80	0.73±0.71	5.37±1.58	1.35±0.39	0.36±0.26
179–245 (N=3)	1047±194	11.72±6.50	9.65±7.30	1.14±0.40	22.51±14.27	3.89±2.05	3.10±2.20
265–337 (N=4)	2618±259	41.25±4.84	39.65±3.88	3.26±0.50	84.15±2.92	12.82±2.13	11.96±1.03

Values for lipids and PUFAs are given as mg g<sup>-1</sup> fresh mass.

Values are means ± S.D.

PUFAs, polyunsaturated fatty acids.

Phospholipid (PL), triacylglycerol (TAG) and cholesterol (Ch) levels were determined for each ovary.

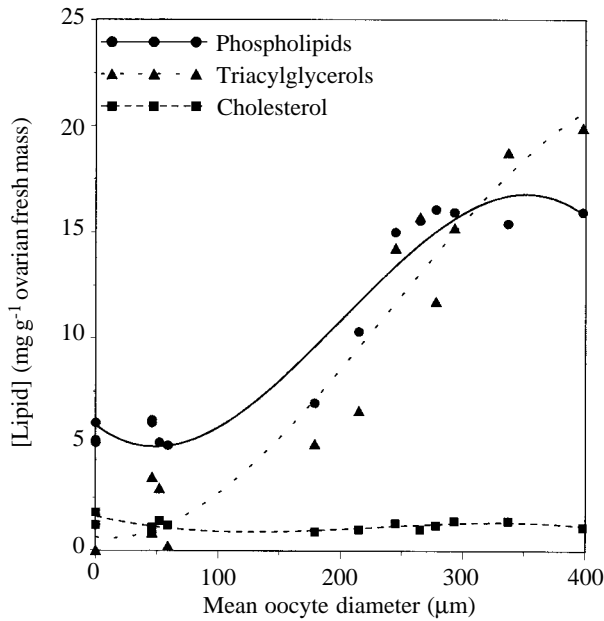


Fig. 2. The accumulation of phospholipids (PLs), triacylglycerols (TAGs) and cholesterol (Ch) ( $\text{mg g}^{-1}$  ovarian fresh mass) during oocyte development in ovaries ( $N=15$ ) related to mean oocyte diameter in *Penaeus semisulcatus*. Regression analysis was performed using SAS/STAT, and lines were plotted using SAS/Graph.  $\text{PL}=5.91-0.044x+0.0005x^2+0.00000085x^3$ ;  $\text{TAG}=0.62-0.0074x+0.0003x^2+0.00000045x^3$ ;  $\text{Ch}=1.60-0.013x-0.00007x^2+0.0000001x^3$ , where  $x$  is mean oocyte diameter.

unsaturated fatty acids (MUFAs; from 20.3 to 31.1 %) during oocyte growth, while polyunsaturated fatty acids (PUFAs) decreased by an average of 21 % (from 38.2 to 30.1 %) because of a decrease in the relative abundance of n-3 (Table 2). The increase in the amount of MUFAs ( $P=0.0001$ ) and the decrease in the amount of PUFAs ( $P=0.0009$ ) were statistically significant. The relative amount of saturated fatty acids (SFAs;  $P=0.35$ ) and the mean ratio of n-3:n-6 ( $P=0.78$ ) did not change during vitellogenesis. The distribution of SFAs, MUFAs and PUFAs did not change in the TAGs during oocyte

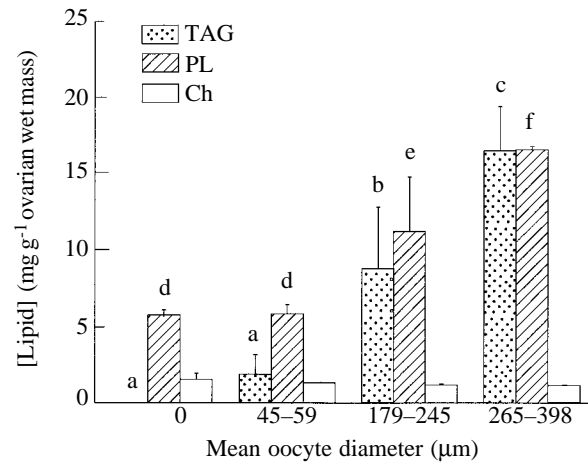


Fig. 3. Statistical differences between the amounts of triacylglycerols (TAGs), phospholipids (PLs) and cholesterol (Ch) accumulated within ovaries of *Penaeus semisulcatus* at various stages of oocyte development. The results shown in Fig. 2 were divided into four groups on the basis of oocyte diameter;  $0\ \mu\text{m}$  ( $N=3$ ),  $46-59\ \mu\text{m}$  ( $N=4$ ),  $179-245\ \mu\text{m}$  ( $N=3$ ) and  $265-398\ \mu\text{m}$  ( $N=5$ ). Bars indicate standard deviations. Mean values with different letters indicate statistically significant differences ( $P<0.05$ ) obtained by ANOVA and Duncan's test for variability.

development, but the mean ratio of n-3:n-6 changed from  $2.4\pm 1.0$  to  $2.0\pm 0.4$  ( $P=0.43$ ;  $t$ -test).

The phospholipids consisted mainly of phosphatidylcholine (PC; approximately 75 % for non-vitellogenic and 80 % for vitellogenic ovaries) and phosphatidylethanolamine (PE; 25 % for non-vitellogenic and 20 % for vitellogenic ovaries). The fatty acid analysis revealed a greater abundance of PUFAs (22.5 % of EPA and 15.8 % of DHA) in PE than in PC (10.8 % of DHA and 11.8 % of EPA), which was also reflected in a higher n-3:n-6 ratio in PE. The PC showed a relatively greater abundance of MUFAs (mainly 16:1) than of PE (results are not shown in detail).

To examine the origin of lipids in the ovary, the total amounts of lipids and of PUFAs (which are not synthesized in

Table 2. The relative distribution of saturated (SFA), mono-unsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and the n-3:n-6 ratio in the phospholipids (PL) and triacylglycerols (TAG) extracted from shrimp ovaries at various stages of oocyte development

Fatty acids	Mean oocyte diameter ( $\mu\text{m}$ )							
	0 ( $N=3$ )		46-59 ( $N=4$ )		179-245 ( $N=3$ )		265-398 ( $N=5$ )	
	PL	PL	TAG	PL	TAG	PL	TAG	
SFA (%)	21.8 $\pm$ 1.5	22.9 $\pm$ 0.9	24.2 $\pm$ 2.6	23.7 $\pm$ 1.0	25.7 $\pm$ 0.8	23.9 $\pm$ 1.8	26.4 $\pm$ 2.8	
MUFA (%)	20.3 $\pm$ 1.0	25.2 $\pm$ 2.2	29.4 $\pm$ 1.5	28.6 $\pm$ 0.7	30.4 $\pm$ 0.3	31.1 $\pm$ 2.6	31.6 $\pm$ 1.6	
PUFA (%)	38.2 $\pm$ 1.8	38.5 $\pm$ 1.7	35.0 $\pm$ 4.0	33.5 $\pm$ 1.3	32.8 $\pm$ 2.3	30.1 $\pm$ 2.7	29.2 $\pm$ 3.1	
n-3:n-6 (%)	2.0 $\pm$ 0.3	1.7 $\pm$ 0.6	2.4 $\pm$ 1.0	2.0 $\pm$ 0.4	2.9 $\pm$ 0.5	1.8 $\pm$ 0.3	2.0 $\pm$ 0.4	
Total ( $\text{mg lipid g}^{-1}$ ovarian fresh mass)	5.6 $\pm$ 0.5	5.7 $\pm$ 0.5	1.8 $\pm$ 1.3	11.1 $\pm$ 3.4	8.5 $\pm$ 4.0	16.3 $\pm$ 0.2	16.2 $\pm$ 2.9	

Previtellogenic ovaries contained negligible amounts of triacylglycerols.

\*Values are means  $\pm$  S.D.

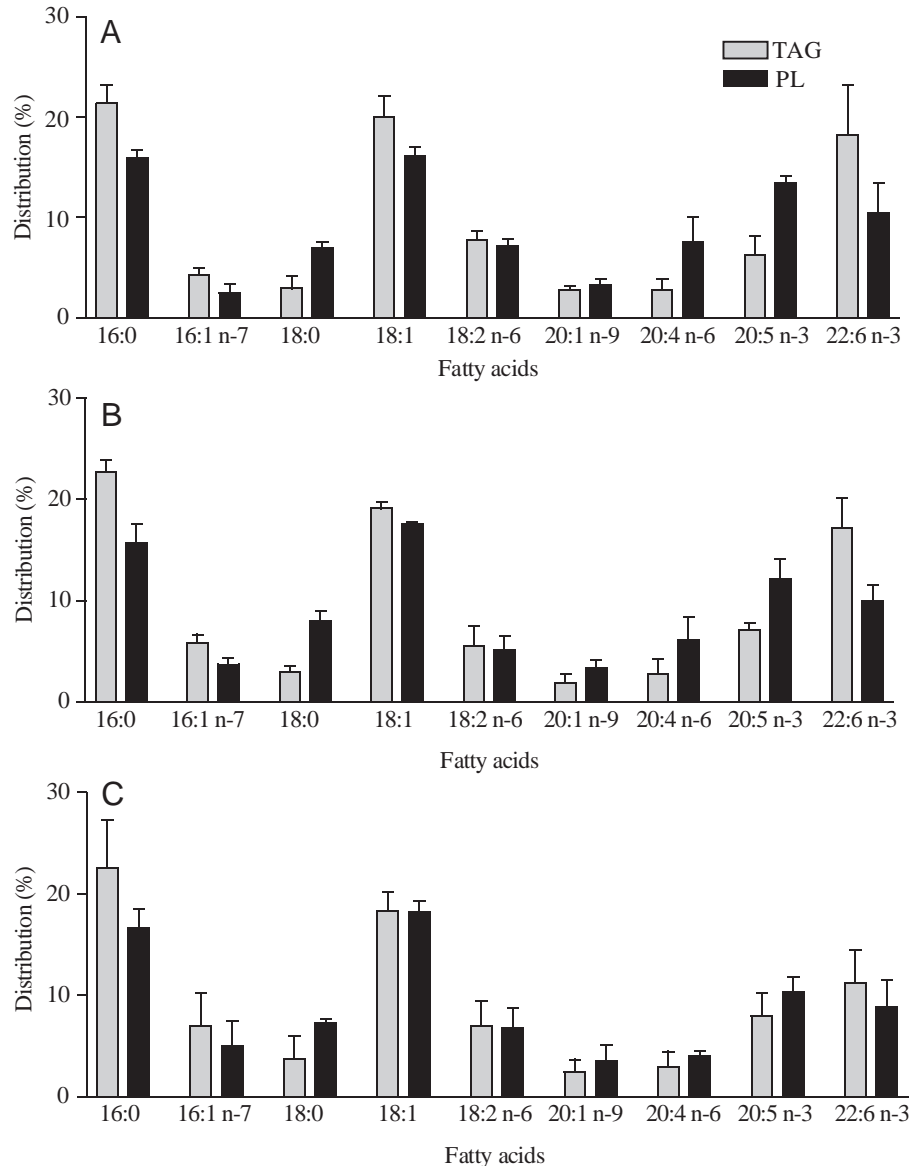


Fig. 4. The distribution of fatty acids (as a percentage) in the phospholipids (PLs) and triacylglycerols (TAGs) extracted from ovaries. Mean oocyte diameter was determined for each ovary, and the results were pooled into three groups: (A) ovaries ( $N=4$ ) in which the mean oocyte diameter (AOD) ranged from 46 to 59  $\mu\text{m}$  (non-vitellogenic); (B) ovaries ( $N=3$ ) in which the AOD was between 179 and 245  $\mu\text{m}$  (vitellogenic) and (C) ovaries ( $N=5$ ) in which the AOD was between 265 and 398  $\mu\text{m}$  (end of vitellogenesis). Bars indicate standard deviation of the means.

the ovary; Shenker et al., 1993) were calculated in ovaries at three stages of oocyte development (Table 1). High variability was observed in the lipid and PUFA contents of ovaries ranging in size from 179 to 245  $\mu\text{m}$ , reflecting the rapid accumulation of lipids in this size range, as shown in Fig. 2. The total lipid content (PLs + TAGs + cholesterol) of an ovary increased 15.7-fold, from a mean value of 5.4 mg to 84.2 mg in mature ovaries, whose mean fresh mass reached 2.6 g. The mean increase in fresh mass of the ovary over this period was only 4.1-fold. Most of the increase in lipid content occurred after the oocytes had reached a diameter of 245  $\mu\text{m}$ , coincident with a large increase in the total mass of the ovary. While the amount of phospholipids increased 11.6-fold during oocyte development, the increase in the amount of PUFAs in the phospholipid was only 9.5-fold. The proportion of phospholipids in the total lipids was reduced from 66 to 49% during vitellogenesis. Triacylglycerols increased 36.4-fold in the total lipids and 33.2-fold in the PUFAs. However, the

proportion of TAGs increased from 20.3% to 47.1% during egg formation in the ovary. The proportion of PUFAs in the total lipids was 31.8% in non-vitellogenic ovaries and 29.4% in mature ovaries.

Fractionation of homogenates from immature and mature ovaries revealed that the main increase in lipid content during development was associated with the cap and infranatant fractions. The floating fat cap contained mainly TAGs and small amounts of cholesterol (Table 3; Fig. 5). Mature ovaries contained significantly greater amounts of TAGs ( $P<0.02$ ; ANOVA) than immature ones. While the mean values for cholesterol showed a fourfold increase, this change was not statistically significant ( $P>0.47$ ; ANOVA) owing to the high variation among samples. The infranatant fraction from mature ovaries containing vitellin showed significantly higher amounts of PLs, TAGs and cholesterol ( $P$  ranging from 0.006 to 0.03). Most of the PLs ( $69.2\pm 0.03\%$ ) and cholesterol ( $62.4\pm 0.23\%$ ) of mature ovaries were found in this fraction.

Table 3. Lipid composition of ovarian homogenates and fractions (floating fat or cap, infranatant and pellet) obtained after centrifugation of immature and mature ovaries

		PL ( $\mu\text{g mg}^{-1}$ )	TAG ( $\mu\text{g mg}^{-1}$ )	Cholesterol ( $\mu\text{g mg}^{-1}$ )
Immature	Homogenates	9.0 $\pm$ 1.84	5.49 $\pm$ 2.76	1.90 $\pm$ 0.37
	Cap	0.01 $\pm$ 0.01	1.74 $\pm$ 0.71	0.05 $\pm$ 0.05
	Infranatant	2.85 $\pm$ 0.91	0.86 $\pm$ 0.50	0.42 $\pm$ 0.18
	Pellet	4.79 $\pm$ 1.69	2.43 $\pm$ 2.16	1.48 $\pm$ 0.23
Mature	Homogenates	20.48 $\pm$ 4.08	25.12 $\pm$ 2.41	2.48 $\pm$ 0.43
	Cap	0.05 $\pm$ 0.09	6.80 $\pm$ 2.24	0.22 $\pm$ 0.38
	Infranatant	12.35 $\pm$ 2.96	4.46 $\pm$ 1.82	1.35 $\pm$ 0.39
	Pellet	5.39 $\pm$ 1.15	2.56 $\pm$ 1.34	0.65 $\pm$ 0.26

Results are shown for phospholipids (PLs), triacylglycerols (TAGs) and cholesterol ( $\mu\text{g lipid mg}^{-1}$  tissue).

Values are means  $\pm$  S.D. for three replicates at each stage.

There were similar amounts of PLs and TAGs in the pelleted fraction of immature and mature ovaries ( $P=0.63$  for PLs and  $P=0.93$  for TAGs; ANOVA). However, most of the lipids of immature ovaries were located in the pelleted fraction, which contained 59.4 $\pm$ 13.7% of the total lipids and had a relatively high abundance of PLs (61.9 $\pm$ 14.1%) and cholesterol (76.9 $\pm$ 7.1%). The pelleted fraction of immature ovaries contained significantly more cholesterol (1.48 $\pm$ 0.23  $\text{mg g}^{-1}$  compared with 0.65 $\pm$ 0.26  $\text{mg g}^{-1}$  tissue) than that of mature ovaries ( $P=0.015$ ).

Squid and shrimp, provided as food for the experimental animals kept in captivity, contained substantial amounts of PUFAs. The food pellets contained 15.8% of 18:2 n-6, while squid contained 0.5% and frozen shrimp and 1.3% (results are not shown in detail). Squid and shrimp were richer in EPA (17.7% for squid and 14.8% for shrimp compared with 8.9% in food pellets) and DHA (33.5% in squid and 17.3% in shrimp compared with 8.5% in food pellets) and shrimp contained greater amounts of 20:4 n-6 (6.7% compared with 1.9% in pellets and 1.7% in squid).

#### Lipid and protein content of the haemolymph, HDL, VHDL and haemocyanin fractions

The total amount of glycerolipids in the female haemolymph sample was 1288.4  $\mu\text{g ml}^{-1}$  at 10.5  $\mu\text{g mg}^{-1}$  protein, and the total amount of cholesterol was 193.7  $\mu\text{g ml}^{-1}$  at 1.6  $\mu\text{g mg}^{-1}$  protein (Table 4). Most of the haemolymph lipids, 62.7% of the glycerolipids and 78.4% of the cholesterol, were recovered in the HDL layer, which contained only 1.4% of the haemolymph proteins (Fig. 1, step IV). Thus, 1 mg of protein contained 453.7  $\mu\text{g}$  of glycerolipids and 85.2  $\mu\text{g}$  of cholesterol. Most of the haemolymph proteins were found in the haemocyanin (Hcy I) fluffy precipitate, and this fraction contained 17.9% of the total glycerolipids and 9.8% of the total cholesterol. The interface layer ('infranatant' in Fig. 1; step IV), between the top HDL fraction and the bottom haemocyanin I layer, contained the VHDL I fraction, which was collected after centrifugation at higher salt density (1.31  $\text{g ml}^{-1}$ ). The VHDL I fraction

contained 5.1% of the total haemolymph lipids at 89.3  $\mu\text{g}$  total lipid  $\text{mg}^{-1}$  protein. This fraction consisted mainly of the clotting protein (visualized as a typical ladder in polyacrylamide gel electrophoresis; PAGE) and small amounts of haemocyanin (Fig. 6). The haemocyanin I fraction, centrifuged at a higher NaBr density (1.31  $\text{g ml}^{-1}$ ), resulted in the separation of a VHDL II fraction which contained 64.4  $\mu\text{g lipid mg}^{-1}$  protein (9.3% of total haemolymph lipids). The haemocyanin II fraction, which was collected after two ultracentrifugations steps, contained only trace amounts of lipids (less than 0.9% of the total haemolymph lipids) and was almost completely devoid of clotting protein (Fig. 6). No lipids were found in haemocyanin extracted from gels after native PAGE. All lipid fractions (HDL, VHDL I and VHDL II) contained mainly PLs (74.1%

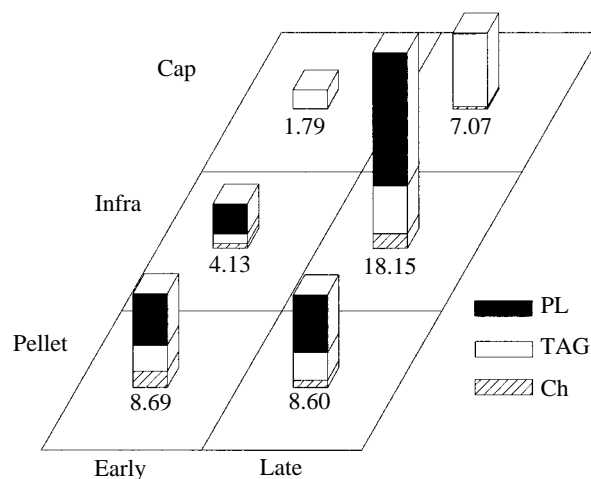


Fig. 5. Distribution of glycerolipids (TAG, PL) and cholesterol (Ch) in the floating fat (cap), infranatant and pellet fractions obtained by centrifugation of ovarian homogenates of immature (Early; mean oocyte diameter ranging from 135 to 160  $\mu\text{m}$ ,  $N=3$ ) and mature (Late; mean oocyte diameter ranging from 450 to 630  $\mu\text{m}$ ;  $N=3$ ) ovaries. The values given below each column show the total amount of lipids (glycerolipids and cholesterol) in each fraction ( $\text{mg g}^{-1}$  ovarian tissue). PL, phospholipid; TAG, triacylglycerol.

Table 4. Distribution of lipids and proteins in the haemolymph and fractions obtained by NaBr gradient ultracentrifugation

Fraction	Proteins (% of total <sup>a</sup> )	Glycerolipids			Cholesterol			Total lipids ( $\mu\text{g mg}^{-1}$ protein)
		( $\mu\text{g ml}^{-1}$ ) <sup>b</sup>	(% of total) <sup>c</sup>	( $\mu\text{g mg}^{-1}$ protein)	( $\mu\text{g ml}^{-1}$ )	(% of total)	( $\mu\text{g mg}^{-1}$ protein)	
Total haemolymph		1288.4		10.5	193.7		1.6	12.1
HDL	1.4	808.3	62.7	453.7	151.9	78.4	85.2	538.9
Hcy I	77.8	230.4	17.9	2.4	19.0	9.8	0.2	2.6
VHDL I	0.7	66.0	5.1	77.6	9.4	4.9	11.7	89.3
VHDL II	1.9	154.3	12.0	59.7	10.7	5.5	4.7	64.4
Hcy II	58.8	11.6	0.9	0.14	0	0	0	0.14

The initial haemolymph volume was 17.5 ml and the protein content was  $123 \text{ mg ml}^{-1}$ .

<sup>a</sup>Only 62.7% of the proteins were recovered after fractionation.

<sup>b</sup>Results were corrected for the initial haemolymph volume.

<sup>c</sup>98.6% of the original glycerolipids and cholesterol were recovered after fractionation.

Fractions are defined in Fig. 1.

in the HDL, 85.4% in VHDL I and 88.7% in VHDL II) and DAGs (11.3–15.9%). Triacylglycerols were found only in the HDL (7.5%) and VHDL I (3.0%) fractions. The distribution of cholesterol among the different lipoprotein fractions is shown in Table 4, which also shows that cholesterol constituted 13.1% of the total lipids in the VHDL I and 7.3% in VHDL II but 15.8% in the HDL fraction. No major differences were found in the fatty acid distribution (results not shown in detail) in the HDL, VHDL I and VHDL II: all contained 20:4 n-6 (2.3–2.5%), 20:5 n-3 (7.1–9.4%) and 22:6 (10.2–14.5%). Most of the lipids (98.6%), but only 62% of the proteins, were recovered during fractionation.

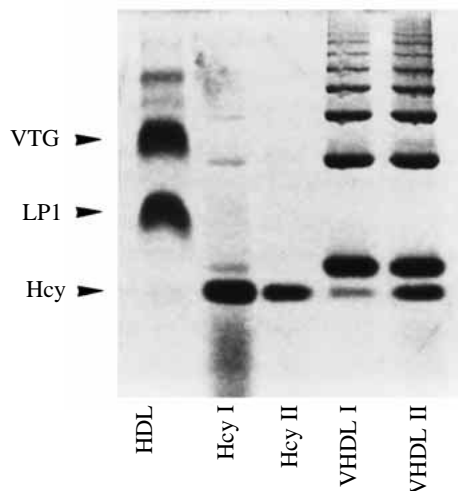


Fig. 6. The protein profiles of the high-density lipoprotein (HDL), very high-density lipoprotein (VHDL I and VHDL II) and haemocyanin (Hcy I and Hcy II) fractions after NaBr density ultracentrifugation (as described in Materials and methods and Fig. 1). Samples were subjected to 5% native polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lipoprotein I (LPI) and vitellogenin (VTG) were found only in the HDL fraction, while the clotting protein (shown as a ladder) and haemocyanin (Hcy) were present in the VHDL I and VHDL II fractions. The Hcy I fraction also contained low levels of clotting protein, but this was absent from the Hcy II fraction.

## Discussion

Approximately 84.2 mg of lipids (fresh mass) accumulate in a mature ovary weighing an average of 2.6 g before egg extrusion (Table 1). The results indicate that most of the lipids were accumulated before the oocytes reached their maximum size, which may be  $650 \mu\text{m}$  or more in diameter at ovulation. These results, together with those published previously (Browdy et al., 1990), indicate that there are four stages of oocyte maturation: (a) a previtellogenic stage in ovaries whose oocytes are less than  $100 \mu\text{m}$  in diameter, (b) an early vitellogenic stage in which oocyte diameter is in the range  $100$ – $200 \mu\text{m}$ , (c) a vitellogenic stage where oocyte diameters range from  $200$  to  $300 \mu\text{m}$  and (d) a late vitellogenic stage where the mean oocyte diameter is greater than  $300 \mu\text{m}$ . Cortical crypts appear in oocytes at late vitellogenic stages (Browdy et al., 1990), but in live samples they could be clearly distinguished during the late stage when AOD exceeded  $450 \mu\text{m}$  (M. Khayat and E. Lubzens, unpublished results).

Approximately equal quantities of PLs and TAGs were found, in addition to cholesterol. Diacylglycerols were not detected, although they have been reported to occur in *P. japonicus* in minute quantities (1.6–3.9%; Teshima and Kanazawa, 1983). The occurrence of DAGs may indicate degradation during lipid extraction. The relative proportion of PLs decreased from 66% to 49% during the course of vitellogenesis, while that of TAGs increased from 20% to 47%. The relative greater abundance of PLs in non-vitellogenic ovaries probably reflects their contribution to ovarian membrane structure. This was clearly demonstrated after fractionation of immature and mature ovarian homogenates (Fig. 5), which showed similar amounts of lipids in the pelleted membrane fraction at the two stages. The increase in the amount of lipids in the infranatant fraction as maturation proceeds (from  $4.13$  to  $18.15 \text{ mg g}^{-1}$ ) reflects the accumulation of vitellin during ovarian maturation. The percentage of PLs (68.5%), TAGs (22.7%) and cholesterol (8.75%) was similar at both stages. Similar data have been reported previously for the lipid composition of purified vitellin (Lubzens et al., 1997). There is also an increase in the

amount of lipid (from 1.79 to 7.07 mg g<sup>-1</sup>) in the floating fat or cap fraction during maturation. This fraction, which is composed almost exclusively of TAGs, reflects the accumulation of TAGs during vitellogenesis. This layer probably did not contain vitellin since it lacked PLs. In mature ovaries, recovery of TAGs is lower than that of PLs and cholesterol, reflecting the difficulty in the quantitative recovery of the semi-liquid cap lipids. Thus, the amount of TAGs in the cap is probably higher than the measured value of 27 %.

These results are in accordance with those reported for the blue crab *Callinectes sapidus*, in which 27.5 % of the total lipids were found in the lipid droplet fraction of mature oocytes (Lee and Walker, 1995). In *P. japonicus*, an increase in PL content from 39 % to 46.4 % occurred during egg development, while only a slight change in the abundance of TAGs (from 24.2 to 27.9 %) was reported (Teshima and Kanazawa, 1983). A relatively large proportion of these lipids in *P. semisulcatus* consist of PUFAs (30.1 % in the PLs and 29.2 % in the TAGs), which are not synthesized in the shrimp and must originate from their diet (Harrison, 1990). The relative abundance of both DHA and EPA decreases during oocyte maturation, indicating intraovarian synthesis of other lipids during this period (Shenker et al., 1993). The food consumed by the shrimp affects the abundance of specific fatty acids in their tissues, including the ovary (Lytle et al., 1990; Alava et al., 1993; Cahu et al., 1994, 1995; Xu et al., 1994). The food supplied to the shrimps examined here contained abundant amounts of PUFAs, which have been shown to be essential for egg development and for the survival of larvae after hatching (Bray et al., 1990; Bray and Lawrence, 1992; Cahu et al., 1995; Xu et al., 1994).

Haemolymph LPs carry mainly PLs (74.1 % in HDL to 88.7 % in VHDL II) and DAGs (11.3–15.9 %). Triacylglycerols were only found in HDL (7.5 %) and VHDL I (3.0 %). We have previously shown (Lubzens et al., 1997) that female HDL is composed of two LPs: LP1 and vitellogenin. These lipoproteins contained PLs (77.8 % in LP1 and 70.9 % in vitellogenin), DAGs (5.0 % in LP1 and 9.5 %, in vitellogenin) and cholesterol (20.3 % in LP1 and 12.0 % in vitellogenin). TAGs were found almost exclusively in vitellogenin. Hall et al. (1995) showed that male HDL/BGBP contains an average of 47.6 % (w/w) lipids, while VHDL (or clotting protein) contains 11.4 % and haemocyanin 0.2 %. They proposed that the clotting protein and haemocyanin may play a significant role in lipid transport in crayfish. In *P. semisulcatus*, most of the haemolymph lipids were recovered in the HDL fraction, and the amount found was within the range reported previously (Lubzens et al., 1997). The HDL fraction of *P. vannamei* has also been reported to carry substantial amounts (52–80 %) of the lipids found in the haemolymph (Vasquez-Moreno et al., 1995; Yepis-Plascencia et al., 1995; Ruiz-Verdugo et al., 1997). The N-terminal amino acid sequence of *P. vannamei* HDL protein has a high homology to the BGBP of crayfish (Ruiz-Verdugo et al., 1997), and similar results were obtained for LP1 of *P. semisulcatus* (M. Khayat, M. Hall, A. Tietz, E. Lubzens and K. Soderhall, unpublished results).

The VHDL fraction of *P. semisulcatus* was composed mainly of the clotting protein as identified by its characteristic

ladder pattern in non-denatured PAGE. The clotting protein was also detected in the Hcy I fraction, and this may explain the abundance of lipids in this fraction before their removal by further ultracentrifugation steps. While lipid analysis of haemocyanin removed from a NaBr density gradient at a density of 1.21 gm ml<sup>-1</sup> indicated the presence of lipids, the haemocyanin lost these lipids after repeated centrifugations at higher NaBr densities. After the second ultracentrifugation step, the haemocyanin fraction contained less than 0.9 % of the total haemolymph lipids, while it still carried 58 % of the proteins found in the original haemolymph sample. Moreover, lipids were not detected in haemocyanin extracted from native gels. The total amount of glycolipids in both VHDL fractions (VHDL I and VHDL II) amounts to 17.3 % of the lipids recovered after fractionation. These fractions contained substantial amounts of PUFAs, but the amount of lipids per milligram protein is significantly lower than in the HDL fraction. While ovarian receptors for vitellogenin have been reported in crustaceans (Jugan and Soyez, 1985; Laverdure and Soyez, 1988; Jugan and Van Herp, 1989), it is not known whether they bind to other lipoproteins such as LP1 or VHDL.

From the data presented here and by Shafir et al. (1992), it is possible to suggest a model for transport of lipids into the ovary. Assuming that almost all lipids are delivered to the ovary by HDLs (LP1 and/or vitellogenin) and VHDLs, two alternative hypothetical routes can be examined: (1) TAGs in the haemolymph are the main source of TAGs in the ovary, (2) PLs in the haemolymph are the source of PLs and TAGs in the ovary.

Considering the first of these possibilities: the ovary accumulated 38.56 mg of TAGs, of which 97 % (37.4 mg) apparently originates from extraovarian sources (as indicated by PUFA abundance; see Results). The mean amount of TAGs in female haemolymph is 45 µg ml<sup>-1</sup> (Lubzens et al., 1997). The mean volume of haemolymph in a female weighing 50 g is 20.15 ml, and the total amount of TAGs in the haemolymph is then 0.906 mg. Approximately 10 days are needed for ovarian development (Browdy, 1988). Assuming a constant and equal rate of ovarian development, an accumulation of 3.7 mg of TAGs per day in the ovary is expected, requiring the TAGs in the haemolymph to be turned over four times per day. Since TAGs in the haemolymph are bound to vitellogenin (Lubzens et al., 1997) and have also been found in the VHDL I fraction, this will indicate a high turnover for vitellogenin and the clotting protein. Alternatively, if TAGs in the VHDL I fraction reflect contamination with vitellogenin, then vitellogenin is the only source of TAGs in the ovary.

Similarly, we can examine the second possibility, that haemolymph PLs are the main source of ovarian lipids. The concentration of PLs in the haemolymph is approximately 1000 µg ml<sup>-1</sup>. The total TAGs and PLs in the ovary is 80.9 mg, of which 76.25 mg accumulates during vitellogenesis. Only 71.1 mg originates from extraovarian sources. This means that the amount accumulated daily is 7.1 mg, requiring that 28 % of PLs in the haemolymph must turn over per day. Because PLs are found in LP1, vitellogenin and the clotting protein, these lipoproteins can theoretically serve as a source for

ovarian lipids. The relatively low lipid content per milligram protein of the clotting protein seems to exclude it from a function in lipid transport.

The rate of turnover of lipids in the haemolymph of shrimps is unknown. A differential turnover rate of haemolymph proteins has been observed in other studies (Khayat et al., 1994b), with a faster turnover of vitellogenin relative to that of LP1. This, however, does not exclude the possibility of a reusable shuttle for lipid transport by one of the haemolymph lipoproteins, which would then transport the lipid moiety without being sequestered from the haemolymph. A lipase has recently been found in ovarian homogenates (A. Tietz, N. Daube and E. Lubzens, in preparation) and could be associated with lipid processing within the ovary during vitellogenesis. The measurement of a lipid turnover rate is complicated by the occurrence of intraovarian lipid synthesis and by the possibility of additional synthesis in the hepatopancreas and oxidation of lipids in all tissues.

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