Mediterranean Marine Science

Vol 15, No 3 (2014)

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doi: 10.12681/mms.436

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
Doi: http://dx.doi.org/10.12681/mms.436

Biochemical and fatty acid composition of *Arca noae* (Bivalvia: Arcidae) from the Mali Ston Bay, Adriatic Sea

I. DUPČIĆ RADIĆ[1], M. CARIĆ[1], M. NAJDEK[2], N. JASPRICA[1], J. BOLOTIN[1], M. PEHARDA[3] and A. BRATOŠ CETINIĆ[4]

[1] Institute for Marine and Coastal Research, University of Dubrovnik, Kneza Damjana Jude 12, 20000 Dubrovnik, Croatia
[2] Ruđer Bošković Institute, Center for Marine Research Rovinj, Giordano Paliaga 5, 52210 Rovinj, Croatia
[3]Institute of Oceanography and Fisheries, Šetalište Ivana Meštrovića 63, 21000 Split, Croatia
[4]Department of Aquaculture, University of Dubrovnik, Ćira Carića 4, 20000 Dubrovnik, Croatia

Corresponding author: iris@unidu.hr

Handling Editor: Pascal Divanach

Received: 17 April 2013; Accepted: 12 Februry 2014; Published on line: 19 March 2014

Abstract

Biochemical and fatty acid composition of the bivalve *Arca noae* were investigated in the Mali Ston Bay in relation to environmental conditions. Sampling was carried out monthly, from December 2001 to November 2002. Wet shellfish meat consists on average of 77.61% water and 22.39% dry matter, while dry shellfish meat consists on average of 89.04% organic and 10.96% inorganic matter. PCA analysis identified temperature, nitrate, silicate, MICRO, Chl *a* and salinity as the most important environmental factors influencing the biochemical composition of *A. noae*. An increase of dry weight content of *A. noae* was observed during the spring when both the sea temperature and food supply increased rapidly. Protein (54.39-62.06% of dry weight), carbohydrate (4.13-8.07% of dry weight) and lipid (3.46-8.58% of dry weight) content varied significantly during the year. Protein and lipid level reached the maximum value in June. The fatty acid profiles of total lipids extracted from *A. noae* showed a high level of unsaturation (UNS/SAT 1.9-3.4). Total polyunsaturated fatty acids (PUFA) represented the majority of total fatty acids (40.3-59.9% of total fatty acids) and the most abundant were eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acid. The n-3/n-6 PUFA ratio value varied between 2.1 and 5.0 and was highest during the spring (April to June). Due to their low lipid and high percentage of healthy polyunsaturated fatty acids *A. noae* can be evaluated as a quality seafood product. The most suitable period of the year for the consumption of *A. noae* is in the spring when it reaches its highest nutritional value.

Keywords: *Arca noae*, Adriatic Sea, biochemical composition, fatty acids, environmental parameters.

Introduction

Noah's Ark (*Arca noae* Linnaeus, 1758) is a commercially important epifaunal bivalve whose distribution covers the eastern Atlantic Ocean, the Mediterranean and Black Seas and the West Indies (Nordsieck, 1969). It is one of the most important edible bivalves in the eastern Adriatic and is harvested from natural populations primarily by SCUBA divers. There are no reliable catch statistics including catch quantities or number of people involved in the exploitation of *A. noae* in Croatia. The majority of harvested *A. noae* is sold on the black market (≈7 EUR / kg) during the tourist season or is consumed by the local population (Župan *et al.*, 2012). Previous studies have shown that this species can obtain a maximum length of between 70 and 90 mm (Hrs-Brenko & Legac, 1996; Poppe & Goto, 2000) and lives for approximately 15 years, requiring 3 to 7 years to reach a commercial length of 50 mm (Peharda *et al.*, 2002, 2003).

Protein, carbohydrate and lipid levels in the soft tissue of marine bivalves undergo seasonal fluctuations, which are controlled primarily by ambient food and temperature conditions and by the reproductive status of the organism (Gabbot, 1983). These macromolecules together with minerals and minor components of hydrophilic and lipophilic nature, contribute to the nutritional value and organoleptic characteristics of bivalves (Orban *et. al.*, 2006).

Lipids represent an important energy reserve because of their high caloric contents; they are mainly used in chronic stress conditions, whereas carbohydrate reserves are generally used during gametogenetic processes when lipids are not available (Gabbott, 1976). Fatty acid profiles of total lipids extracted from various bivalves showed the prevalence of total polyunsaturated fatty acids (PUFA) over saturated (SFA) and monounsaturated (MUFA) acids (Orban *et al.,* 2002, 2006). Changes in fatty acid composition are closely related to available food and high levels of PUFA correspond with good nutritional conditions (Ojea *et al.*, 2004; Dridi *et al.*, 2007). Carbohydrates have two major biological functions: as long term energy storage and as structural elements (Robledo

et al., 1995). In bivalves, glycogen is the most prominent carbohydrate stored in large quantities during their growing season and used over the rest of the year (Beukema, 1997). Protein is the most abundant biochemical component in tissues and it may be an alternative energy reserve in some bivalve species during gametogenesis (Beninger & Lucas, 1984; Galap *et al.*, 1997).

Variations in biochemical composition are closely related to the reproductive cycle, which is demonstrated in various species of bivalves including the mussel *Mytilus galloprovincialis* (Bressan & Marin, 1985), clams *Venerupis philippinarum* and *Venerupis decussata* (Beninger & Lucas, 1984; Robert *et al.*, 1993) and some species of oyster (Gabbott, 1976; Berthelin *et al.*, 2000). In general, energy is stored prior to gametogenesis when food is abundant in the form of lipid, glycogen and protein substrates and subsequently is mobilized and utilized in the production of gametes when metabolic demand is high (Bayne, 1976; Barber & Blake, 1985, 1991; Dridi *et al.*, 2007).

Mali Ston Bay is an important bivalve aquaculture area for the European flat oyster (*Ostrea edulis*) and black mussel (*Mytilus galloprovincialis*) and one of the most studied areas in the Adriatic Sea, with a long tradition of collecting marine organisms and their aquaculture over several centuries. *Arca noae* is one of the main bivalve species traditionally collected in the Mali Ston Bay. This work is a part of intensive experimental investigations carried out from 2001 to 2004, mostly in the area of Mali Ston Bay, by a team of scientists from the Institute of Oceanography and Fisheries, Split-Laboratories Dubrovnik. The aim of the study was to collect data necessary for assessing the aquaculture potential of the bay and potentially initiating commercial production of Noah's Ark shell *A. noae* in the eastern part of the Adriatic Sea (Peharda *et al.*, 2002, 2003, 2006). The reproductive cycle of this species was described by Peharda *et al.* (2006) but no data are available on its biochemical composition and nutritional value. The knowledge of biochemical composition of bivalves is important as an aspect of quality of seafood and sensory attributes. The aim of this work was to study the seasonal variations of the gross biochemical composition and fatty acid profiles in *A. noae* populations from the Mali Ston Bay and to examine the potential influence of environmental parameters on its biochemical composition. The results will be useful for indicating periods of the year that are more suitable for the marketing and consumption of Noah's Ark.

Materials and Methods

Study Area

Mali Ston Bay is an elongated, sparsely populated, unpolluted area and traditionally important shellfish-farming region in the south-eastern part of the Adriatic coast (Fig. 1). It is characterized by strong marine currents, underwater springs ("vruljas") and abundant and constant sedimentation that influence the formation of soft-mud sediments (Šimunović, 1981). This area is also influenced by the freshwater income from the Neretva River at the outer part of the Bay. The pattern of nutrient concentration is in accordance with the freshwater influx (Carić *et al.*, 1992). Analysis of phytoplankton and zooplankton abundance and community structure indicate that the bay is a naturally moderately eutrophic ecosystem (Viličić, 1989; Lučić & Kršinić, 1998). Sampling was conducted at the Bistrina station located in the northern part of the Mali Ston Bay. Samples were collected from December 2001 to November 2002.

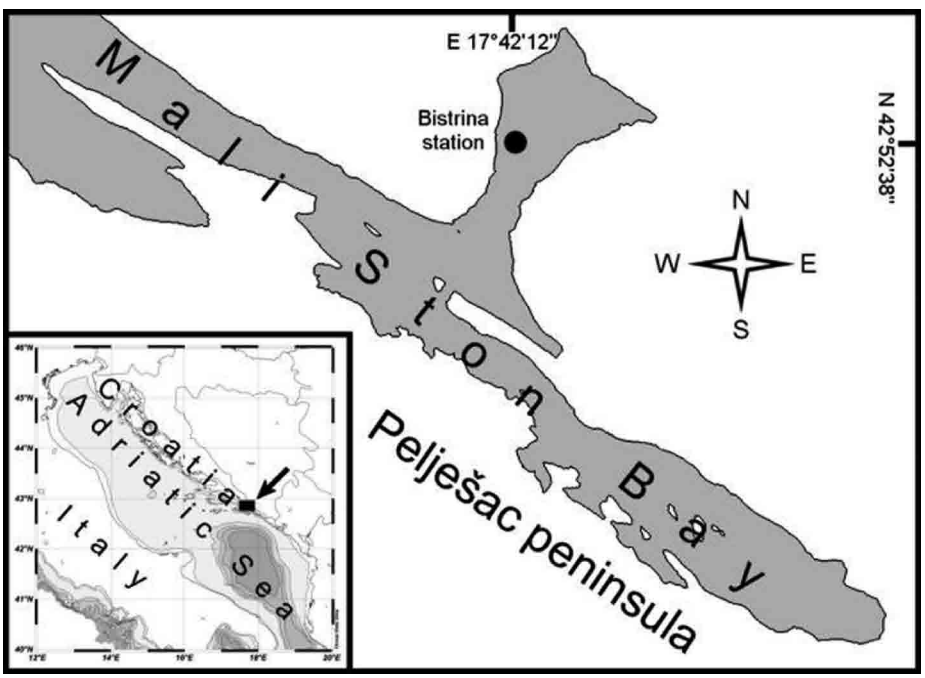

Fig. 1: Geographic location of Mali Ston Bay and the Bistrina station.

Environmental parameters

Temperature and salinity were measured twice a month at a depth of 2 m using a WTW (Ft. Myers, FL) multiline hydrographic probe. Density anomaly (σ_T) values were calculated from temperature and salinity values, on the basis of empirical relations, using oceanographic tables (UNESCO, 1987). Seawater samples for the analysis of nutrient concentrations, chlorophyll *a* (Chl *a*), and phytoplankton were also collected twice a month at the same depth using 5-liter Niskin bottles. Nutrient concentrations were determined according to standard oceanographic procedures (Strickland & Parsons, 1972; Koroleff, 1983). Subsamples of 0.5 dm^3 for determination of Chl *a* were filtered using Whatman GF/F filters, which were subsequently stored at -20°C. Chl *a* concentrations were determined fluorometrically with a Turner TD-700 Laboratory Fluorometer following the method of Parsons *et al.* (1985).

Phytoplankton samples were preserved in 2% neutralized formalin and observed under an inverted microscope (Olympus IX-71) according to the Utermöhl method (Utermöhl, 1958). Sub-samples (25-50 mL) were settled for 24-48 hours in Wild Hydro-Bios counting chambers (Kiel-Holtenau, Germany). Counting was carried out using phase contrast and bright field illumination. Counting of microphytoplankton cells (longer than 20 µm, MICRO) was performed on 1-2 transects along the counting chamber bottom at a magnification of 400x (1 transect) and 200x (2 transects). For counting of taxa larger than 30 µm, the entire chamber was subsequently scanned at 100x magnification. The minimum abundance that can be detected by this method is 20 cells L^{-1}. The entire MICRO community was identified to species or genus, and was divided in four groups: diatoms (Bacillariophyta, BACI), dinoflagellates (Dinophyta, DINO), coccolitophorids (Prymnesiophyceae – Coccosphaerales, COCCO), and other groups (OTHER = Cyanobacteria, Euglenophyta and unidentified cells). Regardless of their size, when filaments or colonies were encountered, single cells were counted and included within MICRO. Nanophytoplankton cells (2-20 µm, NANO) were counted in 30 randomly selected fields-of-view along the counting chamber at a magnification of 400x. NANO cells were not taxonomically identified. Results are expressed as number of cells per litre (abundance).

Biochemical analysis

Thirty individuals of commercial size were collected once a month from the seabed by scuba divers at depths of between 2 and 4 m. Soft tissues were carefully separated from shells and washed in distilled water to remove dirt and extraneous salts. Samples were then left on an absorbent surface for 20 min to remove as much extraneous water as possible. Whole animals were then homogenized and a subsample of homogenized tissue was taken for determination of dry weight and ash content. Dry weight was determined by drying to constant weight at 60°C and ash content after incineration at 800°C (Lovergrove, 1966). For biochemical analysis, homogenized tissue was dried to a constant weight at 60°C and ground to a fine powder in order to ensure efficient extraction of various macromolecules and reduce errors in measurement that may occur due to non-homogenized samples. The pulverized material was stored desiccated at -25°C until analysis (Giese, 1967). Lipids were extracted using a chloroform-methanol (2:1) mixture (Blight & Dyer, 1959) and total lipids were estimated by the sulphophosphovanillin method (Barnes & Blackstock, 1973). TCA was added to the lipid-free pellets to precipitate protein (Holland & Hannant, 1973) and the supernatant was analyzed for total carbohydrates using a phenol-sulphuric acid method (Kochert, 1978), which is based on procedures developed by Dubois *et al.* (1956). Protein content was determined using the method of Lowry *et al.* (1951) after hydrolysis in 1M NaOH for 30 min at 100°C.

Fatty acid analysis

For the analysis of fatty acids, *Arca noae* soft tissues were cold extracted in dichlormethane-methanol (2:1). The dichloromethane phases were purified by adding a salt solution and then evaporated to dryness using rotary evaporation at 30°C. After weighing, the total extracts were saponified following the addition of 1.2M NaOH in 50% aqueous methanol solution. The tubes were placed in a boiling water bath for 30 min. After cooling, the saponificate was acidified with 6M HCL (pH<2), and 12% BF_3 in methanol was added and heated for 10 min in a boiling water bath. After cooling, the fatty acid methyl esters (FAME) were extracted in dichlormethane.

FAME were analyzed by gas-liquid chromatography (GLC) on a 6890N Agilent Network GC System equipped with 5973 Agilent Network Mass Selective Detector with a capillary column (30m x 0.25mm x 0.25µm; cross linked 5% phenylmethylsiloxane) and ultra high purity helium as the carrier gas. The GLC settings were as follows: programmed column temperature 145°C by 4°C/min up to 270°C and constant column pressure 2.17 kPa. Retention times, peak areas and mass spectra were recorded on ChemStation Software. FAME in tissue samples was identified by mass spectral data and family plot of equivalent chain length data (ECL) for GC standards for the GC column used. Fatty acid methyl esters mix C18-C20 and polyunsaturated fatty acids standards (PUFA1 and PUFA3, Supelco, USA), cod liver oil and various individual pure standards of fatty acid methyl esters (Sigma, Germany) were used.

Data analysis

All biochemical analyses were done in triplicate and presented as mean values ± SD. Values for different fatty acids were expressed as a fraction (%) of total identified fatty acids. An analysis of variance (one-way ANOVA) with

Fisher LSD *post-hoc* test was used to test the significance of variations in biochemical components between seasons. The homogeneity of variance among the data was tested using Leven's test. Whenever the assumptions of analysis of variance were not met, the Kruskal-Wallis ANOVA on ranks test was performed. Principal component analysis (PCA) was used to display the relationship between biochemical parameters of shellfish and environmental parameters of Mali Ston Bay. The length of arrows representing a biochemical or environmental variable indicates the degree of correlation with the ordination axes. The arrow's direction represents a variable's gradient and indicates positive and negative correlations. Pearson product-moment correlation was used to test the significance of correlations between environmental parameters and biochemical composition of shellfish. All variables were logarithmically transformed [log (x+1)] to improve correlation among variables (Cassie, 1962). The Kolmogorov-Smirnov test was used for testing normality of distribution. Statistical analyses were performed using STATISTICA 7.0 (StatSoft Inc., 2004), and PC-ORD 5 (McCune & Mefford, 2006).

Results

Environmental parameters

Monthly values of physical-chemical parameters, Chl *a* concentrations, MICRO, NANO and relative contribution of different taxonomic groups to MICRO abundance at the sampling site in Mali Ston Bay are reported in Fig. 2. Seawater temperatures ranged from 7.2°C in January 2002 to 25.8°C in June 2002. Seawater temperatures >20°C were recorded between June and mid-

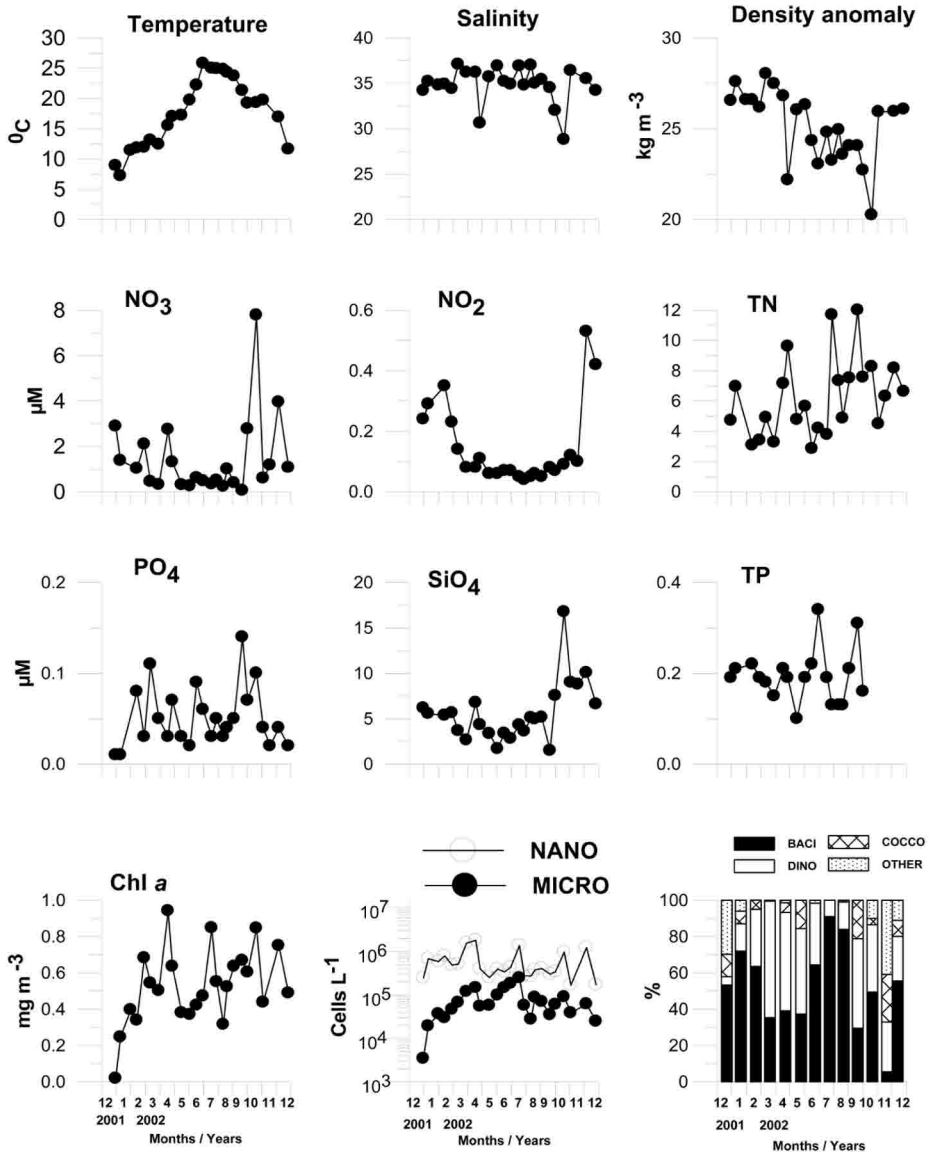

Fig. 2: Distribution of physical-chemical parameters, chlorophyll *a* (Chl *a*), microphytoplankton (MICRO), nanophytoplankton (NANO) and relative contribution of different taxonomic groups to microphytoplankton abundance (BACI – Bacillariophyceae; DINO – Dinophyceae; COCCO - Prymnesiophyceae-Coccosphaerales; OTHER – other MICRO groups).

September. The highest salinity value was recorded in March 2002 (37.1) and the lowest salinity value in October 2002 (28.8). Density anomaly values varied between 20.25 kg m^{-3} (October 2002) and 28.03 kg m^{-3} (March 2002). Nitrite ranged from 0.04 µM to 0.53 µM; nitrate from 0.06 µM to 7.79 µM; total nitrogen (TN) from 2.85 µM to 12.01 µM; ortosilicate from 1.48 µM to 16.77 µM; orthophosphate from 0.01 µM to 0.14 µM; total phosphorus (TP) from 0.10 µM to 0.34 µM. The lowest Chl *a* concentration was recorded in December 2001 (0.02 mg m^{-3}), and the highest in April 2002 (0.94 mg m^{-3}). MICRO ranged from 3.5 x 10^3 cells L^{-1} in December 2001 to 2.5 x 10^5 cells L^{-1} in July 2002, and NANO ranged from 1.7 x 10^5 cells L^{-1} in December 2002 to 1.8 x 10^6 cells L^{-1} in April 2002. Diatoms and dinoflagellates dominated the MICRO abundance.

The PCA diagram demonstrates a significant positive correlation of temperature, MICRO and Chl *a* with the first PCA axis (Fig. 6, Table 2). The strongest positive correlation of the second PCA axis is with nitrate and silicate, and negative with salinity. The PCA diagram illustrates a significant correlation of temperature with MICRO ($r = 0.80$, $P = 0.01$) and Chl *a* ($r = 0.65$, $P = 0.04$). Salinity had a significant negative correlation with nitrate ($r = -0.82$, $P = 0.01$).

Biochemical composition

Wet shellfish meat consists on average of 77.61 ± 1.86% water and 22.39 ± 1.86% dry matter, while dry shellfish meat consists on average of 89.04 ± 2.17% organic and 10.96 ± 2.17% inorganic matter. Monthly variations of dry flesh weight in *Arca noae* during the study, are shown in Fig. 3 (mean values with standard deviation bars). The highest percent of dry flesh weight was in April (25.96 ± 0.63) and the lowest in September (19.46 ± 0.23).

Monthly variations in protein, carbohydrate, lipid and ash contents of *A. noae* are presented in Fig. 4 in percentage dry weight (mean values with standard deviation bars). Percentage of inorganic matter (ash) ranged from 8.10 ± 0.27 in November to 14.02 ± 0.76 in August. The lipid content increased to a maximum value in June (8.58 ± 0.02%). After an abrupt drop in July, gradual decrease continued until September when the minimum value was recorded (3.46 ± 0.18%). Continuous increase of the lipid content was observed until November. The maximum carbohydrate value was recorded in December 2001 (8.07 ± 0.04%) and minimum carbohydrate value in July 2002 (4.13 ± 0.09%). Like in lipids, the peak in protein value was also recorded in June (62.06 ± 2.29%), and was followed by a gradual decrease until September.

Statistically significant differences among seasons were observed for all the biochemical components of *A. noae*. As regards dry weight (one-way ANOVA, $F=9.34$, $P<0.001$) and inorganic matter content (Kruskal-Wallis ANOVA, $H=24.29$, $P<0.001$), major significant differences were between autumn and the rest of the year. For lipid content (Kruskal-Wallis ANOVA, $H=14.87$, $P<0.01$), major differences were between spring and autumn. For carbohydrate content (Kruskal-Wallis ANOVA, $H=29.07$, $P<0.001$), significant differences exist between winter and spring and these values differ sharply from summer

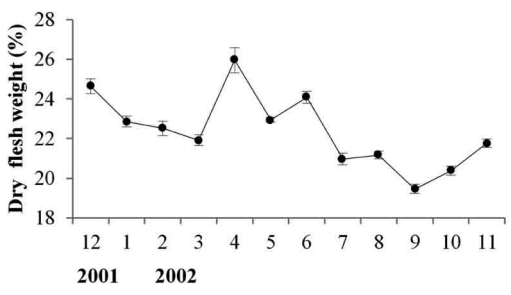

Fig. 3: Monthly variations of dry flesh weight in the ark shell *Arca noae* from Mali Ston Bay.

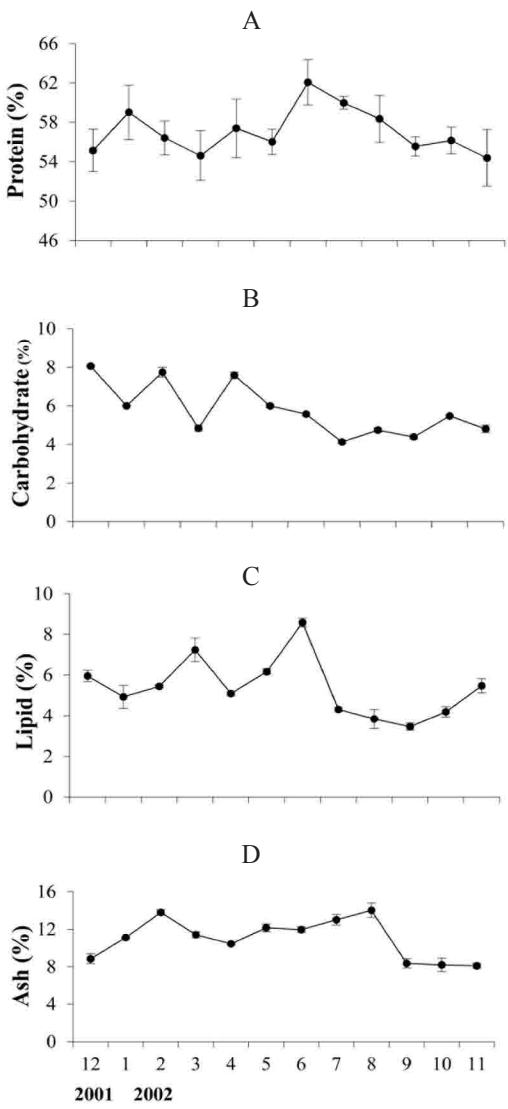

Fig. 4: Monthly variations in protein, carbohydrate, lipid and ash contents (% of dry weight) of the ark shell *Arca noae* from Mali Ston Bay.

and autumn values. As regards protein content (one-way ANOVA, F=3.65, P<0.05), summer values are higher and significantly differ from the rest of the year. PCA analysis indicates that inorganic matter (ash) had a significant correlation with salinity (r = 0.66, P = 0.04). Lipids had a significant negative correlation with total nitrogen (r = -0.79, P = 0.01) and carbohydrates with temperature (r = -0.66, P = 0.04) (Fig. 6).

Fatty acid profiles

Fatty acid composition of the soft tissue of *Arca noae* is shown in Table 1. The palmitic C16:0, palmitoleic C16:1(n-7) and oleic C18:1(n-9) acids dominated among the saturated (SATs) and monounsaturated fatty acids (MUFAs), respectively, while among the polyunsaturated fatty acids (PUFAs), eicosapentaenoic–EPA C20:5(n-3) and docosahexaenoic–DHA C22:6(n-3) prevailed. SFA and MUFA variations were similar during the course of the study but inverse to that of PUFA (Fig. 5).

Total PUFA represented the majority of total fatty acids, ranging from 40.3% to 59.9% (Fig. 5). The percentage levels of 22:6n-3 and 20:5n-3 showed opposite changes in the June-July period; otherwise, their changes were similar. Generally, DHA/EPA ratios were lower in the spring–summer period and higher during autumn–winter. The DHA/EPA ratio varied in an opposite way to the C16:1/C16:0 ratios. Consequently, the highest C16:1/C16:0 ratios were observed in either May or July, and during autumn and winter the ratios were lower. PUFA n-3 was greater than PUFA n-6; their ratio varied between 2.1 and 5.0 and was the highest during the spring (April to June). Minimum values of PUFA n-6 were observed during May and June. In the soft tissue of Noah's Ark, appreciable content of 22:2NMID (non methylene interrupted dienoic fatty acid) was determined. NMIDs were negatively correlated to palmitoleic acid (Fig. 5).

Table 1. Fatty acid composition (% of total fatty acid) of the lipids in the ark shell *Arca noae* in Mali Ston Bay. SFA- saturated, MUFA – monounsaturated, NMID – non methylene interrupted dienoic, PUFA- polyunsaturated fatty acids, UNS/SAT – unsaturated to saturated fatty acids ratio.

Fatty acid	Dec '01	Jan '02	Feb '02	Mar '02	Apr '02	May '02	Jun '02	Jul '02	Aug '02	Sep '02	Oct '02	Nov '02
14:00	3.2±1.2	3.1±1.2	3.9±1.3	3.4±1.3	2.5±1.0	4.9±1.6	4.0±1.4	2.1±0.9	2.1±0.9	2.9±1.2	4.4±1.7	2.7±1.2
16:00	18.3±4.4	10.4±3.1	11.8±0.1	12.1±3.3	10.1±3.0	15.8±3.0	15.4±2.9	11.7±3.9	10.3±2.7	11.1±2.2	13.2±3.9	12.9±4.5
17:00	2.5±0.2	1.9±0.3	2.5±0.3	2.1±0.3	1.9±0.3	2.4±0.3	2.4±0.3	2.1±0.4	2.2±0.5	2.5±0.4	2.0±0.3	2.1±0.0
18:00	6.6±0.7	4.9±0.2	5.9±0.5	5.3±0.8	5.0±0.3	6.3±0.5	6.3±0.4	6.3±0.8	6.2±0.8	6.5±0.9	5.3±0.2	4.8±0.1
20:00	0.3±0.0	0.2±0.0	0.2±0.1	0.2±0.0	0.2±0.1	0.3±0.1	0.2±0.1	0.2±0.0	0.2±0.0	0.2±0.1	0.0±0.0	0.3±0.0
22:00	0.7±0.1	0.3±0.0	0.4±0.0	0.3±0.1	0.3±0.0	0.4±0.1	0.4±0.1	0.4±0.1	0.3±0.03	0.5±0.1	0.1±0.0	0.3±0.0
SFA	32.9±4.2	21.2±4.8	27.2±4.1	26.0±3.4	21.4±3.2	33.1±3.4	31.0±3.9	24.4±4.3	22.5±4.1	25.8±3.6	26.8±4.1	24.6±4.9
16:1(n-7)	8.2±2.0	7.2±1.6	8.9±1.3	7.5±2.6	6.7±1.5	10.7±2.2	9.2±1.9	5.9±2.4	4.5±1.9	6.7±2.4	7.3±2.6	5.3±2.2
18:1(n-7)	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.3	0.2±0.1	0.3±0.1	0.2±0.5	0.2±0.1	0.1±0.1	0.2±0.2	0.2±0.1	0.2±0.1
18:1(n-9)	6.3±0.6	5.3±0.4	6.8±0.4	6.1±0.6	6.1±0.5	8.1±0.6	7.7±0.5	7.0±0.6	4.9±0.4	6.0±0.5	5.7±0.5	5.1±0.9
20:1	5.3±0.0	4.4±0.1	5.0±0.2	4.5±0.1	4.8±0.2	5.3±0.2	5.1±0.1	6.0±0.2	6.5±0.1	6.8±0.1	4.5±0.1	5.9±0.3
22:1	0.6±0.1	0.6±0.0	0.8±0.1	0.3±0.0	0.9±0.1	1.3±0.1	0.4±0.2	0.3±0.1	0.6±0.1	1.1±0.1	0.4±0.0	0.3±0.0
MUFA	20.7±3.3	17.2±1.9	21.3±1.2	18.6±1.8	18.1±1.7	24.8±1.6	22.6±2.3	19.5±1.7	16.3±1.7	20.2±2.6	17.8±1.3	16.8±1.3
18:2(n-6)	2.4±0.9	2.7±0.5	2.8±0.6	2.4±0.4	2.8±0.5	2.1±0.3	1.8±0.3	2.2±0.5	2.5±0.6	3.1±0.5	2.7±0.5	4.6±0.6
18:3(n-3)	3.1±0.2	3.0±0.5	3.0±0.3	2.8±0.4	3.2±0.6	1.4±0.2	2.5±0.4	1.6±0.4	2.5±0.6	2.9±0.5	3.1±0.5	2.3±0.2
18:3(n-6)	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.1	0.2±0.1	0.1±0.1	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0
18:4(n-3)	1.8±0.3	2.2±0.2	1.7±0.4	2.3±0.2	3.0±0.3	1.8±0.1	1.6±0.1	1.5±0.2	1.1±0.2	1.2±0.1	1.5±0.1	2.0±0.4
20:4(n-3)	0.4±0.2	0.4±0.1	0.3±0.0	0.4±0.0	0.5±0.0	0.4±0.0	0.4±0.1	0.4±0.1	0.3±0.1	0.3±0.0	0.3±0.0	0.5±0.1
20:4(n-6)	3.5±0.5	3.2±0.5	3.7±0.5	4.5±0.6	3.6±0.2	2.3±0.4	2.5±0.4	3.9±0.5	6.7±1.0	5.7±0.8	5.0±0.7	5.0±0.6
20:5(n-3)	8.2±1.6	11.7±2.1	8.9±1.9	12.1±2.4	13.1±1.5	9.5±1.1	10.1±1.6	9.6±1.5	7.1±1.0	6.4±1.1	8.4±1.4	9.3±1.4
22:2(NMID)	8.2±1.7	10.9±1.6	8.6±1.6	7.1±1.2	8.4±1.3	7.3±1.3	7.3±1.3	11.5±1.5	16.1±1.9	14.5±2.2	7.2±1.0	8.6±1.5
22:4(n-6)	0.5±0.1	0.7±0.1	0.7±0.1	0.8±0.2	0.5±0.1	0.4±0.1	0.4±0.1	0.7±0.1	1.1±0.1	0.9±0.1	0.9±0.1	1.6±0.1
22:5(n-6)	1.3±0.2	1.0±0.2	1.2±0.2	1.3±0.1	0.4±0.1	0.6±0.1	0.7±0.1	1.2±0.2	1.6±0.2	1.3±0.2	1.3±0.2	1.4±0.2
22:5(n-3)	1.4±0.6	1.4±0.2	1.1±0.2	1.6±0.1	1.6±0.2	1.0±0.2	1.2±0.2	1.6±0.2	1.4±0.2	1.2±0.2	1.1±0.1	0.0±0.0
22:6(n-3)	11.3±2.4	18.3±5.4	11.2±3.6	13.4±3.2	16.1±3.8	9.7±2.1	12.0±2.9	18.0±5.0	17.0±4.6	11.9±3.5	18.7±5.5	19.8±5.8
PUFA	44.1±2.0	58.6±7.2	47.9±4.6	52.7±4.6	56.4±4.7	40.3±2.9	44.4±3.2	55.1±3.8	59.9±3.5	52.1±2.4	54.4±4.7	57.5±5.0
UNS/SAT	1.9±0.3	3.2±0.8	2.4±0.5	2.7±0.6	3.4±0.8	2.0±0.4	2.1±0.5	3.0±0.7	3.3±0.8	2.7±0.6	2.7±0.7	3.0±0.8
n-3/n-6	3.2±0.5	4.2±0.8	2.8±0.4	3.2±0.4	4.4±0.8	3.4±0.7	4.5±0.7	3.6±0.7	2.2±0.3	1.9±0.3	2.9±0.5	2.9±0.6

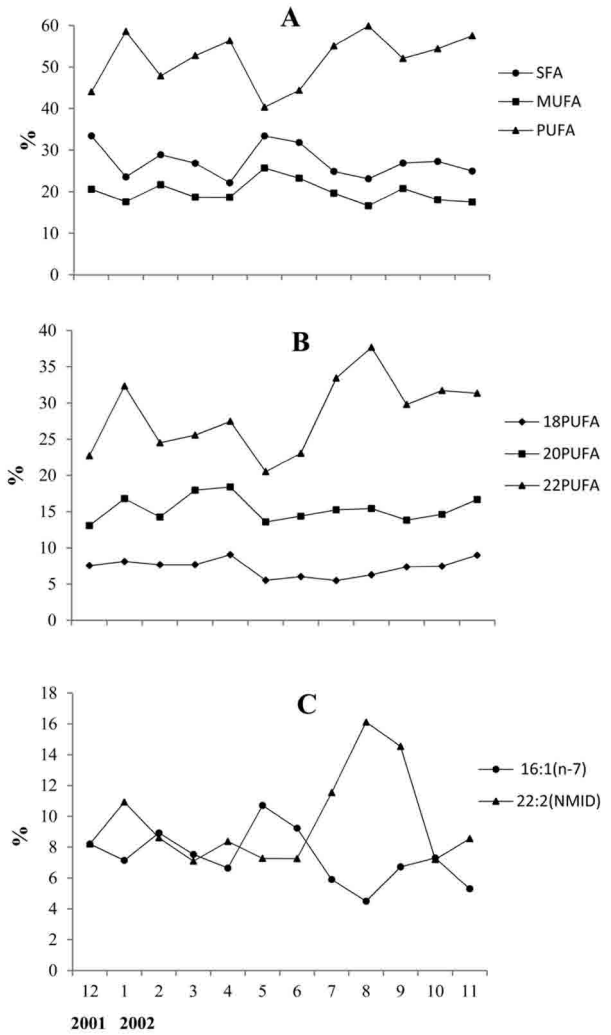

Fig. 5: Monthly variations in fatty acid composition (% of total fatty acid) of the lipids in the ark shell *Arca noae* from Mali Ston Bay.

Table 2. Results of the principal component analysis.

	Axis 1	Axis 2	Axis 3
Eigenvalue	5.311	4.191	1.846
% of Variance	33.192	26.196	11.539
Cumulative % of Variance	33.192	59.388	70.927
Correlation coefficient			
Temperature (T)	0.909	-0.110	0.049
Salinity (S)	-0.037	-0.800	-0.328
Density (D)	-0.755	-0.338	-0.229
Nitrate (NO3)	-0.359	0.891	0.173
Nitrite (NO2)	-0.767	0.316	-0.027
Total nitrogen (TN)	0.459	0.572	-0.057
Phosphate (PO4)	0.595	0.072	-0.071
Silicate (SiO4)	-0.152	0.870	0.084
Chlorophyll *a* (Chl *a*)	0.810	0.202	0.123
Microphytoplankton (MICRO)	0.858	-0.165	0.374
Nanophytoplankton (NANO)	0.172	0.282	0.688
Dry flesh weight (DRY)	-0.571	-0.299	0.655
Inorganic matter (ASH)	0.122	-0.797	0.185
Lipid (LIP)	-0.406	-0.610	0.222
Protein (PROT)	0.340	-0.441	0.497
Carbohydrate (CARB)	-0.751	0.050	0.501

Discussion

Principal component analysis indicates that temperature, nitrate, silicate, MICRO, Chl *a* and salinity are the main environmental factors influencing the biochemical composition of *Arca noae*. Temperature showed a significant correlation with Chl *a* and MICRO because higher sea water temperatures promote photosynthesis and phytoplankton growth. An intensive development of phytoplankton in Mali Ston Bay was noticed during spring and summer. Two annual peaks of NANO and MICRO were in April and July, and diatoms and dinoflagellates were the most abundant MICRO groups. This agrees with previous studies of phytoplankton in the bay (Jasprica, 1989). Since the fatty acid composition of lipids reflects the food sources of bivalves on a longer-term basis (weeks-months) (Dalsgard *et al.*, 2003), our data indicate that phytoplankton is a major food source for *A. noae* in the Mali Ston Bay. Markers of diatoms (16:1, 20:5n-3) and dinoflagellates (16:0, 16:1/16:0<1, 22:6n-3/20:5n-3>1) predominated in the fatty acid composition of Noah's Ark. Zooplankton markers were also increased (18:1n-9, 20:4n-6, 22:6n-3) as evidenced by their presence in the food of bivalves. A recent investigation conducted by Ezgeta-Balić *et al.* (2012) also established that *A. noae*, like the other shellfish from Mali Ston Bay (*Mytilus galloprovincialis*, *Ostrea edulis* and *Modiolus barbatus*), used mixed food; mainly phytoplankton followed by zooplankton and detritus.

Salinity was mainly in the range of 34-37, except at the end of April and in October when some lower values were recorded, which is probably a consequence of higher rainfall, Neretva river inflow in the outer part of the Bay and the activity of submarine springs in the inner part of the Bay. The influence of freshwater on nutrients in Mali Ston Bay was evident in the negative correlation between salinity and concentrations of nitrate and silicate (Fig. 6) - the highest values of nitrate and silicate concentrations coincided with the minimum salinity value (October, 15). During 2002, monthly average flow of the Neretva River was the highest in October (Čalić *et al.,* 2013). Phosphate and nitrite showed no significant correlation with salinity, indicating that parts of PO_4 and NO_2 are decomposition products of organic matter and plankton excretion. Decreased nutrient concentrations, mostly noted during spring and summer months, can be explained by intensive phytoplankton uptake and decreased inflow of freshwater. Nutrient concentrations at Bistrina station were similar with the concentrations at Usko station in Mali Ston Bay (Čalić *et al.,* 2013) and in the Neretva Channel (Vidjak *et al.*, 2007) but lower than in river estuaries in the South Adriatic (Carić *et al.*, 2012; Jasprica *et al.*, 2012). The influence of salinity on the biochemical composition of Noah's Ark was evident in the correlation between salinity and ash percentage. Higher salinity affects the increase of inorganic matter

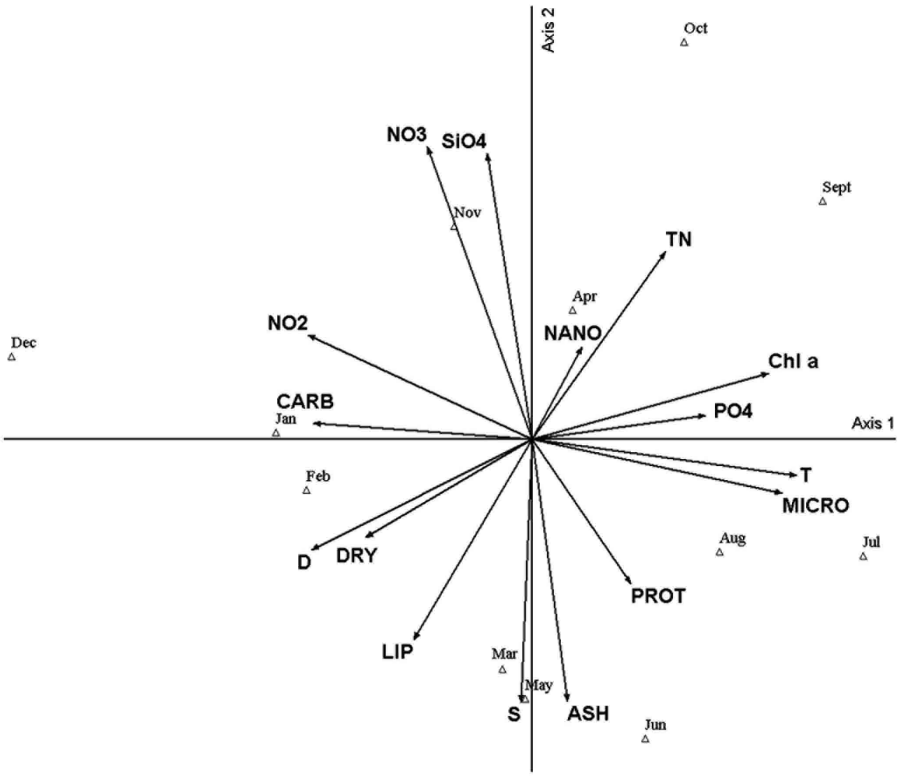

Fig. 6: Principal component analysis ordination graph showing shellfish biochemical composition (DRY: dry flesh weight, ASH: inorganic matter, LIP: lipid, PROT: protein, CARB: carbohydrate) in relation to environmental parameters (T: temperature, S: salinity, D: density, NO3: nitrate, NO2: nitrite, TN: total nitrogen, PO4: phosphate, SiO4: silicate, Chl *a*: chlorophyll *a*, MICRO: microphytoplankton, NANO: nanophytoplankton) in Mali Ston Bay.

in *A. noae*. The same phenomenon was observed for the clam *Chamelea gallina* from the Neretva River Estuary (Dupčić Radić, 2012). NO_3 and SiO_4 in Mali Ston Bay were negatively correlated with inorganic matter, and positively correlated with organic matter (the organic matter vector is not shown in the PCA diagram because it is opposite to the inorganic matter vector and coincides with the SiO_4 vector). Therefore, it can be concluded that during the increased freshwater inflow, shellfish meat enriches with organic matter.

During the winter period (December 2001-March 2002), when sea temperatures and MICRO and NANO abundances were low, there was a gradual decrease of the dry weight content of *A. noae*. An increase of dry weight content started in March and coincided with an increase of temperature, Chl *a*, MICRO and especially NANO abundances. Laruelle *et al.* (1994) also found that the maximum rate of increase in weight of *Venerupis decussata* occurs in the spring when both the sea temperature and food supply increase rapidly. Percent of dry weight of *A. noae* declined in May and July. In May, Chl *a* and phytoplankton abundance were also lower while in July reduced values may be attributed to summer spawning. Peharda *et al.* (2006) found that spawning of *A. noae* from Mali Ston Bay occurred in July-August, like for the related species *Anadara broughtonii* and *Scapharca subcrenata* from the southern coastal bay of Korea (Park *et al.*, 2001; Park *et al.*, 2011). The lowest value of dry weight was in September following recovery of *A. noae* during autumn. *A. broughtonii* and *S. subcrenata* showed similar monthly variations of dry tissue weight during the year (Park *et al.*, 2001; Park *et al.*, 2011). Maximum and minimum dry weight values obtained in this study coincide with the values of the condition index, defined as the ratio between dry flesh weight and wet shell weight of *A. noae* in Mali Ston Bay (Peharda *et al.*, 2003). Dry matter content and condition index are parameters indicating the quality and physiological condition of shellfish, and hence the best time for their consumption. Ojea *et al.* (2004) found that variations in weight, body growth, gonad growth and spawning may occur together in response to changes in environmental conditions, especially in food availability. When food is abundant, surplus energy is used by animals for growth of somatic tissues and also for gonad development (Urrutia *et al.*, 1999).

In this study, values of macromolecule content are reported on a dry weight basis since the true nutrient content of bivalves does not seem to be masked by the moisture fluctuations occurring during the year (Orban *et al.*, 2006). The accumulation and mobilization of energy reserves, according to the stage of the reproductive cycle, is reported in different marine bivalve species (Beninger & Lucas, 1984; Robert *et al.*, 1993; Galap *et al.*, 1997; Berthelin *et al.*, 2000; Dridi *et al.*, 2007). Protein and lip-

id level in *A. noae* reached the maximum value in June, just before spawning, following the decrease in macromolecule content. Protein is a major organic component of bivalve oocytes (Holland, 1978). Therefore, protein maxima prior to spawning (June) may support this hypothesis. The decrease in protein level after spawning (from July to September) was similar to many other marine bivalves, such as *Mytilus galloprovincialis* (Bressan & Marin, 1985) and *Venerupis philippinarum* (Marin *et al.*, 2003) from the Lagoon of Venice, *Crassostrea gigas* from the Atlantic French coast (Berthelin *et al.*, 2000), and *Glycymeris glycymeris* from the coast of southern France (Galap *et al.*, 1997). Protein also provides an energy and material source for gametogenesis after carbohydrate and lipid reserves are depleted (Yan *et al.*, 2010). In marine bivalves, lipids play a crucial role in maturing gonadal tissues and constitute a major component of reproductive material (Zandee *et al.*, 1980). In general, lipid content increases before mass spawning occurs, and then markedly decreases (Racotta *et al.*, 2003; Dridi *et al.*, 2007; Liu *et al.*, 2008; Colaco *et al.*, 2009). Lipids also play a small but appreciable role as maintenance energy reserves during food-limited periods (Beninger & Lucas, 1984). Carbohydrate in *A. noae* is negatively affected by seawater temperature so the highest carbohydrate value was detected in December. The same was observed for glycogen in the clam *Venerupis decussata* (Serdar & Lök, 2009). Glycogen is the main carbohydrate constituent, representing about 50% of total carbohydrates (Robert *et al.*, 1993) and carbohydrate patterns are strongly related to those of glycogen (Park *et al.*, 2001; Marin *et al.*, 2003; Park *et al.*, 2011). In this study, carbohydrate peak also observed in April, coincided with high phytoplankton abundance (especially NANO). Although, we did not observe a statistically significant correlation between carbohydrate and chlorophyll *a* levels and phytoplankton abundances, the seasonal variation in glycogen reserves is strongly influenced by food availability, in addition to reproductive demand (Li *et al.*, 2000; Patrick *et al.*, 2006).

In *A. noae*, lipid variations from February to June are inversely related to carbohydrate content. This relationship is usually attributed to the conversion of glycogen to lipids biosynthesized during the formation of gametes and was also detected in the ark shell *A. broughtonii* from the southern coastal bay of Korea (Park *et al.*, 2001) and in many other bivalves (Gabbott, 1976; Beninger & Lucas, 1984; Robert *et al.*, 1993; Marin *et al.*, 2003; Ojea *et al.*, 2004; Yan *et al.*, 2010). Gabbott (1975) already noted that gonad development in bivalves may involve metabolic conversion of glycogen to lipids.

Due to the different ways of expressing the biochemical composition of shellfish, it is not possible to compare the biochemical composition of *A. noae* with the ark shells from the Korean coast (Park *et al.*, 2001; Park *et al.*, 2011) but it can be compared with some other shellfish of commercial size from the Mediterranean coast. Thus, comparing the biochemical composition of *A. noae* with the flat oyster *Ostrea edulis* from Turkey (Yildiz *et al.*, 2011), the Mediterranean mussel *M. galloprovincialis* from Italy (Orban *et al.*, 2002) and the clam *C. gallina* from Croatia (Dupčić Radić, 2012), some differences can be observed. Values for protein content in *A. noae* were in the same range as those for *O. edulis* and higher than values recorded for *M. galloprovincialis* and *C. gallina.* Lipid content of *A. noae* was similar to that of *C. gallina* and *O. edulis* and lower than in *M. galloprovincialis*. Carbohydrate content was low in comparison with other mussels from the Mediterranean coast and ash content was in the same range as that of *O. edulis* and lower than in *C. gallina* and *M. galloprovincialis*.

The fatty acid composition of *A. noae* corresponds in general to a healthy marine mollusc pattern characterized by a high degree of unsaturation, being similar to those of other adult marine bivalves in temperate waters (Ahn *et al.*, 2000). PUFA content was high mostly according to PUFA of n-3 series, EPA (20:5n-3) and DHA (22:6n-3), nutritionally essential for growth and condition. EPA and DHA primarily derive from food (Sargent, 1976). The variations of the DHA/EPA ratio, oppositely correlated to C16:1/16:0 ratios, suggested that during spring *A. noae* food was mostly composed of microalgae due to their higher level of EPA than DHA (Viso & Marty, 1993). During spring, the highest n-3/n-6 ratio was also observed, giving this period a relevant importance from a nutritional point of view. The increase of the DHA/EPA ratio during summer and autumn might indicate the selective enrichment of DHA with respect to EPA or prevalence of an animal component in the bivalve's diet. Higher contributions made by animal food sources to the bivalve's diet corresponded to a relatively lower abundance of phytoplankton during this period. At that time, *A. noae* soft tissue was also rich in NMIDs. These acids and their precursor palmitoleic acid were negatively correlated implying that synthesis was also stimulated by the presence of C16:1(n-7) in the diet (Zhukova, 1991).

In conclusion, statistical analysis (ANOVA, Fisher LSD *post-hoc* test and PCA) showed that the biochemical composition of Noah's Ark varies with season. Principal component analysis pointed to temperature and salinity as the most important factors indirectly influencing shellfish biochemical composition. Inflow of freshwater causes decrease of salinity, increase of NO_3 and SiO_4 concentrations in seawater and consequently increase of organic matter in shellfish meat. Temperature increase promotes photosynthesis and phytoplankton growth and consequently an increase of dry weight content of *A. noae* occurred during the spring when both the sea temperature and food supply increased rapidly. In that period, the protein content pattern is similar to that of dry weight, with the maximum value reached in June. The decline of macromolecule content in the period June-September is

mostly due to spawning, as confirmed by the study of the reproductive cycle (Peharda *et al.*, 2006). Decreases during winter suggest the occurrence of different sources of physiological stress, related to environmental conditions prevailing in Mali Ston Bay. Fatty acid composition of *A. noae* suggest that it would be an excellent source of n-3 fatty acids, especially eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acid. These results, give an insight into the biochemistry and ecology of this species. Data on the biochemical and fatty acid composition of *A. noae* obtained in this study indicate that it is a quality seafood product and the most suitable period of the year for its consumption is in the spring.

Acknowledgements

This research was supported by the Ministry of Science and Technology of the Republic of Croatia. The authors wish to thanks Rade Garić for designing the figure of the study area.

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