



Effect of fenitrothion on the acylglyceride exchange in crustacean lipoproteins

Fernando García*, María R. González-Baró, Ricardo Pollero

Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de La Plata (UNLP), 1900 La Plata, Argentina

Received 21 September 2004; accepted 18 November 2004

Available online 4 May 2005

Abstract

The effect of fenitrothion (FS) on the functionality of two models of crustacean lipoproteins was studied. Models used were a high density plasma lipoprotein (HDL) that is involved in the lipid–tissue exchange, and a lipovitellin (LV), the main source of embryo nutrients, which presents two forms only distinguishable in its lipid composition. The relative influence of lipid and apoprotein compositions on the FS effect in lipid transfer was comparatively evaluated using liposomes prepared with lipids obtained from both lipoproteins. FS treatment of HDL modified the transfer of lipids to and from hepatopancreas, diminishing the uptake of phosphatidylcholine and triacylglycerols and increasing its capacity to release both lipids to the tissue. Likewise, FS increased the transfer of phosphatidylcholine to the embryonic tissue from both LVs and from liposomes. It was assessed that apoproteins are involved in the transfer of phosphatidylcholine to tissues, though they are not involved in the changes produced by FS concerning the affinity of this lipid to lipoprotein systems.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Fenitrothion; Transfer of lipids; Crustacean lipoproteins; Embryos

1. Introduction

Decapod *Macrobrachium borellii*, like other crustaceans [1,2], exhibits a high density lipoprotein (HDL) which transports plasma lipids and has a high content of phospholipids and moderate

amounts of triacylglycerides (TAG). The HDL protein portion contains three polypeptides of 124, 26, and 23 kDa, respectively [3]. The function of this circulating lipoprotein in the exchange of lipids with hepatopancreas was studied; its capacity to transfer free fatty acids (FFAs) and phosphatidylcholine (PC) to tissue as well as to uptake acylglycerides from the latter was assessed [4].

* Corresponding author. Fax: +54221 4258988.

E-mail address: cfgarcia@atlas.med.unlp.edu.ar (F. García).

Lipovitellin (LV), another lipoprotein system studied in *M. borellii*, is the main nutritional source in the vitellus which is consumed during the embryogenesis [5]. Proteins and lipids of LV are both consumed by the growing embryo, being the latter consumed selectively. This leads to different LV forms (LVe and LVl) which appear at early and late stages of the embryonic development, respectively. Their apoprotein compositions and lipid/protein ratios are quite similar, but not their lipid compositions, being the PC and TAG contents higher in the latest stages of development [6].

The effect of the organophosphorous insecticide fenitrothion (FS) on these crustacean lipoproteins was studied, assuming that, as it modifies membrane structures in *M. borellii* [7] and in some other organisms [8–10], it would also affect lipoprotein structures. Then, it was corroborated that FS penetrates both lipoproteins, producing failures in the lipid package, and this structural change markedly alters its physiological behavior like the free fatty acid transfer [3,6]. We have found that FS affects in a similar fashion arachnid lipoproteins that exert a double function, lipid transport and respiratory pigments [11].

The present study shows further data on the structural changes caused by FS, on the functions of circulating and vitellinic lipoproteins in *M. borellii*. It is mainly focused on the uptake and release of different lipids between circulating HDL and hepatopancreas, as well as from the vitellinic LVs (at early and late stages) to the embryonic tissue. The influence of lipid and apoprotein compositions upon lipid transfer was comparatively evaluated using liposomes built up with lipids of both lipoproteins.

2. Materials and methods

2.1. Biological and chemical materials

Adult males and ovigerous females were collected during spring and summer (October–February) from a water course close to the Rio de la Plata river, Argentina, and kept in glass aquaria containing tap water at room temperature (20–25 °C) until used.

[1-¹⁴C]Palmitic acid (57.0 mCi/mmol and 99% radiochemically pure) and phosphatidylcholine, L- α -dipalmitoyl, [dipalmitoyl-1-¹⁴C] (111 mCi/mmol and 97% radiochemically pure) were purchased from New England Nuclear (Boston, MA). All chemicals were of analytical grade.

2.2. Hepatopancreas and plasma HDL lipid labeling

Radioactive palmitic acid was administered to groups of 4–8 adult shrimps at intermoult stage, which were maintained in aquaria at room temperature. Each animal was injected with 2 μ Ci (35 nmol) radioactive fatty acid as ammonium salt in water into the cephalic sinus [4]. After 16 h incubations, hepatopancreas was dissected and hemolymph extracted. Hemolymph was obtained by puncture in the cephalic sinus using a thin-needled syringe containing 0.1 N sodium citrate as anticoagulant at 4 °C [4]. Hematic cells were separated by centrifugation at 100g for 10 min. Labeled plasma and hepatopancreas were used for lipid analysis in the experiments of lipid transference.

2.3. Lipoprotein isolation and liposome preparation

Hemolymph from adult shrimps was obtained as described [4]. Eggs were obtained from gravid females, and the embryonic developmental stage was determined as described by Lavarias et al. [12]. They were discriminated at early and late stages.

Eggs were homogenized in 3 ml phosphate buffer, 50 mM, pH 7.4, and sequentially ultracentrifuged at 10,000g for 20 min, and then at 100,000g for 60 min. Lipovitellins were isolated from the final supernatant (cytosolic fraction). Plasma and egg lipoproteins were isolated by density gradient ultracentrifugation. One milliliter of plasma or egg cytosolic fraction was overlaid on 3 ml NaBr solution (density 1.26 g/ml) containing 0.01% sodium azide, and centrifuged at 178,000g at 10 °C for 24 h in a Beckman L8 70 M centrifuge, using an SW 60 Ti rotor. Saline solution of the same density as that of samples was centrifuged in parallel to determine relative densities, and to check the appropriate gradient formation. The total volume of the tubes was frac-

tionated from top to bottom into 0.2 ml aliquots, and the protein content of each fraction was monitored spectrophotometrically at 280 nm. The zone in the gradients containing the plasma lipoprotein (HDL) (density 1.10–1.14 g/ml) or the lipovitellin (density 1.18–1.19 g/ml) was separated as a whole fraction. Two forms of a lipovitellin, LVe and LVI, were obtained from eggs collected at the early and late stages of embryo development, respectively.

Total lipids were extracted from HDL, LVe, and LVI, and used for liposome preparation (LP-HDL, LPe, and LPI, respectively). Extracts containing 6 mg lipid and 0.5 μCi ^{14}C PC (4.5 nmol) were dry evaporated, hydrated with 1 ml of 50 mM potassium phosphate buffer, pH 7.4, vortexed, and extruded through polycarbonate membranes with pore diameters of 50 nm (Avestin, Ottawa, Canada).

2.4. *In vitro* labeling of LV and HDL lipid

Eighteen milligrams of lipoproteins, LVs, and HDL, was incubated with [^{14}C]PC liposomes at 37 °C for 30 min in 1 ml of 50 mM potassium phosphate buffer, pH 7.4. After incubation, plasma and vitellus labeled lipoproteins were isolated from the remaining liposomes (LPs) by density gradient ultracentrifugation as described. The protein content of each fraction was monitored spectrophotometrically at 280 nm. Radioactivity in each fraction was measured by liquid scintillation counting in a Wallac 1214 Rack Beta apparatus. One tube containing a NaCl solution (density 1.04 g/ml) instead of plasma was centrifuged simultaneously and fractionated to determine the density of the fractions by monitoring the refraction indexes. Fractions corresponding to densities 1.10–1.14 and 1.18–1.19 g/ml, showing an increase of A_{280} and radioactivity, were pooled and their protein content was measured colorimetrically [13].

2.5. Embryo isolation

Embryos from late developmental stage were isolated from yolk by breaking the vitellin sac under a stereoscopic microscope (Nikon). Early stage embryos were further purified by Percol discontin-

uous density gradient (Pharmacia LKB, Uppsala, Sweden), with solutions of 100, 50, and 25% diluted with 75 mM NaCl, according to Heras et al. [5].

2.6. Effect of FS on lipid transfer between HDL and hepatopancreas

A fraction of 250 μl complete hemolymph containing HDL as the only lipoprotein was pre-incubated with 20 and 40 ppm FS with agitation and at room temperature for 2 h. To study the effect of FS on the uptake of different lipids by HDL, the labeled hepatopancreas (donor) was incubated with unlabeled hemolymph plasma containing FS-treated HDL (acceptor). Incubations were done in 50 mM potassium phosphate buffer, pH 7.4, 0.25 M sucrose, with the addition of 5 μl (50 KIU) aprotinin as protease inhibitor in a final volume of 330 μl . To determine the effect of FS on the release of lipids from FS-treated HDL, labeled plasma (donor) was incubated under the same conditions described above with unlabeled hepatopancreas (acceptor). Both experiments were carried out in a shaker bath at 27 °C for 30 min, being the donor/acceptor ratio 70–90 mg hepatopancreas/125 μl hemolymph. Hemolymph without FS treatment was incubated simultaneously as control.

2.7. Effect of FS on phosphatidylcholine transfer from LVs to embryos

Labeled LVs (3 mg protein) and labeled LPs (1 mg lipids) were preincubated with 20 ppm of FS in 50 mM potassium phosphate buffer, pH 7.4, for 2 h at room temperature. Pools of 30 embryos isolated at early and late developing stages were incubated with the FS-treated LVs and LPs at 27 °C. Afterwards, embryos were removed and washed with cold potassium phosphate buffer.

Another series of experiments was designed to study the probable role of HDL apoproteins in PC transfer. For this reason, isolated native HDL and liposomes from HDL lipids, both of them labeled with [^{14}C]PC, were utilized. Also, labeling transfer from these systems to hepatopancreas was compared, as described previously.

2.8. Lipid extraction and analysis

Total lipids from hepatopancreas and plasma HDL were extracted following the procedure described by Folch et al. [14]. Lipid classes were separated by HP-TLC on Merck plates, using hexane–diethyl ether–acetic acid (80:20:1.5 v/v) for neutral lipids, and chloroform–methanol–acetic acid–water (65:25:4:4 v/v) for polar lipid analysis. Radioactivity distribution in different lipid classes was detected by scanning proportional counting using a Berthold LB-2723 Dunnschicht Scanner II apparatus (Germany). Appropriate standards, run simultaneously, were visualized by exposure to iodine vapors.

Embryos containing [^{14}C]PC were homogenized, lipids were extracted, and their label as well as that in the remaining LVs and LPs were quantified by scintillation counting.

3. Results

3.1. Effect of FS on the HDL capacity to uptake and release lipids

Shrimps were incubated *in vivo* with radioactive palmitic acid to obtain hepatopancreas and hemolymph HDL labeled in their lipid moiety. Both tissue and lipoprotein were used as donors/acceptors in the experiments of lipid transfer.

Results on the transfer of labeled lipids from hepatopancreas to HDL showed that FS-treated lipoprotein significantly decreased the uptake of PC and TAG by the hemolymph (Fig. 1). The uptake of such lipids was decreased around 57 and 68%, respectively, in samples pre-treated with 20 ppm FS. When the pre-treatment was done using 40 ppm of FS (Fig. 1), the fall in the PC and TAG uptake was 58 and 80%, respectively.

Likewise, the lipoprotein treatment using the same insecticide concentrations produced a dose-dependent lipid release increment, from the labeled HDL to the acceptor hepatopancreas (Fig. 2). In this regard, the release increment for PC and TAG was 19 and 157%, respectively, when treating the lipoprotein with 20 ppm FS. PC and TAG release in samples pre-treated with 40 ppm FS was 274 and 328% higher than in controls (without treatment with the insecticide).

Free fatty acid and diacylglyceride transfer, although quantitatively important, were unaltered by the FS presence, under our experimental conditions.

3.2. Labeling *in vitro* of lipoproteins with radioactive PC

When [^{14}C]PC liposomes were incubated with purified HDL and LV, a portion of the radioactivity was transferred to lipoproteins. Figs. 3 and 4 show the incorporation of [^{14}C]PC to LV and

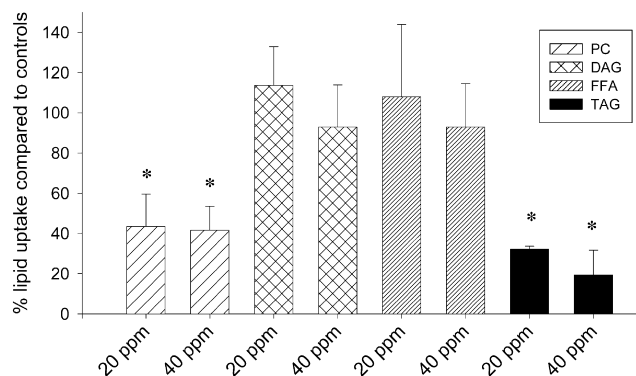


Fig. 1. Effect of 20 and 40 ppm FS on the uptake of different lipids by HDL from the hepatopancreas. (Values represent the average of three experiments \pm SD.) Control without FS is considered 100%. Student's *t* test was used to compare the significance of the differences with respect to control: * $P < 0.05$.

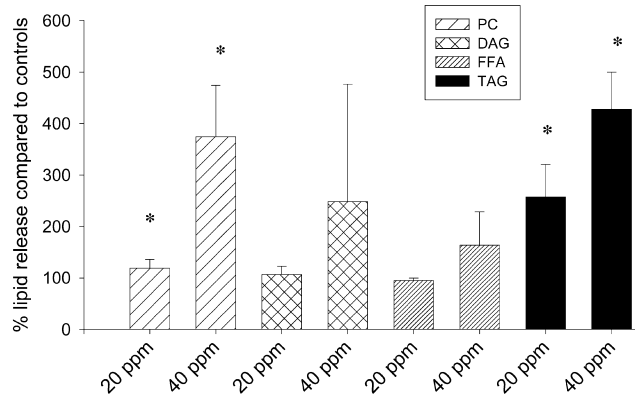


Fig. 2. Effect of 20 and 40 ppm FS on the release of different lipids by HDL to the hepatopáncreas. (Values represent the average of three experiments \pm SD.) Control without FS is considered 100%. Student's *t* test was used to compare the significance of the differences with respect to control: **P* < 0.05.

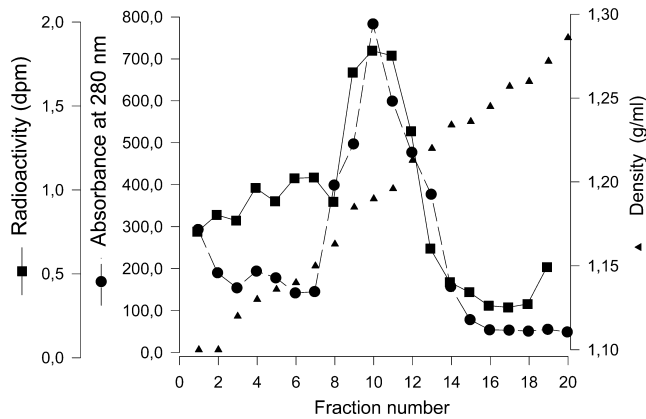


Fig. 3. Total proteins (● absorbance at 280 nm), radioactivity (■), and density (▲) distribution after ultracentrifugation of LVs preincubated with [¹⁴C]PC liposomes.

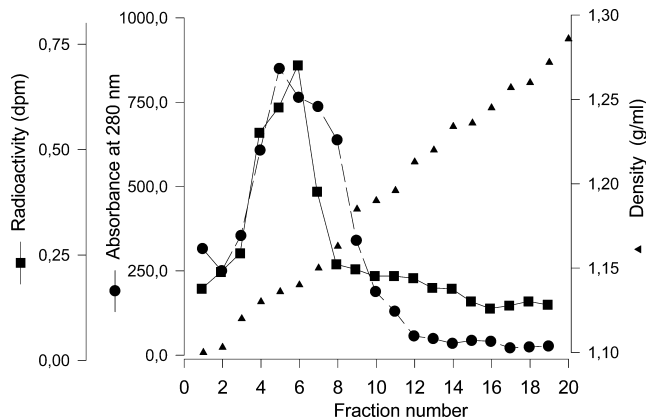


Fig. 4. Total proteins (● absorbance at 280 nm), radioactivity (■), and density (▲) distribution after ultracentrifugation of HDL preincubated with [¹⁴C]PC liposomes.

HDL, respectively. In both cases, the protein and radioactivity profiles obtained after ultracentrifugation in density gradient showed consistent maxima which evidenced the PC uptake by the lipoproteins. The transfer of PC from the so-labeled lipoproteins to either embryos or hepatopancreas was studied.

3.3. Effect of FS on PC release from LV to embryos

Treatment of lipovitellins with FS led to an increase of PC transfer to the acceptor embryonic tissue, disregarding the fact that lipoprotein or embryo was obtained at early or late development stages (Fig. 5). A similar behavior was noted in the PC transfer from labeled and FS-treated liposomes to acceptor embryos. In all these cases, in either LVs or LPs at early (LVe and LPe) or late (LVI and LPI) stages, the transfer of phospholipid to tissue (early or late embryos) increased around 8–40% compared to controls.

3.4. Effect of lipoprotein components on the PC release

LVe, LVI, HDL, and liposomes prepared with their lipids (LPe, LPI, and LP-HDL) were incubated, without FS treatment, with embryos and

hepatopancreas. Our aim was to determine whether either the lipid composition or the presence of apoproteins affected per se the PC transfer from lipoproteins to tissues. Results are shown in Fig. 6. There was a marked increase in the amount of PC released by whole lipoproteins when compared to that released by liposomes, though their lipid compositions were the same. Such a difference was observed in both lipovitellin and HDL.

4. Discussion

Hemolymph circulating lipids in *M. borellii* are exclusively associated to the high density plasma lipoprotein (HDL) which exchanges free fatty acids and acylglycerides with the hepatopancreas [4]. Incubations in vivo using labeled fatty acids resulted in radioactivity-incorporated lipids such as PC, DAG, FFA, and TAG in hepatopancreas as well as in HDL; then they were used to evaluate the effect of fenitrothion (FS) upon the lipid transfer in the hemolymph-hepatopancreas binary system. The treatment of HDL with FS led to an altered lipoprotein capacity for the uptake and release of lipids. The decreased uptake in parallel with the increased release of these lipids by HDL under FS administration suggests an affinity dimi-

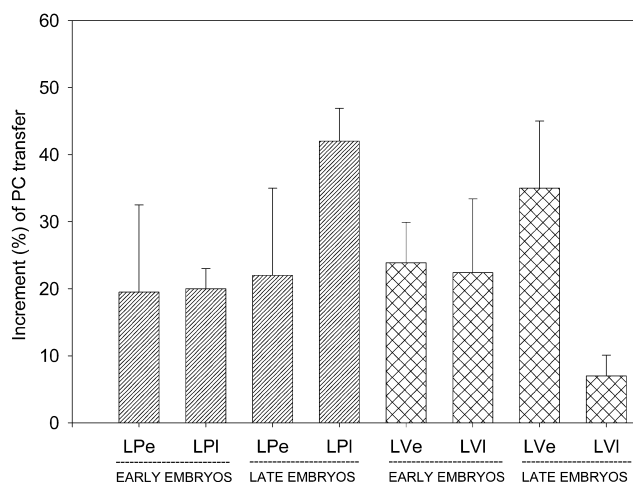


Fig. 5. FS effect on PC transfer from lipoproteins and liposomes to embryos. LVs were isolated at early (LVe) and late (LVI) stages of embryo development. LPe and LPI were prepared using LVs lipids. Early and late embryos were PC acceptors. Experiments were performed in triplicate.

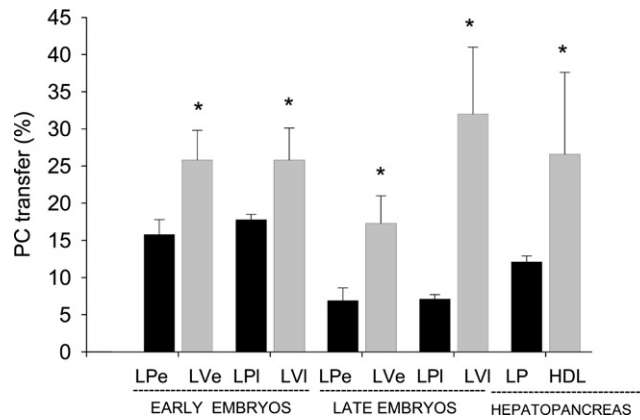


Fig. 6. Percent of PC transfer from lipoproteins and liposomes to tissues. Error bars represent SD of the mean ($n = 3$). Student's t test was used to compare the significance of the differences between LVs and LPs: $*P < 0.05$.

nution of both lipids for the HDL when the insecticide damages its structure. We have already observed that FS penetrates into the HDL of *M. borellii*, causing structural alterations evidenced by changes in the parameters that show the degree of lipid package. Although such alteration is produced in the core as well as in the peripheral area, its effect is more notorious in the inner part of the lipoprotein [3]. Both lipids in which affinity for the lipoprotein is apparently altered show different responses to structural changes produced by FS. Thus, TAG affinity seems to be more affected than that of PC, especially at high concentrations of the insecticide. The fact that TAGs, due to their polarity, are located in the lipoprotein hydrophobic core, and that this region is the most affected by FS, may explain the greater release and lesser uptake of TAG compared with PC. Results also indicated that FS action on the behavior of both lipids is dose-dependent. Although FS modifies the behavior of the two acylglycerides above-mentioned, the uptake and release of FFA and DAG by the lipoprotein are not markedly modified. These data are not consistent with previous results which evidenced a lower uptake of palmitic acid when administered as ammonium salt [3]. However, it must be noted that in the present experiments, the donor was not a soluble salt but a tissue.

Recently, we have demonstrated that PC is one of the lipids supplied by LV to the embryo. Be-

cause of this fact, at different embryogenesis stages, two forms of this lipoprotein (LVe and LVI) that have different lipid compositions arise [6]. Also, that study evidenced that FS alters the lipid package in both LV forms as well as in liposomes prepared with its lipids (LPe and LPI). We have just demonstrated that the transfer of PC from either LVs or LPs to the embryos is altered by FS. Results show that this alteration is quite similar to that discussed above, concerning the lipid release from HDL to hepatopancreas. This means an apparent diminution of the lipoprotein affinity for PC and consequently, a greater transfer of this lipid to the tissue. The fact that the pesticide could alter PC uptake in the tissue; may be discarded since when embryos were treated with FS and incubated with labeled LVs, the lipid uptake was not increased as compared with the non-treated embryos. Increases in the transfer of PC from LPe and from LPI to the embryo in liposomes pre-incubated with FS were similar. This shows that differences in the lipid composition of LPs are not enough to modify any physiological alteration caused by the insecticide. Also the effect of FS upon PC release in lipoproteins does not seem to be influenced by the presence of apoproteins, since the pesticide similarly affects PC release either from lipoproteins or liposomes.

Those control systems without FS administration showed that net amount of PC transferred from lipoproteins to the tissue was always greater

than the one transferred from liposomes to the tissue. This occurrence was observed in PC transfers either from LVs to embryos or from HDL to the hepatopancreas. Although apoproteins do not affect the alteration produced by FS on PC affinity of these systems, but they influence the transfer of this lipid, which under physiological conditions is released from lipoproteins to tissues. This possible lipid–protein interaction might imply an ordering effect of the lipid phase exerted by the apoproteins, similarly to the one already described in other lipoprotein systems [15,16]. This seems to indicate that an appropriate structure of the lipoprotein is necessary to release a larger amount of PC to tissues.

In brief, the present work shows that a liposoluble insecticide may disturb the usual lipid exchange, not only in hemolymph but also in vitellus leading to gross alterations in its normal functioning.

Acknowledgments

F.G. is a postdoctoral fellow of CONICET, M.G.-B. is a member of Carrera del Investigador CONICET, Argentina. R.P. is a member of Carrera del Investigador CICBA, Argentina. This work was partially funded by CONICET PIP No. 02101.

References

- [1] G. Yepis-Plascencia, F. Vargas-Albores, I. Higuera-Ciara, Penaeid shrimp hemolymph lipoproteins, *Aquaculture* 191 (2000) 177–189.
- [2] R.F. Lee, Lipoproteins from the hemolymph and ovaries of marine invertebrates, in: R. Gilles (Ed.), *Advances in Comparative and Environmental Physiology*, vol. 7, Springer-Verlag, London, 1991, pp. 187–208.
- [3] C.F. Garcia, M.L. Cunningham, M.R. González-Baró, H. Garda, R. Pollero, Effect of fenitrothion on the physical properties of crustacean lipoproteins, *Lipids* 37 (2002) 673–679.
- [4] C.F. Garcia, M. Gonzalez-Baró, R. Pollero, Transfer of lipids between hemolymph and hepatopancreas in the shrimp *Macrobrachium borellii*, *Lipids* 37 (2002) 581–585.
- [5] H. Heras, M. Gonzalez Baró, R. Pollero, Lipid and fatty acid composition and energy partitioning during embryo development in the shrimp *Macrobrachium borellii*, *Lipids* 3 (2000) 645–651.
- [6] C.F. García, M. Gonzalez-Baró, H. Garda, M. Cunningham, R. Pollero, Fenitrothion-induced structural and functional perturbations in the yolk lipoproteins of the shrimp *Macrobrachium borellii*, *Lipids* 39 (2004) 389–396.
- [7] M. González-Baró, H. Garda, R. Pollero, Effect of fenitrothion on hepatopancreas membrane fluidity in *Macrobrachium borellii*, *Pestic. Biochem. Physiol.* 58 (1997) 133–143.
- [8] M.C. Antunes-Madeira, R.A. Videira, V.M. Madeira, Effects of parathion on membrane organization and its implications for the mechanisms of toxicity, *Biochim. Biophys. Acta* 1190 (1994) 149–154.
- [9] M.C. Antunes-Madeira, L.M. Almeida, V.M. Madeira, Depth-dependent effects of DDT and lindane on the fluidity of native membranes and extracted lipids. Implications for mechanisms of toxicity, *Bull. Environ. Contam. Toxicol.* 51 (1993) 787–794.
- [10] M.C. Antunes-Madeira, V.M. Madeira, Effects of DDE on the fluidity of model and native membranes: implications for the mechanisms of toxicity, *Biochim. Biophys. Acta* 1149 (1993) 86–92.
- [11] M.L. Cunningham, C.F. Garcia, M.R. González-Baró, H. Garda, R. Pollero, Organophosphorous insecticide fenitrothion alters the lipid dynamics in the spider *Polybetes pythagoricus* high density lipoproteins, *Pestic. Biochem. Physiol.* 73 (2002) 37–47.
- [12] S. Lavarias, H. Heras, S. Demichelis, E. Portiansky, R. Pollero, Morphometric study of embryonic development of *Macrobrachium borellii* (Arthropoda: Crustacea), *Invert. Reprod. Dev.* 41 (2002) 157–163.
- [13] O.H. Lowry, N.J. Rosebrough, A.R. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [14] J. Folch, M. Lees, G.H. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem.* 226 (1957) 497–509.
- [15] C. Castuma, R. Brenner, The influence of fatty acid unsaturation and physical properties of microsomal membrane phospholipids on UDP-glucuronyltransferase activity, *Biochem. J.* 258 (1989) 4733–4738.
- [16] H. Garda, A. Bernanconi, R. Brenner, Possible compensation of structural and viscotropic properties in hepatic microsomes and erythrocyte membranes of rats with essential fatty acid deficiency, *J. Lipid Res.* 35 (1994) 1367–1377.