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COMPARATIVE EXAMINATION OF PHOSPHOLIPIDS AND FATTY ACIDS FROM SOME CASPIAN INVERTEBRATES

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Abstract—1. Phospholipid compositions, plasmalogen included, of three invertebrate species—crabs, *Rhithropanopeus harrisii*, crayfish, *Astacus leptodactylus eichwaldi* and Bivalvia, *Mytilaster lineatus*, were examined.

2. Eighty-six fatty acids, including 23 saturated, 27 monoenoic, 10 dienoic and 26 polyenoic acids, were identified by capillary GC–MS.

3. A number of unusual fatty acids such as 4,8,12-trimethyl-13:0, pristanic, 3-*trans*-16:1, as well as polyenoic acids 18:5 (n-3), 25:5 (n-6) and 24:6 (n-3), were identified.

INTRODUCTION

The Caspian Sea, the world's largest salt lake, became isolated from other seas in the post-glacial period; it preserved rich late-Tertiary brackish-water fauna of marine origin formed about 30 or 40 million years ago in isolated Tertiary waterpools with water of much-varied salinity, in the southern parts of present-day Russia. The isolated position and low salinity of the water prevented many invertebrates from inhabiting the Caspian Sea, including some carnivorous species like starfish, large molluscs and jelly-fish, and Actinia; as well as "weedy" species, like large porifers, the majority of hydroids and Bryozoa (Birshtein *et al.*, 1968; Grimm, 1876, 1877; Mordukhai-Boltovsky, 1960).

Considering the above details it may be of interest to investigate the lipid compositions of invertebrates which inhabit such brackish water (the salinity in the sampling site was 13.4‰).

Lipids from invertebrates, including molluscs and crustaceans, in both the marine and freshwater forms (Ackman, 1981, 1989; Cossins, 1976; Dembitsky, 1979; Dembitsky and Vaskovsky, 1976; Gardner and Rilley, 1972; Joseph, 1982, 1989; Kostetsky, 1985; Kostetsky and Shchipunov, 1983; Liang and Strickland, 1969; Vaskovsky, 1989) have been described in the literature; however, the present comparative biochemical examination of lipid compositions of Caspian invertebrates may considerably add to our knowledge of evolutional and adaptive mechanisms.

MATERIALS AND METHODS

All the invertebrates—crab, *Rhithropanopeus harrisii* Gould, crayfish, *Astacus leptodactylus eichwaldi* Bott; and bivalvia, *Mytilaster lineatus* Gmel. (= *Mytilus minilus* Poli = *Mytilus lineatus* Gmel.), were sampled during September 1991 at a site located 70 km south of Krasnovodsk on the western coast of the Caspian Sea (Republic of Turkmenia) from depths of 2–3 m. The temperature of the water was 20°C.

The animals were treated, immediately after sampling, as described elsewhere (Dembitsky, 1979; Dembitsky and Vaskovsky, 1976).

Lipid analysis

Qualitative and quantitative determination of lipid from extracts was done by micro-thin-layer chromatography as described previously (Dembitsky, 1979; Dembitsky and Vaskovsky, 1976; Dembitsky *et al.*, 1992). Plasmalogen contents were determined using the method of Dembitsky (1988).

Preparation of fatty acid methyl esters

Methyl esters of corresponding FAs were prepared by alkaline hydrolysis, and reaction of free acids with BF_3-methanol (Rezanka *et al.*, 1990). Fatty acid methyl esters were purified by thin-layer chromatography with a silica gel G-plate of 0.5 mm thickness by developing with *n*-hexane/ether (85:15 v/v).

The oxazolines and/or their trimethylsilyl (TMS) ethers were prepared by modification of the reference methods (Tulloch, 1985; Yu *et al.*, 1988): 5 mg dicyclohexylcarbolimide was added to a solution of 5 mg FAs in 1 ml CH_2CI_2. After stirring (10 min) 5 mg 2-amino-2-methylpropanol was added (20°C; 4 hr). The evaporated mixture was dissolved in 1 ml diethylether and treated with 0.5 ml $SOCI_2$ (20°C; 1 hr), washed in ice-cold water and eluted through a column with anhydrous sodium sulfate and silica-gel. The eluate was evaporated, dissolved in pyridine, and heated with trimethylsilylchloride (50°C; 4 hr).

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Table 1. Phospholipid composition of invertebrates

	PE	PS	CAEP	SPH	LPC	PC	PI	DPG	PA	X	TL
Mollusc *Mytilaster lineatus*	41.4 ± 1.1	4.9 ± 0.3	2.2 ± 0.1	—	2.2 ± 0.1	43.8 ± 0.9	3.4 ± 0.2	2.1 ± 0.2	—	—	48.0
Crayfish *Astacus leptodactylus eichwaldi*	24.0 ± 0.8	6.8 ± 0.4	—	8.4 ± 0.6	—	51.1 ± 1.2	6.0 ± 0.3	2.9 ± 0.1	0.9 ± 0.1	—	42.0
Crab *Rhithropanopeus harrisii*	22.1 ± 0.5	5.5 ± 0.3	—	6.3 ± 0.4	5.3 ± 0.3	53.7 ± 1.4	3.0 ± 0.2	1.9 ± 0.2	—	2.3 ± 0.3	31.0

Abbreviations—PE, phosphatidylethanolamine; PS, phosphatidylserine; CAEP, ceramide aminoethylphosphonate; SPH, sphingomyelin; LPC, liso-PC; PC, phosphatidylcholine; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; PA, phosphatidic acid; X, non-identified phospholipid; TL, total lipids (mg/g dry wt).

Methyl esters were identified in a Shimadzu QP-1000 apparatus with a fused silica capillary column (60 m × 0.32 mm ID/SPB-1; Supelco, Bellefonte, PA), using splitless injection, and helium as the carrier gas. The oven temperature was programmed from 100 to 320°C, at a rate of 4°C/min. Ionization energy was 70 eV, and electron multiplier voltage was 2.5 kV. TMS-ethers of oxazolines were identified under conditions similar to those of the methyl ester identification, the only difference being that the column temperature was programmed from 150 to 300°C at the rate 5°C/min.

RESULTS AND DISCUSSION

The mollusc *Mytilaster lineatus*, according to Birshtein *et al.* (1968) and Bogachev (1928) was brought over to the Caspian Sea during the 1920s from the Black Sea and became adapted to new conditions. The species occurs in the Southern and middle parts of the Caspian Sea where the water salinity is below 7–8‰. The lipid composition of *Mytilaster lineatus* is given in Table 1. Amine-containing lipids comprise 48.5%, while choline-containing lipids make up 46.0%. PI and DPG are also found at levels of 3.4 and 2.1%, respectively. Aminolipid content is similar to that of freshwater Bivalvia. As reported in our earlier studies, *Dreissena polymorpha* contained 47.2%, while *Unio* sp. contained 51.7% aminolipids (Dembitsky *et al.*, 1992). Choline-containing lipids in *M. lineatus*, at the level of 46.0% were higher compared to those of the freshwater *Dreissena polymorpha* (38.0%) and *Unio* sp. (33.5%) species. The cited figures make *M. lineatus* closer to the marine species. The same results from the data were obtained by various authors (Dembitsky, 1979; Dembitsky and Vaskovsky, 1976; Kostetsky, 1985; Vaskosky, 1989).

Of particular interest is fatty acid (FA) composition of the total lipid extract from *M. lineatus*. Among saturated FAs, we identified 4,8,12-trimethyl-13:0 (0.50%), pristanic (0.47%) as well as iso-(the sum acids) (0.42%) and anteiso- (0.8%) acids (Table 2). Acids 4,8,12-trimethyl-13:0 and pristanic are likely to be of plant origin.

Among monoenoic acids (Table 3), four families of FAs are worthy of note. Acid 16:1 has four isomers with one *trans*-isomer (3-*trans*-16:1) and amounts to 0.19%; this is an acid of low occurrence in molluscs. The families 18:1, 20:1 and 22:1 possess five isomers each.

Dienoic acids are invariably interesting in that they contain non-methylene-interrupted (NMI) FA (Ackman and Hopper, 1971; Ackman *et al.*, 1973; Ackman, 1981; Johns *et al.*, 1980; Joseph, 1982, 1989; Naumenko and Kostetsky, 1986a). To these belong acids 5,11–20:2, 5,13–20:2, 7,13–22:2 and 7,15–22:2. Marine Bivalvia usually contain large amounts of NMI FAs. As reported by Zhukova (1986) who studied 17 species of marine Bivalvia, total 20:2 NMI FAs varied from 0.1 to 4%, while 22:2 NMI FAs showed variation from 0.6 to 20.7%. Naumenko and Kostetsky (1986b) who studied the seasonal distribution of individual phospholipid classes, pointed out 1.8% and 22.8% of 20:2 NMI FAs in PC and PE, respectively, during summertime in contrast to 2.5% and 18.8%, respectively, in winter samples. The contents of 22:2 NMI FA in the marine Bivalvia *Crenomytilus grayanus* were 2.6% and 10.6% in the summer season, and 1.1% and 5.6% in

Table 2. Plasmalogen content at individual classes of phospholipids of invertebrates (%)

	Phospatidylethanolamine		Phosphatidylcholine	
	plasmalogen	diacyl + alkylacyl	plasmalogen	diacyl + alkylacyl
Mytilaster lineatus	86.2	13.8	12.4	87.5
Astacus leptodactylus eichwaldi	41.2	58.8	7.3	92.7
Rhithropanopeus harrissi	44.4	55.6	13.5	86.5

Table 3. Saturated fatty acids from invertebrates

	Rhithropanopeus harrisii	*Astacus leptodactylus eichwaldi*	*Mytilaster lineatus*
iso-13:0	0.01	0.02	0.05
anteiso-13:0	0.03	0.04	0.03
n-13:0	0.09	0.06	0.04
iso-14:0	0.05	0.01	0.04
n-14:0	2.57	3.61	1.19
4,8,12-triMe-13:0	0.40	0.25	0.50
iso-15:0	0.10	0.01	0.03
anteiso-15:0	0.12	0.08	0.02
n-15:0	0.27	0.20	0.09
iso-16:0	0.04	0.08	0.07
Pristanic	0.35	0.19	0.47
n-16:0	12.20	10.02	10.08
iso-17:0	0.03	0.01	0.01
anteiso-17:0	—	0.12	0.02
n-17:0	0.95	0.68	0.31
iso-18:0	–	0.13	0.16
n-18:0	2.31	2.08	0.84
iso-19:0	—	—	0.06
anteiso-19:0	—	—	0.01
n-19:0	0.12	0.16	0.19
n-20:0	0.82	0.40	0.35
n-22:0	0.06	0.22	0.07
n-24:0	0.03	0.06	0.15
Total	20.55	18.44	14.78

the winter season, contained in PC and PE, respectively. As suggested by Zukova (1986), NMI FA distribution in Bivalvia is related to their systematic position; different species within one family have similar NMI contents. Our own results (Table 5) point to very moderate levels of NMI FA not exceeding 1%.

Among the identified polyenoic acids of particular interest are acids 18:5 (n-3) (0.36%) as well as 5,11,14–20:3 and 5,11,14,17–20:4. In addition, we identified a number of acids which rarely occur in invertebrates: 24:4 (n-6), 24:5 (n-6) and 24:6 (n-6). These three acids had been previously found in the fat of marine fish, where their amounts were less than 2% (Linko and Karinkanta, 1970), in prawn *Penaeus japonicus* (Guaru *et al.*, 1974) and in some marine echinodermata (Ackman, 1989). Significant amounts of these FAs were found in different coelenterates (Joseph, 1979; Cham *et al.*, 1981). Vysotskii and Svetashev (1991), in the recent screening analysis of

Table 4. Momoenoic fatty acids from invertebrates

	Rh. harrisii	*A. leptodactylus*	*M. lineatus*
14:1 (n-7)	0.09	0.10	0.19
14:1 (n-5)	0.04	0.06	0.01
16:1 (n-9)	15.71	13.83	9.21
16:1 (n-7)	1.04	0.15	3.09
3-*trans*-16:1*	—	—	0.19
16:1 (n-5)	0.20	0.22	0.26
17:1 (n-8)	0.12	—	0.03
18:1 (n-13)	0.20	0.59	0.33
18:1 (n-11)	0.71	2.19	3.38
18:1 (n-9)	3.80	0.32	2.25
18:1 (n-7)	0.12	0.02	0.27
18:1 (n-5)	0.15	0.25	0.31
19:1†	—	—	0.03
19:1 (n-8)	0.02	0.05	0.10
20:1 (n-15)	0.03	—	0.03
20:1 (n-13)	0.09	0.30	0.20
20:1 (n-11)	0.33	0.18	0.29
20:1 (n-9)	0.90	0.69	1.18
20:1 (n-7)	0.23	0.18	0.39
21:1 (n-9)	—	—	0.08
22:1 (n-15)	0.06	0.02	0.19
22:1 (n-13)	0.02	0.05	0.13
22:1 (n-11)	0.69	0.59	0.41
22:1 (n-9)	0.23	0.27	0.28
22:1 (n-7)	—	—	0.09
23:1 (n-9)	0.33	0.37	0.37
24:1 (n-9)	0.54	0.22	0.37
Total	25.65	20.65	23.66

*Identified only on the basic retention time and mass spectrum of methyl ester.

†Unknown position of double bond, identified by retention time and value of molecular ion of methyl ester.

Table 5. Dienoic fatty acids from invertebrates

	Rh. harrisii	*A. leptodactylus*	*M. lineatus*
16:2 (n-4)	0.42	0.90	0.91
18:2 (n-9)	9.35	7.38	7.86
18:2 (n-6)	1.01	0.60	1.88
18:2 (n-4)	—	—	0.14
5,11–20:2	0.53	0.44	0.41
5,13–20:2	0.43	0.29	0.15
20:2 (n-9)	—	0.06	0.09
20:2 (n-6)	0.67	0.56	0.24
7,13–20:2	0.23	0.38	0.45
7,15–22:2	0.11	0.25	0.22
Total	12.75	10.86	12.35

the above-mentioned acids' contents in coelenterates, have shown that, in the five studied species, the content of acid 24:4 varies from 0.1 to 0.4%, that of acid 24:5 (n-6) varies from 0.4 to 15.6%, and that of 24:6 (n-6) varies from 1.5 to 18.6%. The respective contents of acids C-26 and C-28, found in the mollusc *M. lineatus*, are reflected in Table 6.

Out of the two crustacean species belonging to the family Astacidae (genus *Astacus*), living in the Caspian Sea, one was sampled for our analysis. This genus comprises freshwater and marine subspecies. Our sample crayfish, *A. leptodactylus eichwaldi*, belongs to the latter. It is endemic and occurs only in the Bay of Krasnovodsk of the Caspian Sea (Bokova, 1948; Kessler, 1875).

The crab, *Rh. harrisii*, belongs to the family Xanthidae (genus *Rhithro*). It is the only species of the only genus living in the Caspian Sea. The species originates from the Atlantic coasts of North and South America, from Canada to Brazil, where it inhabits the marine salt and brackish waters (Rathbun, 1930). The species was brought over to the Caspian Sea on vessel bottoms, as supposed by Reznichenko (1967) in the 1950s, and became well-adapted to the conditions of the Caspian Sea.

In their phospholipid compositions, the two analyzed crustaceans are very similar. The major phospholipids found are PC and PE (Table 1). In contrast to the mollusc *M. lineatus*, containing CAEP, the crustaceans contain sphingomyelin; 8.4% sphingomyelin was found in *A. leptodactylus eichwaldi*, and 6.3%, in *Rh. harrisii*; sphingomyelin is present in many crustacean species, varying from 1.6 to 13.4% (Cossins, 1976; Kostetsky, 1982, 1985; Vaskovsky, 1989). The presence of CAEP is characteristic of Mollusca where its content varies, as a rule, within 6.8–15.8% (Vaskovsky, 1989), though the compound is known to be detected in a few crabs such as *Cyclograpsus punctatus* (De Koning, 1970) at a level of about 1%. Some authors report on sphinghomyelin detected in Bivalvia molluscs (Haley *et al.*, 1978; De Moreno *et al.*, 1976, 1980; *Octopus* Sp. in particular, are reported to contain 9.3% sphingomyelin and 6.8% CAEP (Vaskovsky, 1989). The crab, *Rh. harrisii*, was found to contain an unidentified phospholipid (2.2%) with a position on the chromatographic plate between PE and PC.

Table 6. Polyenoic fatty acids from invertebrates

	Rh. harrissii	*A. leptodactylus*	*M. lineatus*
16:3 (n-4)	0.93	0.96	1.16
16:3 (n-6)	0.95	1.51	1.63
16:4 (n-1)	1.48	1.11	1.44
18:3 (n-6)	11.94	19.34	10.93
18:3 (n-3)	16.91	15.03	14.63
18:4 (n-3)	0.13	0.11	0.96
18:5 (n-3)	—	—	0.36
20:3 (n-9)	0.53	0.25	0.70
5,11,14–20:3	—	—	0.27
20:3 (n-6)	0.09	0.20	0.50
20:4 (n-6)	3.02	4.06	4.93
20:3 (n-3)	0.45	1.04	1.31
5,11,14,17–20:4	—	—	0.62
20:4 (n-3)	0.13	0.43	0.64
20:5 (n-3)	0.23	0.34	0.31
21:5 (n-3)	0.01	0.00	0.19
22:3 (n-9)	0.07	0.08	0.11
22:3 (n-6)	0.27	0.41	0.41
22:4 (n-6)	0.17	0.27	0.28
22:5 (n-6)	0.14	0.26	0.25
22:5 (n-3)	0.49	0.94	0.91
22:6 (n-3)	1.41	1.84	1.74
24:4 (n-6)	0.42	0.22	0.45
24:5 (n-6)	0.46	0.18	0.65
24:6 (n-3)	0.40	0.69	1.90
NIFA*	0.42	0.78	0.93
Total	41.05	50.05	49.21

*Non-identified fatty acids (NIFA), the sum of furanoid acids.

Plasmalogens were found in PE and PC (Table 2). The plasmalogen in PE of the mollusc, *M. lineatus*, amounts to 86.2%%. A high plasmalogen content in PE is characteristic of marine Bivalvia. In our earlier reports (Dembitsky, 1979; Dembitsky and Vaskovsky, 1976) we pointed out that the average plasmalogen in the PE of 40 studied mollusc species was equal to 80 and 70% in freshwater species (Dembitsky *et al.*, 1992). Plasmalogen in the crayfish, *A. leptodactylus*, and crab, *Rh. harrisii*, was twice as low and made up to 41.2 and 44.4%, respectively. Marine crustaceans contain the same amount of plasmalogen in PE; plasmalogen levels in PC of the studied species, as a rule do not exceed 15% (Dembitsky, 1979).

Saturated acids are reflected in Table 3. Similarly to the molluscs, the crustaceans contain 4,8,12–13:0 and pristanic acids, though in lower amounts, while there anteiso-acid totals are higher than those of the mollusc. Acid 3-*trans*-16:1 is absent from the monoene group of crustaceans (Table 4). Small amounts of NMI FAs (Table 5) are found also in the crayfish and crab. As reported by Naumenko (1987), who studied FAs in PE and PC from the crab, *Hemigrapsus sanguineus*, the content of 20:2 NMI FA varies seasonally from 0 to 1.5% while 22:2 NMI FA was not detected. Neither 5,11,14–20:3 nor 5,11,14,17–20:4 acids are detected in crustaceans (Table 6). Out of polyenoic acids, the major acids 18:3 (n-6) and 18:3 (n-3) are found to be present in crustaceans, while these acids are known to be absent from the crab, *Hemigrapsus sanguineus*, altogether. The unusual acids, 24:4 (n-6), 24:5 and 24:6, are detected in amounts of less than 0.5%.

The present comparative study of some invertebrate species sampled from the Caspian Sea has shown that marine invertebrates inhabiting brackish water are well-adapted to lower salinity conditions; the lipid composition of such inverebrates differs from that of both their marine and freshwater relatives.

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