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Activation of lipid catabolism by the water-soluble fraction of petroleum in the crustacean *Macrobrachium borellii*

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Abstract

Little is known about the effect of the water-soluble fraction of crude oil (WSF) on lipid metabolism in invertebrates. The effect of the WSF on the triacylglycerol (TAG) mobilization, fatty acid activation and degradation was evaluated in the decapod *Macrobrachium borellii,* exposing adult and eggs at different stages of development for 7 days to a sublethal concentration of WSF. Using radioactive tracers, mitochondrial palmitoyl-CoA synthetase (ACS), triacylglycerol lipase (TAG-lipase) and fatty acid β-oxidation system activities were assayed.

Before studying the effect of WSF, the kinetic parameters of ACS were determined in purified mitochondria. Its optimal temperature and pH were 32 °C and 8.0, respectively, the apparent K_m 2.48 μmol l^{-1}, and its V_{max} of 1.93 nmol min^{-1} mg $protein^{-1}$. These kinetic parameters differed significantly from this shrimp's microsomal isoform.

After 7 days exposure to a sublethal concentration of WSF (0.6 mg/l), changes were observed in the enzymatic activity of all enzymes or enzymatic system assayed in adult midgut gland as well as in stage 5 eggs, a period of active organogenesis.

An increase in the mobilization of energy stores was detected as early as stage 4, where TAG-lipase activity increased by 27% in exposed eggs. The increase was even more marked in exposed eggs at stage 5 where a three-fold rise (154%) was determined. Exposed adult shrimp also showed an augmented lipase activity by 38%.

Fatty acid β-oxidation increased by 51.0 and 35.5% in midgut gland and eggs at stage 5, respectively, but no changes were observed at less-developed stages. Mitochondrial fatty acid activation by ACS also increased in adults and stage 5 eggs by 7.4 and 52.0%, respectively.

A similar response of the lipid catabolic pathways to WSF contamination in both adult and eggs, suggests that the exposure to this pollutant causes an increase in the energy needs of this shrimp. When validated by field studies, these catabolic enzymes could be employed as early pollution biomarkers.

Keywords: Shrimp; Triacylglycerol; Toxicity; WSF; Hydrocarbon; Lipid; Lipase; Fatty acid β-oxidation; Mitochondrial acyl-CoA synthetase

1. Introduction

Among the numerous forms of environmental pollution, the impact of anthropogenic hydrocarbons in aquatic and terrestrial ecosystems is probably the one that has caught major public attention (i.e. GESAMP, 1993; Mendes Da Silva et al., 1997; Neff et al., 2000). Aquatic pollution by petroleum is usually caused by spills from tankers or industrial discharges and is especially critical in rivers and coastal waters. Although the most visible part of an oil spill is the surface slick or mousses, the fraction really responsible for the toxic effects is the water-soluble hydrocarbon fraction (WSF). The biological response to sublethal exposure (biomarkers) in sensitive indicator species is particularly valuable to monitor environmental contaminant levels (Reid and MacFarlane, 2003). As water-soluble hydrocarbons will preferentially accumulate in membranes and other lipid stores of cells, lipid levels, composition and dynamics are critical factors controlling the uptake, bioaccumulation and toxicity of hydrocarbons in aquatic organisms. Freshwater crustaceans are one of the groups currently employed to monitor environmental pollution because of their advantageous characteristics: They constitute the major component in most aquatic ecosystems, their populations are often numerous and they are easily cultured in the laboratory (Fossi et al., 2000; Gerhardt et al., 2002; Shigehsa and Shiraishi, 1998). One such species, representative of the benthic community in Río de La Plata river area (Argentina), is the autochthonous freshwater shrimp *Macrobrachium borellii* (Decapoda: Palaemonidae) Boschi, 1981

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which was selected as model for the present study. In previous studies, the lipid composition and metabolism of *M. borellii* were investigated. Phospholipids (PL) and triacylglycerols (TAG) were identified as the main lipid classes in adult tissues (González-Baró and Pollero, 1988) and eggs (Heras et al., 2000), respectively. This shrimp has an active lipid metabolism (i.e., González-Baró and Pollero, 1998; González-Baró et al., 2000; Irazú et al., 1992). This species had also been studied regarding the bioaccumulation and biodepuration rates of WSF. It was observed that lipid compartments retain polyaromatic hydrocarbons for long periods of time (Lavarías et al., 2004). However, there are few reports available about alterations in crustacean lipids by petrogenic hydrocarbons (Capuzzo et al., 1984; Wang and Stickle, 1988) as well as on their effect on lipid catabolism (Elumalai and Balasubramanian, 1999).

The present study was focused on the effect of the WSF of hydrocarbons on shrimp lipid catabolism at the critical points of fatty acid activation, lipolysis and mitochondrial β-oxidation of fatty acids. These pathways are discussed in the context of the central role triacylglycerol (TAG) plays in the energy metabolism of shrimp. Adults and eggs were included to determine if such alterations are associated with a particular stage of the life cycle.

2. Materials and methods

2.1. Sample collection

Adults of *M. borellii* were sampled during spring and summer (October–February) in a non-polluted watercourse close to La Plata river, Argentina (20 km SW from La Plata). They were taken to the laboratory and kept in dechlorinated running water at 22 ± 2 °C, and 14:10 h L:D photoperiod for at least a week before experiments were performed. Eggs were removed from the pleopods of ovigerous females and checked under a stereoscopic microscope to determine stage of development.

2.2. Preparation and analysis of the WSF of crude oil

Punta Loyola light crude oil, obtained from Santa Cruz, Argentina, stored at 4 °C, was used to prepare the water-soluble fraction. It was stirred in a 10-L stainless steel mixing vessel equipped with a mechanical stirrer, a bottom drain, and kept in a cold room at 4 °C. Crude oil and freshwater in a ratio of 1:100 (v/v) were stirred at low speed for 24 h and allowed to settle for additional 48 h (lower settling times rendered a fraction with dispersed oil droplets). WSF was collected daily using the bottom drain. During experiments, fresh WSF batches were prepared every 2 days using several 10-L vessels to compensate the volatilization of lighter hydrocarbons (Heras et al., 1995). Analysis of the WSF was performed on water samples from the WSF stock every time a WSF batch was prepared. The HCs in the WSF were extracted according to Murray extraction procedure (Murray et al., 1984). Analyses were conducted by capillary gas chromatography. The specimen components were identified by comparison of retention times with those of external standards and with a WSF identified by GC–MS (Heras et al., 1992). The WSF was mainly composed of single ring aromatic hydrocarbons. The detailed composition has been described elsewhere (Lavarías et al., 2004).

2.3. Adult and embryo toxicity tests

Exposure assays were performed based on the 96-h LC50 concentrations of WSF as determined previously for this shrimp (1.56 mg/ml, Lavarías et al., 2004). Groups of eight adults and six ovigerous females carrying stage 4 or stage 5 eggs were exposed. Embryogenesis lasts 39 days in this prawn, and it has been divided into seven stages according to major morphological characteristics, being stages 4 and 5 where the major yolk consumption was observed. (Lavarías et al., 2002).

Animals were exposed to a sublethal level of WSF (0.6 mg/l) at 22 ± 2 °C and a 14 h light:10 h dark cycle. This concentration was chosen as the maximum with sublethal effects and also because it falls within the levels usually found in *M. borellii* environment. Control groups were held in clean water. Shrimps were held in a 2.5 l aquarium with the lid sealed to avoid hydrocarbon loss, and exposed for 7 days, with daily change of media. Temperature, pH and dissolved oxygen were measured in the control containers.

2.4. Preparation of total cellular homogenate, mitochondrial and cytosolic fractions

After 1 week exposure to WSF, egg clutches and midgut gland (hepatopancreas) from adults were immediately removed, weighed and cooled on ice, then homogenized in 0.25 mol l^{-1} sucrose containing 1.4 mmol l^{-1} *N*-acetyl-L-cysteine, 0.15 mol l^{-1} ClK, 5 mmol l^{-1} $MgCl_2$, 0.4 mmol l^{-1} EDTA and 62 mmol l^{-1} potassium phosphate buffer, pH 7.4, using a glass–Teflon potter homogenizer (Thomas, Philadelphia, PA) as described by González-Baró et al. (1990). The crude homogenate was first centrifuged in a Sorvall RC-2 (Newtown, CT) refrigerated centrifuge at 600 × *g* at 4 °C for 7 min to remove egg envelopes, cellular debris and nuclear fraction. The supernatant was centrifuged at 10,000 × *g* for 20 min to obtain the mitochondrial fraction. The pellet was then resuspended and immediately used for β-oxidation and palmitoyl-CoA synthetase assays. The supernatant was centrifuged at 100,000 × g for 50 min in a Beckman L8M ultracentrifuge (Beckman, Palo Alto, CA) to obtain the cytosolic fraction. Total protein was determined by the method of Bradford (1976).

2.5. Long-chain fatty acyl-CoA synthetase assay

Before starting the exposure assays, an experiment to find the optimal values for protein concentration, incubation time, pH, temperature and substrate concentration was performed in adults. The procedure used was a modification of that applied by Singh et al. (1988). The reaction mixture contained 0.18 μCi (6.4 μmol l^{-1}) [1-^{14}C]-palmitic acid under the conditions previously described for a microsomal ACS (González-Baró et al., 1990). The reaction was started by the addition of mitochondria. After stopping the reaction (Dole, 1956), a partition was

produced and the aqueous phase was washed with *n*-hexane to remove the free fatty acid. Radioactivity present in the aqueous phase was quantified by liquid scintillation counting in a Pharmacia LKB Wallac 1219 Rackbeta liquid scintillation counter (Uppsala, Sweden).

2.6. β-Oxidation assays

The basic procedure used for β-oxidation assays was a modification of that applied by Ruidera et al. (1988) and Hashmi et al. (1986).

The substrate used was [1-^{14}C] palmitic acid (0.22 μCi, 7.72 μmol l^{-1}), and the reaction was carried out in the presence of suitable cofactors and conditions and mitochondrial fraction (120 μg protein/ml of reaction mixture), at 32 °C for 20 min as detailed in a previous work (Irazú et al., 1992). After stopping the reaction by the addition of 1.25 ml of KOH 10% in ethanol, samples were saponified and the fatty acid that did not oxidize was removed, washing the samples with organic solvents. The labeled acetate was quantified in the aqueous phase by liquid scintillation counting.

2.7. Triacylglycerol lipase assays

Lipase activity was assayed according to the method described by Arrese and Wells (1994) with some modifications (González-Baró et al., 2000). Briefly, a mixture of [carboxyl-^{14}C]-triolein (0.2 μCi/ml of reaction mixture, final concentration 1.78 μmol l^{-1}) and unlabelled triolein (final concentration 10 mmol l^{-1}) was used as substrate. In order to form a micellar triolein substrate, Triton X-100 (final concentration 5 mmol l^{-1}) was added. After addition of 0.3 ml of reaction mixture (20 mmol l^{-1} Tris–HCl buffer, pH 7.5 containing 0.5 mol l^{-1} NaCl and 0.1% (w/v) bovine serum albumin), the substrate was dispersed by vortexing for 1 min. The reaction was initiated by adding 1 mg cytosolic protein/ml of reaction mixture, and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 2.5 ml chloroform–methanol (2:1, v/v), and extracted lipids from the chloroform phase were separated by high performance thin-layer chromatography (HPTLC) on silica gel 60 plates (Merck, Darmstadt, Germany) using hexane–ethyl ether–acetic acid (80:20:1.5, v/v/v) as developing solvent. The radioactivity associated with each lipid class was quantified by proportional scanning counting as previously described (González-Baró et al., 2000).

One-way analysis of variance was used to determine significant group differences between treatments ($p < 0.05$).

3. Results

3.1. Characterization of shrimp mitochondrial palmitoyl-CoA synthetase activity

Adult midgut gland and stages 4 and 5 eggs were assayed for mitochondrial ACS activity. Eggs at these developing stages were selected as this is the period of maximal lipid metabolism as determined in a previous work (González-Baró et al., 2000), likewise midgut gland was selected as it is the major site of lipid synthesis and degradation in adults (González-Baró et al., 1990; Irazú et al., 1992).

The palmitoyl-CoA synthesis as a function of protein concentration is depicted in Fig. 1A. The enzyme activity shows a linear response of at least up to 200 and 160 μg/ml mitochondrial protein in midgut gland and eggs, respectively. Therefore, the assays were performed using 120 μg/ml of mitochondrial protein concentration. Enzyme activity as a function of incubation time was linear for the first 20 min in eggs and for the first 10 min in midgut gland (Fig. 1B), so an incubation time of 10 min was selected for the assays as both samples were usually analyzed at the same time. Optimal temperature for adults was found to be 32 °C (specific activity 1120 pmol min^{-1} mg protein^{-1}), and it was observed that the specific activity decayed at higher temperatures as it usually happens in thermo labile enzymes (Fig. 1C). Finally, we observed that the activity increased as a function of pH up to pH 8 (results not shown) which was selected for the assays.

Using the assay conditions above described, the synthesis of palmitoyl-CoA was assayed as a function of substrate concentration. This followed a normal Michaelis–Menten kinetics in the substrate concentration range analyzed. Lineweaver–Burk plots were used to calculate the kinetic parameters (Fig. 1D). The calculated apparent Michaelis–Menten constant (K_m) and maximal velocity were 2.48 μmol l^{-1} and 1.93 nmol l^{-1} min^{-1} mg protein^{-1}, respectively. For the bioassays, we selected a substrate concentration that did not interfere with membrane stability due to fatty acid detergent properties.

3.2. Response of lipid catabolic pathways to WSF exposure

Using the previously determined assay condition for TAG-lipase and fatty acid β-oxidation (see methods), and once we had characterized and established the optimum condition for mitochondrial ACS assay in shrimp (see above), we studied the effect of the WSF on the lipid catabolism pathway of adults and eggs. After a 7-day exposure to 0.6 mg/l WSF we observed an increase in all of these lipid catabolic pathways.

3.3. Fatty acid activation

The effect of WSF on mitochondrial fatty acid activation, measured by the ACS activity, was evidenced by an increase of the enzyme activity in adult midgut gland by 7.4% compared to controls, and a more pronounced increase in eggs at stage 5 (52%). When eggs at stage 4 were exposed to WSF, they did not show significant differences in ACS activity (Fig. 2).

3.4. Degradation of lipids and fatty acids

Lipid store mobilization by TAG-lipase was studied in both eggs and adult midgut gland. Although this enzyme activity increased in adult by 38.4% compared to controls, it was particularly stimulated in eggs. A 27% increase was observed in stage 4 eggs, but the most noticeable increase was found in eggs at stage 5 (154% increase of activity) (Table 1).

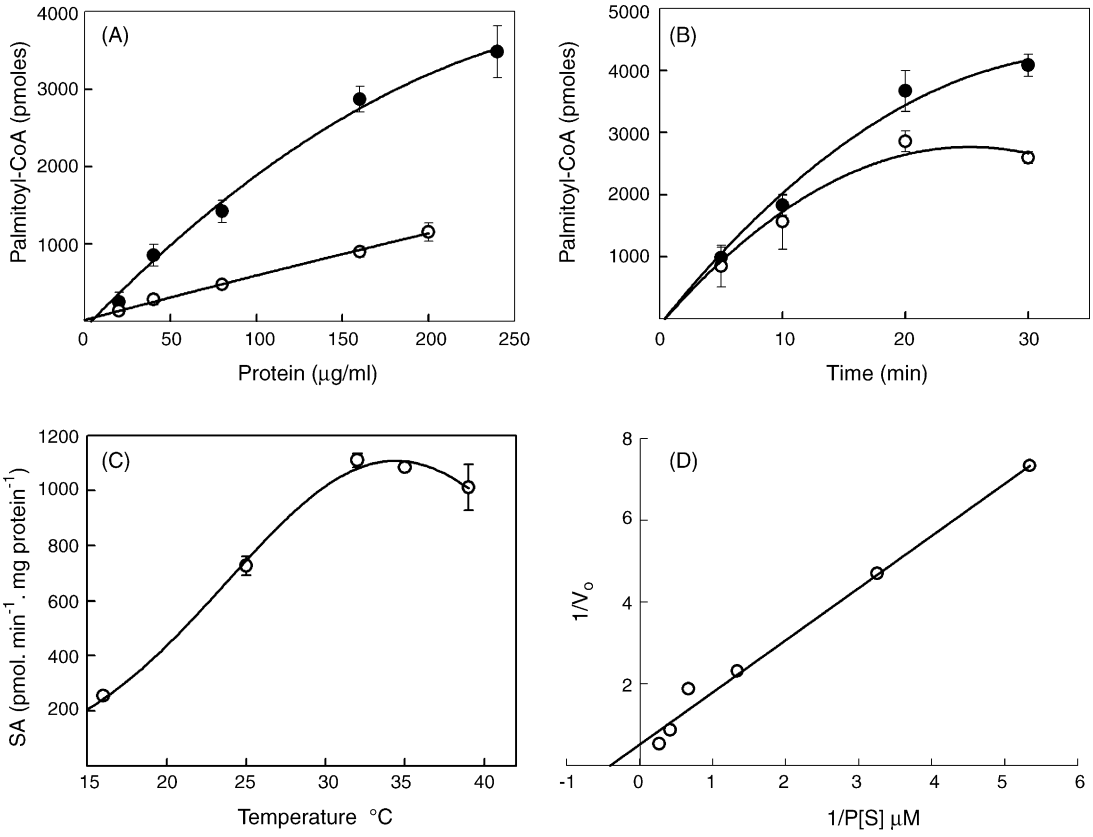

Fig. 1. Effect of protein concentration (A), incubation time (B), temperature (C) and substrate concentration (Lineweaver–Burk plot) (D) on the activity of mitochondrial palmitoyl-CoA synthetase in adult midgut gland and eggs of *M. borellii*. Temperature and substrate concentration were assayed only in adults. (●) Eggs; (○) midgut gland. Values represent the mean of three determinations ± one S.D. SA: specific activity V_0: initial velocity (nmol l^{-1} min^{-1} mg $protein^{-1}$).

The fatty acid β-oxidation system is one of the most important in generating the reductive power for ATP synthesis, and when adults and eggs were exposed to WSF contamination we also observed significant changes in this system. Unlike the effect on lipase and ACS activities, the most affected life-stage were adult shrimp (51% increase) followed by eggs at the end of development (35.5%), while eggs at stage 4 were not significantly affected by the pollutant (Fig. 3).

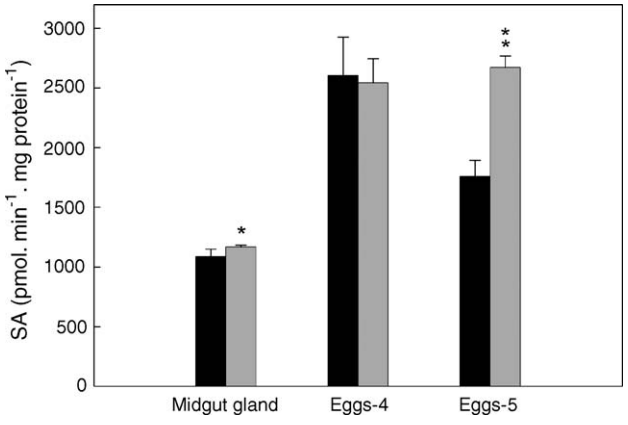

Fig. 2. Effect of a 7-day exposure to 0.6 mg/l WSF on the activity of mitochondrial palmitoyl-CoA in adult midgut gland and eggs of *M. borellii*. ■: Control; ▒: WSF exposed. Values represent the mean of three determinations ± one S.D. Bars marked on top are significantly different $^{*}p<0.05$ $^{**}p<0.001$. Eggs-4: stage 4 embryos; Eggs-5: stage 5 embryos. SA: specific activity.

4. Discussion

4.1. Mitochondrial palmitoyl-CoA synthetase activity

One of the objectives of this study was to investigate if the WSF could affect shrimp lipid catabolism. We therefore analyzed key enzymes involved in TAG degradation, fatty acid activation for incorporation into mitochondria, and the mitochondrial fatty acid oxidation itself. However, as there was no information on mitochondrial Acyl-CoA synthetase in crus-

Table 1
Effect of a 7-day exposure to 0.6 mg/l WSF on TAG-lipase activity in adult midgut gland and eggs of *M. borellii*

	Control	WSF
Midgut gland	49.5 ± 9.8	68.5 ± 11.1*
Eggs-4	51.0 ± 4.0	65.0 ± 8.0*
Eggs-5	37.0 ± 14.0	94.0 ± 21.0**

Values are expressed as pmol min^{-1} mg $protein^{-1}$ and represent the mean of three determinations ± one S.D. Eggs-4: stage 4 embryos; Eggs-5: stage 5 embryos.
* $p<0.05$.
** $p<0.001$.

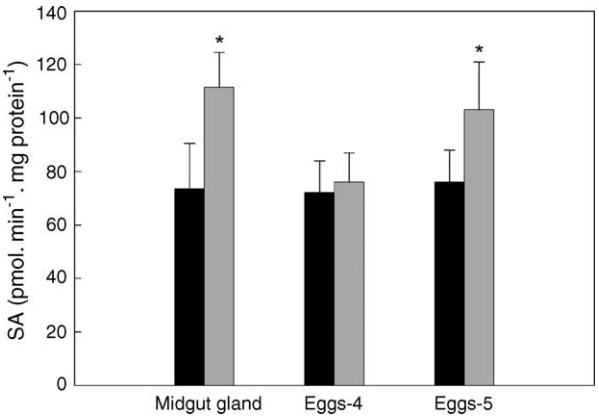

Fig. 3. Effect of a 7-day exposure to 0.6 mg/l WSF on fatty acid β-oxidation in adult midgut gland and eggs of *M. borellii*. ■: Control; □: WSF exposed. Values represent the mean of three determinations ± one S.D. Significantly different $^{*}p < 0.05$. Eggs-4: stage 4 embryos; Eggs-5: stage 5 embryos. SA: specific activity.

taceans, prior to toxicity bioassays, we searched for its presence. We observed that the activity of this mitochondrial enzyme in midgut gland showed kinetic differences compared with the microsomal isozyme we had previously studied (González-Baró et al., 1990), namely significantly higher apparent K_m and V_{max}. These kinetic differences could be attributed to several reasons: (a) most probably they are encoded by different genes or by the same gene with alternative splicing (Lodish et al., 2003); (b) its membrane location is different, and it is known that the membrane environment can affect enzymatic activity (e.g. Castuma and Brenner, 1983); (c) they may have different regulation as it occurs in mammalian liver isoforms (Coleman et al., 2002).

These long-chain ACSs may play a role in channeling fatty acids toward oxidation or complex lipid synthesis and storage. Thus, mitochondrial ACS may provide acyl-CoA for mitochondrial oxidation while microsomal ACS for acylglycerols synthesis or both isoforms may provide acyl-CoA to a single pool. The simultaneous increase in activity of both, ACS and β-oxidation favors the first option that is fatty acids activated by mitochondrial ACS isoform are channeled toward the production of energy.

Moreover, the presence of ACSs isoforms with different characteristics indicates they should play an active role in the regulation of lipid metabolism in shrimp as current data in mammals suggest (Coleman et al., 2002). The presence of more than one ACS in midgut gland and eggs could also represent a redundant genetic mechanism to ensure this key function is expressed in shrimp cells.

Ongoing research deals with the effect of WSF on lipid anabolism including microsomal ACS activity and membrane properties, which will help to better understand fatty acid pathways. No other reports on ACS isoforms in other invertebrate species are available, precluding any generalization.

4.2. Effect of WSF on lipid catabolism

There are few reports on the effect of hydrocarbon in invertebrate lipid metabolism. Elumalai and Balasubramanian (1999) reported a decrease of total esterase activity in muscle and ovary of the crab *Scylla serrata* exposed to naphthalene. In a more specific study, other authors have focused on the esterase subgroup of lipases, hydrolytic enzymes involved in the catalysis of a wide array of lipids (Ramana-Rao and Surendranath, 1991). They reported in the shrimp *Metapenaeus conoceros* exposed to kelthane, an induction of three groups of pH-dependent lipases, paralleled with a decrease of total lipids and an increase of free fatty acids. In the present study on *M. borellii* we used a radioactive TAG substrate to selectively quantify hydrolytic TAG-lipase activity and to differentiate it from other lipases. We had previously observed that total lipids decreased and the triacylglycerol/phospholipid ratios increased in midgut gland and decreased in eggs of *M. borellii* exposed to WSF, suggesting alterations either in the mobilization of triacylglycerols to phospholipid pools or in the energy balance (Lavarías et al., 2005). Lipase activity increased in all exposed organisms regardless of life-stage, suggesting that organisms face an increasing need of energy, probably to cope with the stress situation triggered by hydrocarbons as it has been suggested for this shrimp (Lavarías et al., 2005) and for crabs (Rafi et al., 1991). In this line of thought, we observed a major increase in TAG lipase activity in WSF-exposed eggs at stage 5. This developing stage shows the highest activity in the lipid anabolic and catabolic pathways of the whole period of embryogenesis, supplying energy and structural lipids for this period of active organogenesis (González-Baró et al., 2000). As a result the TAG pool is significantly depleted during this stage (Heras et al., 2000). The exposure of these eggs to a stressor such as WSF augmented even more the reduction of TAG stores (Lavarías et al., 2005). The WSF-induced increase in TAG lipase positively correlates with the activities of mitochondrial palmitoyl-CoA synthetase and fatty acid β-oxidation system in eggs, pointing to an increase in fatty acid oxidation for the production of energy. This is in agreement with the works on *Callinectes sapidus* (Wang and Stickle, 1988) and *Homarus americanus* (Capuzzo et al., 1984), where the lipid composition analysis of crabs and lobsters exposed to WSF showed an alteration in the pattern of energy utilization and a decrease in the lipids used for energy storage.

Interestingly, ACS and β-oxidation were not affected in stage 4 eggs. This difference could be related to the observation that the eggshell permeability to hydrophobic molecules increases at more advanced stages (unpublished results), and the fact that a chronic exposure of eggs to WSF affects only those about to hatch (Lavarías et al., 2004). Midgut gland lipid catabolism of WSF-exposed organisms responded with a pattern similar to that of stage 5 eggs, and we observed a significant but not so marked increase in mitochondrial ACS and β-oxidation activities, positively correlated with TAG degradation.

In summary, shrimp exposed to WSF showed an increased flow of metabolites through the lipid catabolic pathway which could be due to an activation of available enzymes, or a transcription induction. Regardless of the mechanism, this result suggests that adults as well as eggs have an increased energy need triggered by the contaminant. The sublethal WSF concentration employed in the present study is within the range of hydrocar-

bon concentrations usually found in the environment (Colombo et al., 1989) though after an oil spill, hydrocarbon concentrations can reach several times this level (Gallego et al., 1995; Ritchie and O'Sullivan, 1994). Therefore, changes observed in the present study could be employed as a complementary early biochemical biomarker, indicating the presence of hydrocarbon pollution. Nevertheless, further work is needed to validate the suitability of lipid catabolic pathways as biomarkers in the field.

Ongoing research will be focused on the effect of WSF on lipid composition and other metabolic pathways to achieve a more comprehensive view of the consequences of hydrocarbon pollution on crustaceans at a biochemical level.

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