Transfer of Lipids Between Hemolymph and Hepatopancreas in the Shrimp *Macrobrachium borellii*

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ABSTRACT: Crustacean lipids are transported in the hemolymph by an HDL. The hepatopancreas is the most important and active organ regarding lipid metabolism, so we studied the interchange of FA and acylglycerols between both components of the hepatopancreas–hemolymph system in the decapod crustacean *Macrobrachium borellii*. The hepatopancreas and a sole plasma lipoprotein were labeled by *in vivo* incubations with ^{14}C palmitic acid injected into the hemolymph. Then they were incubated *in vitro* with unlabeled hepatopancreas and hemolymph, and the transfer of lipids between them was measured by radiochromatographic techniques. It was determined *in vivo* that more than 80% of the circulating palmitic acid was taken up by the hepatopancreas and incorporated into PC and TAG. Both classes of lipids, but mainly PC, were transferred back from tissues to the hemolymph. Lipid transfer was also demonstrated *in vitro*. The transfer of PC (30% of labeling) as well as that of FFA (48% of labeling) from hemolymph to hepatopancreas was determined. On the other hand, FFA were released more efficiently than the acylglycerols from intact hepatopancreas to hemolymph, and they were the only lipid transferred when the hepatopancreas had been previously washed.

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For several years, we have carried out certain metabolic studies using the hepatopancreas of the shrimp *Macrobrachium borellii* as a model. We first reported that the seasonal distribution of lipids was closely related to the diet (1). Afterward, we explored the consumption of lipids under stressful conditions (2), the effect of temperature upon the FA β-oxidation system (3), and the metabolism of palmitic acid as a precursor of TAG (4). We therefore postulated that the hepatopancreas is the main organ in *M. borellii* responsible for the synthesis and the degradation of TAG. Liver and fat body are the organs analogous to the hepatopancreas in vertebrates and insects, respectively. The hepatopancreas also performs digestive functions, enzyme secretion, and excretion of waste materials (5,6).

Although the occurrence of very high density lipoproteins (VHDL) has been reported in hemolymph of some crab and shrimp species (7,8), plasma lipid transport in crustaceans is mainly carried out by HDL. These lipoproteins are structurally much simpler than the ones present in vertebrates, and up to now there has been no evidence for the presence of any LDL in crustacean plasma (9). Recently, we characterized the plasma lipoproteins of *M. borellii* and found that their compositions are similar to those of other crustaceans (Garcia, F., unpublished results). We observed two different lipoproteins: one HDL found in animals of both sexes, and another HDL, found exclusively in the hemolymph of females during the reproductive season, that is involved in vitellogenesis.

These studies led to the hypotheses that the hepatopancreas, as a biosynthetic organ, transfers lipids to the hemolymph HDL for distribution among other tissues and that the hemolymph transfers to the hepatopancreas the lipids resulting from absorption across the digestive tract. In this way, biochemical interactions must be involved. The mechanism of interchange of lipids between hemolymph lipoproteins and tissues in crustaceans is still unknown. The results reported here deal with investigations performed *in vitro* on the transfer of FFA and other lipids between plasma HDL and the hepatopancreas of *M. borellii*, as well as between isolated lipoprotein and artificial membranes.

MATERIALS AND METHODS

Biological and chemical materials. Male and nonovogenic female adult specimens of *M. borellii* were collected in summer from a water course close to the Río de la Plata, Argentina. They were kept in glass aquaria containing tap water at room temperature (20–25°C) until used for the experiments. 1-^{14}C Palmitic acid (57.0 mCi/mmol, 99% radiochemically pure) and PC, L-α-dipalmitoyl (dipalmitoyl-1-^{14}C, 111 mCi/mmol, 97% radiochemically pure) were purchased from New England Nuclear Corp. (Boston, MA). All chemicals were of analytical grade.

In vivo *labeling with radioactive FA.* Radioactive palmitic acid was administered to groups of 4–8 shrimp. They were maintained in aquaria at room temperature for 1, 4, 7, or 16 h. The cephalic sinus of each animal was injected with 5 μL of an aqueous solution containing 2 μCi (35 nmol) radioactive FA as the ammonium salt. A syringe with a needle designed for animal injection (Hamilton Co.) was used. After incubations, hepatopancreas and hemolymph were separated. Labeled hepatopancreas were used either for radioactive lipid analysis or for the transference studies between hepatopancreas and hemolymph. Hemolymph was obtained by puncturing the cephalic sinus using a syringe containing 0.1 N sodium citrate as anticoagulant. Hematic cells were separated by centrifugation at 100 × *g* for 10 min. Labeled plasma was used for lipid

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analysis in the experiments of transference between plasma and hepatopancreas as well as for lipoprotein isolation.

Plasma lipoprotein isolation. Plasma lipoprotein was isolated by density gradient ultracentrifugation. Aliquots of plasma were overlayered on sodium bromide solutions (density 1.25 g/mL) containing 0.01% sodium azide and centrifuged at 178,000 × *g* for 24 h in a Beckman L8 70M centrifuge, using an SW 60 Ti rotor. The total volume of the tubes was fractionated from top to bottom into 0.2-mL aliquots. The protein content of each fraction was monitored spectrophotometrically at 280 nm. Radioactivity in each fraction was measured by liquid scintillation counting in Wallac 1214 Rack Beta equipment. One tube containing a NaCl solution (density 1.04 g/mL) instead of plasma was centrifuged simultaneously and fractionated in order to determine the density of the fractions by monitoring the refractive indices. Fractions corresponding to densities of 1.10–1.14 g/mL and showing increases in absorbance at 280 nm and in radioactivity were pooled, and the protein content was determined colorimetrically (10).

Lipid extraction and analysis. Lipids from hepatopancreas and hemolymph plasma were extracted following the procedure of Folch *et al.* (11), and the extracts were utilized for lipid separation by TLC. Lipid classes were separated by high-performance-TLC on Merck plates (Darmstadt, Germany), using hexane/diethyl ether/acetic acid (80:20:1.5 by vol) for neutral lipids and chloroform/methanol/acetic acid/water (65:25:4:4 by vol) for phospholipids.

Radioactivity distribution in different lipid classes was detected by scanning proportional counting using a Berthold LB-2723 Dunnschicht Scanner II apparatus (Wildbad, Germany). Appropriate standards, run simultaneously, were visualized by exposure to iodine vapors.

Lipid transfer assays between hepatopancreas and hemolymph. To explore the transfer of lipids from the hepatopancreas to the hemolymph, the palmitic acid-labeled hepatopancreas (donor) was incubated with unlabeled hemolymph plasma (acceptor). Incubations were done in 50 mM potassium phosphate buffer pH 7.4, 0.25 M sucrose, with the addition of 5 µL aprotinin as protease inhibitor in a final volume of 330 µL. Assays were carried out at 27°C for 30 min, with shaking. The donor/acceptor ratio was a whole hepatopancreas (70–90 mg) /125 µL hemolymph. On the other hand, palmitic acid-labeled plasma (donor) was incubated under similar conditions with unlabeled hepatopancreas (acceptor). In another series of experiments, labeled hepatopancreas extensively rinsed with 3% albumin in 50 mM potassium phosphate buffer pH 7.4, were incubated with unlabeled hemolymph. After incubations, tissue and medium were separated, and lipids were extracted and analyzed as described above. All transfer experiments were done at least in triplicate.

Data collected from the transfer experiments were analyzed by a Mann–Witney nonparametric test. It was applied to radioactivity values in original and remaining lipids. Differences were considered with a significance level of 0.05.

Lipid transfer between liposomes and HDL. Liposome (multilamellar vesicle) preparations were done basically as described previously (12). A stock solution of radioactive (45 nCi) and unlabeled (1 mg) PC in chloroform/methanol (2:1 vol/vol) was placed in round-bottomed flasks, and the solvent was completely evaporated. Dry phospholipid was hydrated with 1 mL 50 mM Tris-HCl buffer, pH 8.4, and thoroughly vortexed for 30 s 10 times in a bath at 60°C. Liposomes (donor) and isolated plasma lipoprotein (acceptor) in a ratio of 500 µg phospholipid/450 µg protein were incubated in a final volume of 1 mL at 27°C for 2 h. After incubation, the liposomes were pelleted by centrifuging the sample twice at 167,000 × *g* for 90 min in a 70.1 Ti rotor as above. Lipoprotein remained in the supernatant. A tube containing labeled liposomes and buffer was centrifuged simultaneously as a blank.

RESULTS

Palmitic acid uptake and incorporation into the hepatopancreas and hemolymph. Different classes of circulating radioactive lipids in hemolymph were detected after having injected labeled palmitic acid into the cephalic sinus and sacrificing the animals after different incubation times. The results obtained are shown in Figure 1. A marked decrease of the label detected in FFA was observed that, after 16 h, reached less than 20% of its initial values. At the same time, radioactivity in the PC frac-

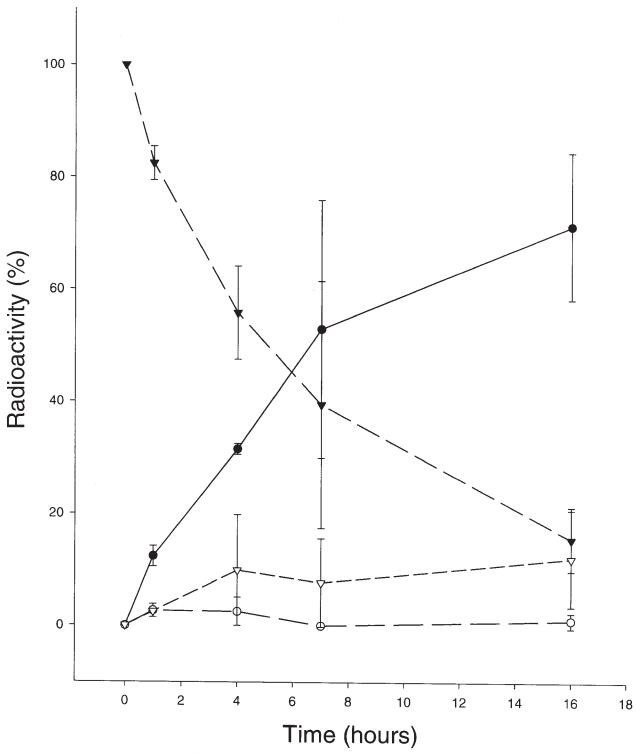

FIG. 1. Distribution of radioactivity into lipid classes after injection of ^{14}C palmitate into the hemolymph of *Macrobrachium borellii*. Radioactive palmitate as ammonium salt (2 µCi: 35 nmol) was injected into the cephalic sinus and incubated *in vivo* for different times. Error bars indicate SD of the mean (n = 3). ●, PC; ○, DAG; ▼, FFA; ▽, TAG.

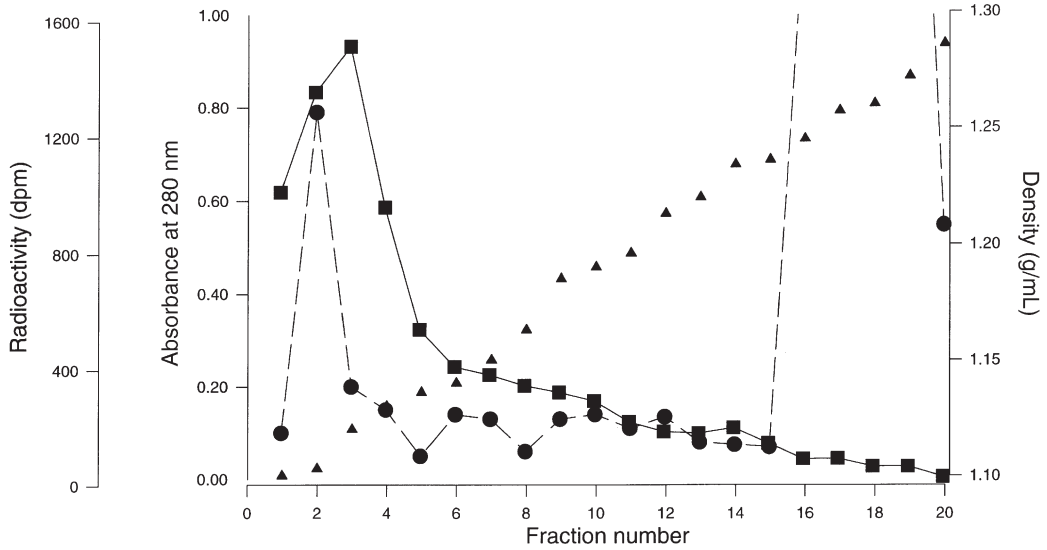

FIG. 2. Total proteins (●, absorbance at 280 nm), radioactivity (■), and density (▲) distribution in plasma fractions of *M. borellii*. Shrimp were incubated *in vivo* with 2 μCi (35 nmol) ^{14}C-palmitate for 5 h. Plasma was ultracentrifuged in a NaBr gradient and fractionated. For abbreviation see Figure 1.

tion increased with the incubation time, whereas the labeling in neutral lipids remained nearly constant.

To elucidate the role of the plasma lipoprotein in the uptake of lipids, the plasma was fractionated by ultracentrifugation in a density gradient. Radioactivity and protein content were measured in each fraction. The results are shown in the profiles in Figure 2, in which the highest labeling correlates with a protein peak at a density of 1.10–1.14 g/mL. This corresponded to the sole plasma lipoprotein present in the hemolymph of the specimens of *M. borellii* that we analyzed and is consistent with the density of the HDL.

Palmitic acid injected into the hemolymph was rapidly taken up by the hepatopancreas and then incorporated into several acylglycerols. The percentage of the label incorporated into different lipid classes as a function of time is presented in Figure 3. An even distribution of radioactivity between the neutral and glycerophospholipids occurred with short-time incubations, whereas at longer times, the label accumulated mainly in PC and to a lesser proportion in neutral acylglycerols. It is evident that DAG, with the minimal labeling concentration, are only intermediates in the synthesis of the other lipids.

Lipid transfer between the hemolymph and the hepatopancreas. When unlabeled hepatopancreas was incubated for 30 min with hemolymph labeled mainly in the FFA fraction, around one-half of the radioactivity was transferred to the tissue (Fig. 4A). At longer *in vivo* incubation times (16 h), the hemolymph contained the label distributed in PC as well as in FFA and TAG. When it was used as donor in incubations with unlabeled hepatopancreas, only the transfer of PC was observed (Fig. 4B).

Figure 5 shows the results from experiments in which radioactive hepatopancreas was incubated *in vitro* with unlabeled hemolymph under two different experimental conditions. After 30 min of incubation, a substantial amount of PC, most of the labeled FFA, and a small amount of TAG were transferred from intact hepatopancreas to hemolymph (Fig. 5A). In another set of experiments, labeled hepatopancreas were washed prior to incubation with BSA, which removes the FA associated with the external face of the hepatopancreas membrane. Figure 5B shows that the FFA is the only lipid transferred to hemolymph, whereas the total radioactivity of

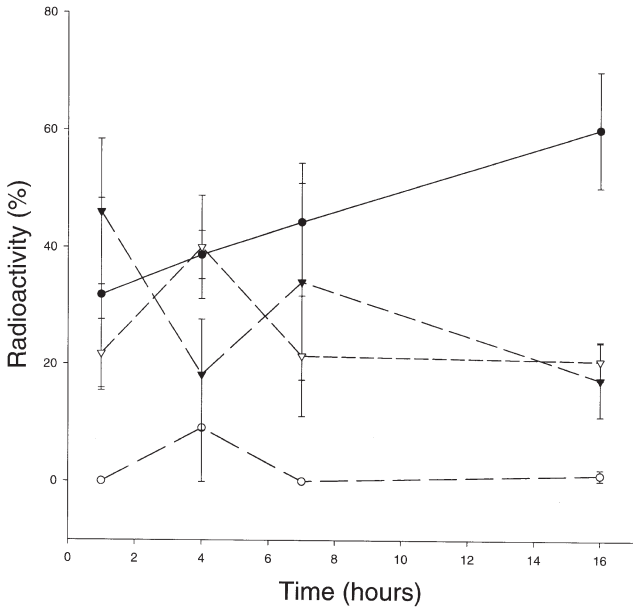

FIG. 3. The distribution of radioactivity into lipid classes in the hepatopancreas of *M. borellii* under the conditions indicated in Figure 1 ($n = 3$). Error bars represent SD. For abbreviation and key for lipid classes see Figure 1.

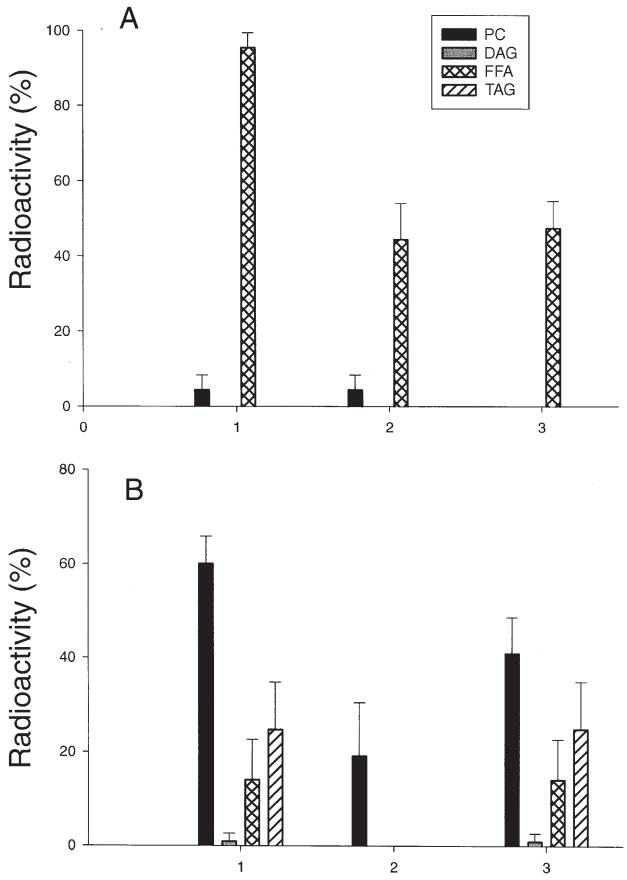

FIG. 4. *In vitro* transfer of labeled lipids from the hemolymph to the hepatopancreas. Whole unlabeled hepatopancreas were exposed to labeled hemolymph for 30 min. (A) Transfer from hemolymph mainly labeled in the FFA ($P < 0.05$). (B) Transfer from hemolymph labeled in the FFA and acylglycerols (PC: $P < 0.05$). 1, Labeled lipids in original hemolymph. 2, Labeled lipids transferred to the hepatopancreas. 3, Labeled lipids remaining in hemolymph. Error bars indicate SD of the mean ($n = 3$).

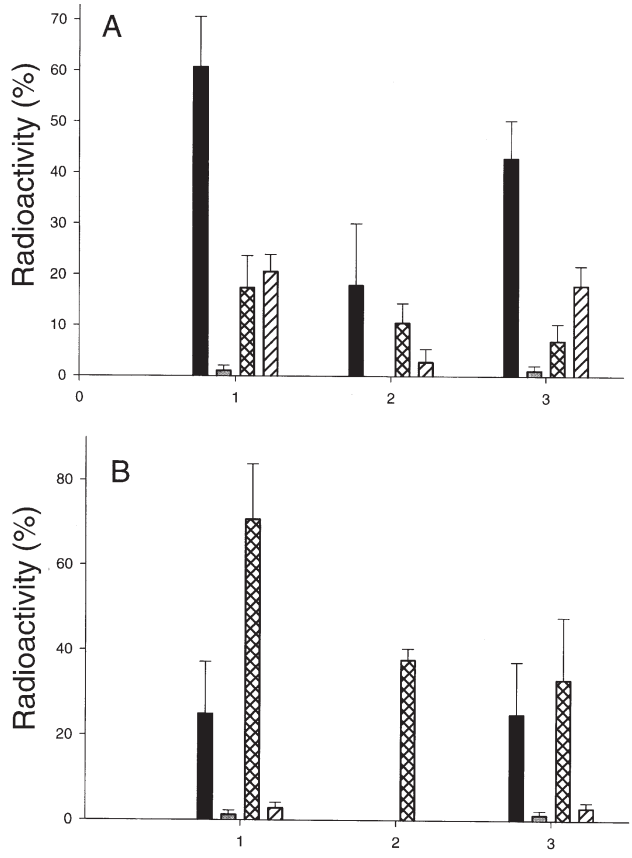

FIG. 5. *In vitro* transfer of labeled lipids from the hepatopancreas to the hemolymph. Whole labeled hepatopancreas were exposed to unlabeled hemolymph for 30 min. (A) Transfer from hepatopancreas without treatment (FFA and PC: $P < 0.05$). (B) Transfer from hepatopancreas extensively rinsed with albumin-buffer solution (FFA: $P < 0.05$). For key see Figure 4. Error bars represent SD of the mean ($n = 3$).

phospholipids and neutral lipids remains in the tissue. In another experimental model, an artificial liposome preparation containing labeled PC was used as donor of lipids to the hemolymph. Under these conditions, no transfer of phospholipid from the liposomes to the lipoprotein was observed; in fact when we reisolated the lipoprotein after the incubation with labeled liposomes, no radioactivity was detected in the lipoprotein fraction.

DISCUSSION

The decrease of the FFA circulating in the hemolymph during the incubation, together with an increase of phospholipids and neutral lipids, clearly indicates that the precursor injected into hemolymph is rapidly incorporated into tissues that synthesize acylglycerols, which then release them back to circulation. The increase in total circulating lipids is mainly attributable to PC, whereas the content of TAG and DAG remains unaltered. It may be inferred that the phospholipid synthesis in tissues is more active than that of neutral lipids. However, the radioactivity distribution among hepatopancreas lipids after *in vivo* FFA incorporation suggests that this organ is active in PC synthesis at longer times, although at shorter times it is able to synthesize both lipids. A similar observation was previously reported when we studied the lipid metabolism of *M. borellii* hepatopancreas (4). Also, the tissues are likely to retain the TAG synthesized, preferentially transferring PC to hemolymph. This fact is coincident with the results obtained from studies on the lipid composition of this and some other crustaceans, where PC was found to be the predominant circulating lipid (13) and TAG are accumulated by the hepatopancreas (1). These observations led us to suggest that those phospholipids synthesized in the hepatopancreas could be easily transferred to hemolymph. The presence of the circulating label only in the high-density plasma fraction demonstrated the role of HDL in taking up lipids from tissues. All the animals used in these experiments were males and nonvitellogenic females; thus, plasma did not contain any vitellogenin, but only the HDL common to both sexes (14).

The study of the *in vitro* transfer of lipids from hemolymph

to the hepatopancreas showed a clear trend of hemolymph to supply the tissue with FFA as well as PC, depending on their relative labeling in hemolymph. The transfer of FFA corroborates the preceding observations dealing with the components of the hemolymph–hepatopancreas system, where hemolymph is likely to provide the FA necessary for the synthesis of acylglycerols, a task that is performed by a tissue of greater activity like the hepatopancreas. *In vitro* experiments were carried out for short times in order to avoid the possibility of lipid synthesis within the hepatopancreas masking the transfer results. In other organisms, the release of the circulating FFA to tissues is produced by HDL structures that are poor in lipids. This is the case of the albumin–FFA complex in vertebrates or the VHDL in certain molluscs (15) and in insects (16). It is evident that this role in *M. borellii* is done by the HDL since it is the only circulating lipoprotein.

In vivo-labeled hepatopancreas, where radioactivity was mainly accumulated in PC and also in smaller amounts in TAG and FFA, were used for *in vitro* experiments. The transfer of these lipids to the hemolymph was demonstrated. In this case, the FFA were comparatively the most efficiently transferred lipid group. Even though PC was highly labeled, it was less efficiently transferred than FFA, whereas the transference of TAG was slight. This also shows a markedly different behavior between glycerophospholipids and neutral lipids, coincident with a large amount of PC in the circulating lipoprotein and an accumulation of TAG in the hepatopancreas under natural conditions. There is a marked change when the experiments are performed using washed hepatopancreas since only the FFA are transferred. As a consequence, it appeared that no carrier would be involved in the FFA transfer, whereas that of glycerophospholipids could be mediated by a transfer factor. If this is the case, this factor could be associated with the hepatopancreas membrane, and it could be removed by the albumin buffer used for washing the tissue. These results led us to another experiment using an artificial membrane as donor. Thus, we tried to transfer labeled PC from liposomes to hemolymph; we found plasma HDL was not able to take up the phospholipid under our experimental conditions. This result reinforces the above assumption about the occurrence of a carrier that would transport lipids from tissue membranes to circulation.

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REFERENCES

1. González-Baró, M.R., and Pollero, R.J. (1988) Lipid Characterization and Distribution Among Tissues of the Freshwater Crustacean *Macrobrachium borellii* During an Annual Cycle, *Comp. Biochem. Physiol. 91B*, 711–715.
2. Pollero, R.J., González-Baró, M.R., and Irazú, C.E. (1991) Lipid Classes' Consumption Related to Fasting and Thermal Stress in the Shrimp *Macrobrachium borellii*, *Comp. Biochem. Physiol. 99B*, 233–238.
3. Irazú, C.E., González-Baró, M.R., and Pollero, R.J. (1992) Effect of Environmental Temperature on Mitochondrial β-Oxidation Activity in Gills and Hepatopancreas of the Freshwater Shrimp *Macrobrachium borellii. Comp. Biochem. Physiol. 102B*, 721–725.
4. González-Baró, M.R., and Pollero, R.J. (1993) Palmitic Acid Metabolism in Hepatopancreas of the Freshwater Shrimp *Macrobrachium borellii, Comp. Biochem. Physiol. 106B*, 71–75.
5. O'Connor, J.D., and Gilbert, L.I. (1968) Aspects of Lipid Metabolism in Crustaceans, *Am. Zool. 8*, 529–539.
6. Al-Mohanna, S.Y., and Nott, J.A. (1986) B-Cells and Digestion in the Hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda), *J. Mar. Biol. Assoc. UK 66*, 403–414.
7. Komatsu, M., Ando, S., and Teshima, S.I. (1993) Comparison of Hemolymph Lipoproteins from Four Species of Crustacea, *J. Exp. Zool. 266*, 257–265.
8. Hall, M., Vanheusden, M.C., and Söderhäll, K. (1995) Identification of the Major Lipoproteins in Crayfish Hemolymph as Proteins Involved in Immune Recognition and Clotting, *Biochem. Biophys. Res. Commun. 216*, 939–946.
9. Yepiz-Placencia, G., Vargas-Albores, F., and Higuera-Ciapara, I. (2000) Penaeid Shrimp Hemolymph Lipoproteins, *Aquaculture 191*, 177–189.
10. Lowry, O.H., Rosebrough, N.J., Farr, A., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
12. González-Baró, M.R., Garda, H., and Pollero, R.J. (2000) Effect of Fenitrothion on Dipalmitoyl and 1-Palmitoyl-2-oleoylphosphatidylcholine Bilayers, *Biochim. Biophys. Acta 1468*, 304–310.
13. Lee, R.F., and Puppione, D.L. (1988) Lipoproteins I and II from the Hemolymph of the Blue Crab *Callinectes sapidus*: Lipoprotein II Associated with Vitellogenesis, *J. Exp. Zool. 248*, 278–289.
14. Lee, R.F. (1991) Lipoproteins from the Hemolymph and Ovaries of Marine Invertebrates, in *Advances in Comparative and Environmental Physiology* (Gilles, R., ed.), Vol. 7, pp. 187–208, Springer-Verlag, London.
15. Heras, H., and Pollero, R. (1990) Occurrence of Plasma Lipoproteins in Octopods. Partial Characterization and Interorgan Transport of Lipids, *J. Exp. Mar. Biol. Ecol. 140*, 29–38.
16. González, M.S., Rimoldi, O.J., and Brenner, R.R. (1995) Studies on Very-High-Density Lipoprotein of *Triatoma infestans* Hemolymph in Relation to Its Function as Free Fatty Acid Carrier, *Comp. Biochem. Physiol. 110B*, 767–775.

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