Biochemical composition and digestive enzyme activity during naupliar development of *Artemia* spp from three solar saltworks in Greece

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A B S T R A C T

Three parthenogenetic brine shrimp *Artemia* populations from saltworks in Greece (Messolongi, Milos and Polychnitos) were studied with regard to their biochemical composition and the activity of digestive enzymes in four developmental stages from decapsulated cysts to nauplii instar III. The content of protein and RNA was highest in nauplii instar II (65.10 and 6.23% dry weight, respectively), while that of total lipid was highest in nauplii instar I (11.40% dw). The carbohydrate content was highest in decapsulated cysts (10.30% dw) and about one half that value in nauplii instar I. The DNA content was ten-fold higher in nauplii instar I than in decapsulated cysts with the highest levels in nauplii instar III (3.67% dw). The activity of alkaline phosphatase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, β-glucosidase, β-glucosidase, esterase lipase (C8), esterase (C4), N-acetyl-β-glucosaminidase, α-fucosidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase increased significantly from decapsulated cysts to nauplii instar II and remained constant or decreased in nauplii instar III. In nauplii instar II, leucine aminopeptidase, β-galactosidase, alkaline phosphatase and esterase (C4) demonstrated higher activity levels (140.90, 133.62, 66.01 and 45.47 nmoles/100 µg dw within 5 min, respectively) of the aforementioned enzymes. The *Artemia* population of Messolongi had significantly higher levels of DNA, protein and alkaline phosphatase activity per individual, as well as body weight, compared to the *Artemia* populations of Milos and Polychnitos. The latter population showed the lowest level of lipid and the highest esterase activity (C4) per individual. There was a reverse relationship between the protein content and leucine aminopeptidase activity, lipid content and esterase (C4) activity, carbohydrate content and β-glucosidase activity among the three *Artemia* populations. The variations observed in the biochemical composition and the activity of some specific enzymes should be taken into consideration in selecting these *Artemia* populations from Greece for application in aquaculture.

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1. Introduction

Among the live diets used in larviculture of fish and shrimp, nauplii of the crustacean *Artemia* sp constitute the most widely used food item worldwide (Bengtson et al., 1991). The nutritional quality of *Artemia* populations varies considerably. This variation might be related to the geographical origin of *Artemia*, or to differences among different batches of cysts from the same origin. Greater changes in biochemical composition might be expected among different strains of *Artemia* (Leger et al., 1986).

Hines et al. (1980) and García-Ortega et al. (1998) followed the biochemical composition (protein, lipid and carbohydrate content) during the development of both cysts and nauplii in bisexual populations of *A. franciscana*. They found differences in biochemical composition during development from cysts to nauplii of *A. franciscana* (% dw and µg ind⁻¹). To determine the nutritional quality of *Artemia*, it is important to study changes in biochemical composition in relative terms (% dw) and on an individual weight basis (García-Ortega et al., 1998). Furthermore, according to Helland et al. (2000) the individual protein content was quite different not only between *A. franciscana* and *Ar. parthenogenetica* but among the parthenogenetic populations as well, due to different ploidy levels. Specifically, the diploid *A. parthenogenetica* of Namibia had the lowest protein content, while the tetraploid of Megalon Embolol (Greece) had the highest amount of protein (Helland et al., 2000).

The nucleic acids RNA and DNA are involved in protein synthesis and cellular multiplication required for growth, and thus affects the nutritional quality of *Artemia*. The ratio of RNA to DNA has also been regarded as an index of protein synthetic capacity per cell, whereas the RNA/DNA ratio variability is stage- and species-specific (Watts et al., 1994; Gorokhova and Kyle, 2002). Increases in RNA/DNA ratio indicates cell enlargement (hypertrophy) and decreases indicate cell proliferation (hyperplasia) (Gwak et al., 2003). Few studies have been performed on the nucleic acid composition only in *A. franciscana* cysts or/and nauplii.
inter I (Watts et al., 1994; Gorokhova, 2005). There is no previous study following the entire biochemical composition in protein, lipid, carbohydrate, RNA and DNA during the developmental stages of Artemia from decapsulated cysts to nauplii instar III.

In Artemia, the enzymatic content depends on the nutritional state and developmental stage (Munilla-Moran et al., 1990). The first steps in the developmental program take place in the absence of nutritional supply. The components necessary for the intense synthetic process come from the degradation of maternal reserves. The dependence on maternal reserves suggests that the degradation of yolk should be a controlled process (Perona et al., 1988). In encysted embryos of Artemia, few enzymes have been detected, while still others may be present in inactive or masked forms (Warner, 1987). A burst in enzymatic activity, concomitant with the hatching of the nauplii, has been reported in A. franciscana (Perona and Vallejo, 1985; Raineri, 1987; Pan et al., 1991; Warner and Matheson, 1998). The increase in enzymatic activity with an alkaline pH optimum (range 7.5–8.5) during post-hatch development may be the result of an unmasking process or enzyme induction (Warner, 1987). The developmental changes, both quantitative as well as qualitative, of alkaline phosphatase activity may be useful to investigate genetic expression and its regulation (Raineri, 1987). So far no further studies have been reported about digestive enzymes activity in parthenogenetic populations of Artemia during further development of cysts and nauplii.

Several studies have suggested the applicability of exogenous digestive enzymes (A. franciscana) for growth, digestibility and assimilation of sea bream and sea bass (Kolkovski et al., 1997; Koven et al., 2001), species that are widely cultured in Greece. It has been shown in sea bream larvae that during the first month post-hatching food is digested mainly by the action of alkaline proteases and the gastrointestinal pH is alkaline (Yúfera et al., 2004). Food is digested mainly by the action of alkaline proteases and the concentration of protein nitrogen (ammonium sulphate), total lipid (tripalmitin), total carbohydrate (glucose), RNA (RNA yeast) and DNA (calf thymus) were determined from standard curves. The protein value was obtained by multiplying the nitrogen value by 6.25.

2.4. Enzymatic activity

Enzymatic activity was determined for the aforementioned 12 treatments (at least two replicates for each treatment). Each sample consisted of 1000 decapsulated cysts or 200 nauplii, which were collected with a pipette and put in Eppendorf polystyrene reaction tubes. The seawater was removed from the tubes and the samples were homogenized in 200 µl physiological saline solution (0.9% NaCl). In each sample, physiological saline was added to make the volume to 1.6 ml. The homogenate was centrifuged for 5 min at 5000 × g. The supernatant was used immediately for enzyme activity assays. Nineteen different enzymes (alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucosidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase) were estimated with an Api-Zym enzymatic assay kit (M. Moraiti-Ioannidou et al. / Aquaculture 286 (2009) 259–265)

2.3. Biochemical composition

Protein, total lipid, total carbohydrate, RNA and DNA levels were determined for 12 treatments, i.e. three Artemia populations at four developmental stages: decapsulated cysts, nauplii instar I, II and III (at least two replicates for each treatment). Each sample consisted of 1000 decapsulated cysts or nauplii, which were collected with a pipette and transferred to Eppendorf polystyrene tubes. Seawater was removed from the tubes and the samples were washed with 0.9% aqueous ammonium formate, isotonic with seawater, in order to avoid lysis of nauplii or cysts. Samples were lyophilized (Freezone 4.5, Labconco), weighed on a Sartorius electron balance (precision±0.1 µg) and stored at −80 °C prior to analysis. The individual dry weight (dw) was also estimated.

Protein, total lipid, total carbohydrate, RNA and DNA analyses were assayed according to the micro-analytical method described by Holland and Gabbott (1971) as modified by Holland and Hannant (1973). The end product analyses were carried out in triplicate for protein, total lipid and RNA and in duplicate for total carbohydrate and DNA. The extinction was measured with a spectrophotometer. The concentrations of protein nitrogen (ammonium sulphate), total lipid (tripalmitin), total carbohydrate (glucose), RNA (RNA yeast) and DNA (calf thymus) were determined from standard curves. The protein value was obtained by multiplying the nitrogen value by 6.25.

Table 1

<table>
<thead>
<tr>
<th>Cysts</th>
<th>Nauplii instar I</th>
<th>Nauplii instar II</th>
<th>Nauplii instar III</th>
<th>S.E.M.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>50.27±***</td>
<td>49.25±***</td>
<td>65.10±***</td>
<td>53.51±***</td>
<td>1.139***</td>
</tr>
<tr>
<td>Lipid</td>
<td>8.27±</td>
<td>11.40±</td>
<td>8.81±</td>
<td>8.58±</td>
<td>0.380***</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>10.30±***</td>
<td>5.38±</td>
<td>6.33±</td>
<td>3.64±</td>
<td>0.149***</td>
</tr>
<tr>
<td>RNA</td>
<td>5.02±</td>
<td>4.52±</td>
<td>6.23±</td>
<td>4.83±</td>
<td>0.104***</td>
</tr>
<tr>
<td>DNA</td>
<td>0.23±</td>
<td>2.82±</td>
<td>3.10±</td>
<td>3.67±</td>
<td>0.131***</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>25.54±***</td>
<td>1.64±</td>
<td>2.09±</td>
<td>1.32±</td>
<td>1.879***</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>16.4±</td>
<td>2.09±</td>
<td>1.32±</td>
<td>0.092±</td>
<td>0.001***</td>
</tr>
</tbody>
</table>

Means in the same row with different letters in superscript are significantly different. ***P<0.001; n=6.
S.E.M.: standard error of grand mean.

2.2. Cysts processing and nauplii selection

The collected cysts were processed according to Sorgeloos et al. (1978) and kept in bottles with crude salt that were stored in a dark and cool place. The decapsulation of cysts was conducted according to Bruggeman et al. (1980). The hydration of the cysts and hatching of the nauplii instar I were performed at conical glass tubes. The incubation conditions were: 28 °C, 35% natural seawater, continuous illumination of 1200 lux at the water surface, and sufficient aeration from the bottom of the tank to maintain the cysts in suspension. Nauplii instar I were separated and kept at the same conditions without illumination and feeding. Nauplii instar I, II, III were distinguished according to Abatzopoulos (1988).

2.1. Cysts origin

Cysts were collected in July and August from the following three saltworks: Milos Island, complex of Cyclades (36°41’N 24°28’E) in 1990 – Polychnitos, Lesvos Island (39°06’N 26°10’E) in 1996 and 1998 – Messolongi, prefecture of Aitolocarnania (38°24’N 23°23’E) in 1997 and 1998.

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<table>
<thead>
<tr>
<th>Cysts</th>
<th>Nauplii instar I</th>
<th>Nauplii instar II</th>
<th>Nauplii instar III</th>
<th>S.E.M.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
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<td>2.82±</td>
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<td>3.67±</td>
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</tr>
<tr>
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<td>RNA/DNA</td>
<td>16.4±</td>
<td>2.09±</td>
<td>1.32±</td>
<td>0.092±</td>
<td>0.001***</td>
</tr>
</tbody>
</table>

Means in the same row with different letters in superscript are significantly different. ***P<0.001; n=6.
S.E.M.: standard error of grand mean.

In view of the important interest in Artemia for use in local aquaculture, we performed the following study of three strains of Artemia from different solar saltworks in Greece. The present study aims to provide data on the biochemical composition (protein, total lipid, carbohydrate, RNA and DNA content) in A. parthenogenetica of Milos, Polychnitos and Messolongi at four developmental stages, from decapsulated cysts to nauplii instar III, without any food supply. Furthermore, the activity of 19 digestive enzymes in the above four developmental stages were investigated to evaluate the stage-specific expression of the genes or genetic differentiations among the three strains and the nutritional value of these Artemia as live food in aquaculture.

2. Materials and methods

The collected cysts were processed according to Sorgeloos et al. (1978) and kept in bottles with crude salt that were stored in a dark and cool place. The decapsulation of cysts was conducted according to Bruggeman et al. (1980). The hydration of the cysts and hatching of the nauplii instar I were performed at conical glass tubes. The incubation
Table 2

Biochemical composition (% dw) estimated at different developmental stages (decapsulated cysts, nauplii instar I, II and III; least squares means) in three Greek Artemia populations: Milos, Polychnitos and Messolongi

<table>
<thead>
<tr>
<th></th>
<th>Milos</th>
<th>Polychnitos</th>
<th>Messolongi</th>
<th>S.E.M.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>54.03a</td>
<td>51.83a</td>
<td>57.74a</td>
<td>0.968</td>
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</tr>
<tr>
<td>Lipid</td>
<td>7.68a</td>
<td>7.86a</td>
<td>9.37a</td>
<td>0.327</td>
<td>***</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>6.95b</td>
<td>6.31a</td>
<td>6.10a</td>
<td>0.129</td>
<td>**</td>
</tr>
<tr>
<td>DNA</td>
<td>2.51a</td>
<td>2.22a</td>
<td>2.63b</td>
<td>0.112</td>
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</tr>
<tr>
<td>RNA/DNA</td>
<td>8.07</td>
<td>6.64</td>
<td>7.47</td>
<td>1.598</td>
<td>NS</td>
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</tbody>
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Means in the same row with different letters in superscript are significantly different. *P<0.05; **P<0.01; ***P<0.001; NS: not significant (P>0.05); n=8.

S.E.M.: standard error of grand mean.

2.5. Statistical analysis

Our experimental design was a factorial type (3×4) in which the treatments (12) consisted of three Artemia populations (p) at four developmental stages (s). The main effects of both factors and their interaction effects were estimated by two-way ANOVA for each biochemical constituent and digestive enzyme. When the interaction effects were not significant (Pp×Ps>0.05), the least squares mean of each population independently of the developmental stage, as well as of each developmental stage independently of the Artemia population, was calculated for each biochemical constituent and digestive enzyme. The 95% Duncan HSD (honesty significant difference) intervals for means were calculated and the homogeneous groups were confirmed by multiple range analysis, for determining which means were significantly different. Differences were considered significant at P<0.05. Regression analysis was used for investigating the dependence of biochemical content (Y) on enzymatic activity (X). The correlation of coefficient (r) and the probability of slope (P) were estimated for each regression equation. The statistical analysis was carried out on a PC, using Statgraphics statistical package.

3. Results

The protein, total lipid, total carbohydrate, RNA and DNA content (% dw), as well as the RNA/DNA ratio, estimated in decapsulated cysts and nauplii instar I, II, III of three Artemia populations of Milos, Polychnitos and Messolongi are given in Table 2. There were no significant interaction effects among the three Artemia populations and the four developmental stages (Pp×Ps<0.05), with regard to the percentage levels for the six above mentioned biochemical parameters. The protein and RNA contents increased considerably in nauplii instar I compared to decapsulated cysts and nauplii instar I and decreased significantly in nauplii instar III. The lipid content increased notably in nauplii instar I compared to decapsulated cysts, declined significantly in nauplii instar II and thereafter remained constant in nauplii instar III. The carbohydrate content in nauplii instar I was about one half of that observed in decapsulated cysts, increased significantly in nauplii instar II and decreased sharply in nauplii instar III. The DNA content increased almost ten fold in nauplii instar I compared to decapsulated cysts, remained at similar levels in nauplii instar II and then increased significantly in nauplii instar III. A remarkable decrease in the RNA/DNA ratio occurred in the naupliar stages compared to the cysts. Further analysis of variance, which was performed only among the naupliar stages, showed that nauplii instar II had a significantly higher RNA/DNA ratio compared to nauplii instar I and III.

The protein, total lipid, total carbohydrate, RNA and DNA content (% dw) estimated in the Artemia populations of Milos, Polychnitos and Messolongi at different developmental stages (decapsulated cysts and nauplii instar I, II and III) are given in Table 2. The population of Messolongi showed the highest percentage content of protein and DNA, but the lowest of RNA. The population of Milos exhibited the highest percentage content of lipid and carbohydrate.

The individual content (Fig. 1a) of protein and DNA in the population of Messolongi was significantly higher than that in the populations of Milos and Polychnitos. The populations of Messolongi and Milos had similar individual lipid content, but significantly higher compared to that in the population of Polychnitos. No significant differences were found for the individual content of carbohydrate and RNA, as well as for the RNA/DNA ratios, among the three Artemia populations.

The enzymatic activities (nmole/100 µg dw) determined in decapsulated cysts and nauplii instar I, II, III of three Artemia populations (Milos, Polychnitos and Messolongi) are presented in Table 3. There were no significant interaction effects among the three Artemia populations and the four developmental stages (Pp×Ps<0.05) for the relative activity of all the enzymes studied. The activity of trypsin and lipase (C14) was observed at very low levels; the former in nauplii instar II and III and the latter only in nauplii instar III. Chymotrypsin, α-galactosidase, β-glucorondase, α-glucosidase and α-mannosidase were not detected at the lower detection limit of 2.5 nmole substrate metabolized by the 65 µl supernatant. The activity of alkaline phosphatase, esterase (C4), leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, β-galactosidase and β-glucosidase increased from decapsulated cysts to nauplii instar I and then increased further in nauplii instar II. The activity of esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase and α-fucosidase showed a gradual increase in the populations.
Enzymatic activities (nmol/100 µg dw within 5 min) determined in three Greek Artemia populations (Milos, Polychnitos and Messolongi; least squares means) at different developmental stages: decapsulated cysts, nauplii instar I, II and III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Cysts</th>
<th>Nauplii instar I</th>
<th>Nauplii instar II</th>
<th>Nauplii instar III</th>
<th>S.E.M.</th>
<th>P</th>
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<tr>
<td>Alkaline phosphatase</td>
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<td>38.31</td>
<td>47.69</td>
<td>67.08</td>
<td>4.128</td>
<td>NS</td>
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<td>Trypsin</td>
<td>8.5</td>
<td>0.81</td>
<td>0.88</td>
<td>0.65</td>
<td>0.65</td>
<td>0.768</td>
<td>NS</td>
</tr>
<tr>
<td>Leucine amionopeptidase</td>
<td>7.5</td>
<td>86.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.94</td>
<td>4.816</td>
<td>**</td>
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<tr>
<td>Valine amionopeptidase</td>
<td>7.5</td>
<td>13.83</td>
<td>12.56</td>
<td>15.02</td>
<td>2.661</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Cystine amionopeptidase</td>
<td>7.5</td>
<td>12.20</td>
<td>9.52</td>
<td>11.90</td>
<td>4.61</td>
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<tr>
<td>Lipase (C14)</td>
<td>7.5</td>
<td>1.92</td>
<td>2.21</td>
<td>1.83</td>
<td>1.409</td>
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<tr>
<td>Esterase lipase (C8)</td>
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<td>9.77</td>
<td>15.98</td>
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<td>0.34</td>
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<td>Esterase (C4)</td>
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<td>18.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.08</td>
<td>4.779</td>
<td>*</td>
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<tr>
<td>Acid phosphatase</td>
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<td>19.71</td>
<td>22.10</td>
<td>20.25</td>
<td>4.61</td>
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<tr>
<td>β-galactosidase</td>
<td>5.4</td>
<td>62.58&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>5.56</td>
<td>7.71</td>
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<td>4.779</td>
<td>NS</td>
<td>**</td>
</tr>
</tbody>
</table>

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S.E.M.: standard error of grand mean.

There were no significant differences among the three Artemia populations at each of the four developmental stages (decapsulated cysts, nauplii instar I, II and III), with regard to the percentage level for any biochemical component or of the enzymes studied.

The mean individual dry weight estimated for the decapsulated cysts, nauplii instar I, II and III in the Artemia populations of Milos (54.03% dw, 2.23 µg ind<sup>-1</sup>), Polychnitos (51.83% dw, 2.12 µg ind<sup>-1</sup>) and Messolongi (57.74% dw, 2.7 µg ind<sup>-1</sup>) was much higher than that in the Artemia populations of San Francisco Bay (32.33% dw, 0.64 µg ind<sup>-1</sup>; Hines et al., 1980). Furthermore, the mean individual protein content of the three Greek parthenogenetic Artemia populations in decapsulated cysts (2.74 µg ind<sup>-1</sup>) and nauplii instar I (1.95 µg ind<sup>-1</sup>) was higher than that found in other Artemia populations, parthenogenetic (1.5 and 1.56 µg ind<sup>-1</sup>, respectively) and bisexual (1.0 and 0.82 µg ind<sup>-1</sup>, respectively) studied by Helland et al. (2000). In
addition, it was higher compared to that in the bisexual A. franciscana of Great Salt Lake (1.73 and 1.3 µg ind\(^{-1}\), respectively; García-Ortega et al., 1998). The present results point out that the three studied Artemia populations had relatively high protein content. Thus, in conjunction with the high digestibility of Artemia protein (García-Ortega et al., 2000), makes the above populations the first choice, as a source of protein, for the nutrition of fish and crustacean larvae.

The mean percentage of lipid and carbohydrate content, from decapsulated cysts to nauplii instar III, in the Artemia populations of Milos (10.57 and 6.95% dw), Polychinots (7.86 and 6.31% dw) and Messolongi (9.37 and 6.10% dw) was lower than that found in A. franciscana of San Francisco Bay (18 and 9.48% dw, respectively; Hines et al., 1980). However, the mean individual lipid and carbohydrate content of these developmental stages in A. parthenogenetica of Milos (0.44 and 0.30 µg ind\(^{-1}\), respectively), Polychinots (0.33 and 0.27 µg ind\(^{-1}\), respectively) and Messolongi (0.43 and 0.29 µg ind\(^{-1}\), respectively) was similar or higher than that in A. franciscana of San Francisco Bay (0.39 and 0.23 µg ind\(^{-1}\), respectively; Hines et al., 1980). Furthermore, in nauplii instar I, the mean individual content of the three studied Artemia populations in lipid (0.45 µg ind\(^{-1}\)) was similar, while that in carbohydrate (0.21 µg ind\(^{-1}\)) was much higher, compared to that found in A. franciscana of Great Salt Lake (0.40 and 0.09 µg ind\(^{-1}\); García-Ortega et al., 1998). The decapsulated cysts of Milos contained high amount of HUFA's, particularly of 20:5n-3 (11.2%) and 20:4n-6 (0.7%) (Moraiiti-Ioanidou et al., 2007), that make this population suitable as live food for sea bass, turbot and halibut larvae (Sargent et al., 1999). The decapsulated cysts of Artemia populations of Polychinots and Messolongi were rich in 18:2n-6% (4.9 and 10%, respectively) (Moraiiti-Ioanidou et al., 2007), and therefore are suitable for freshwater organisms (Kara et al., 2004). Consequently, the three Greek Artemia populations have an advantage in regards to their individual content of protein, lipid and carbohydrate compared to other Artemia populations that are used extensively in aquaculture, even those having a similar size such as A. franciscana of Great Salt Lake (Vanhaecke and Sorgeloos, 1980).

The mean percentage RNA content, from decapsulated cysts to nauplii instar III, in the Artemia population of Messolongi (4.81% dw) was lower than that in the Artemia populations of Milos (5.32% dw) and Polychinots (5.46% dw), and slightly higher than that found in A. franciscana of San Francisco Bay (4.50% dw; Watts et al., 1994). However, the mean RNA content per individual was similar in the three Greek Artemia populations, as the former had a higher body weight and size (Moraiiti-Ioanidou et al., 2007). In addition, the population of Messolongi had the highest mean DNA content while the population of Polychinots the lowest. It is possible that the population of Messolongi could be a mixture of different ploidy levels, as has been found for other parthenogenetic Artemia populations (Triantaphyllidis et al., 1995). The increase in size seems to be correlated with increased ploidy levels and protein content (Holland et al., 2000). This could be associated with the observed variations in the DNA and protein content among A. parthenogenetica of Messolongi, Milos and Polychinots. The mean individual RNA and DNA content (0.18 and 0.11 µg ind\(^{-1}\), respectively) in nauplii instar I of these populations – the latter has been found to be tetraploid (Triantaphyllidis et al., 1993) – was almost two-times higher than that recorded in the same stage (0.08 and 0.05 µg ind\(^{-1}\), respectively) of A. franciscana, which is bisexual and diploid (Gorokhova, 2005).

In nauplii instar I of the three studied Artemia populations, the percentage content of lipid increased, of protein remained constant and of carbohydrate reduced, showing that the latter was the main source of energy in this stage of development. A reduction of the carbohydrate content to one half from decapsulated cysts to nauplii instar I has also been reported in A. franciscana of Great Salt Lake (García-Ortega et al., 1998). The present results corroborate the previous suggestion for carbohydrate utilization in metabolic processes, leading to the emergence of the swimming nauplii from the cyst of Artemia (Sada et al., 1990). According to Clegg and Conte (1980), the Artemia embryos during hatching use carbohydrate as substrate for respiratory. Furthermore, the weight of nauplii instar I in the studied Artemia populations was lower than that of decapsulated cysts, as has been reported for A. franciscana by García-Ortega et al. (1998), presumably due to the degradation of yolk and particularly of its carbohydrate content. The observed increase of the percentage lipid content in nauplii instar I was similar to that previously recorded in other Artemia populations (García-Ortega et al., 1998; Navarro et al., 1991). This indicates a converting of lipids that are used in the formation of membrane lipids (Navarro et al., 1999). Vallejo et al. (1996) indicated that the cells are doubled in nauplii instar I from decapsulated cysts of the bisexual A. franciscana.

Simultaneously, in nauplii instar I of this study, the DNA content increased and the RNA content decreased, so the RNA/DNA ratio decreased compared to that in decapsulated cysts. An increase of DNA synthesis has been observed in A. franciscana by Watts et al. (1994) until late emergence and hatching of nauplii instar I, as well as in Daphnia pulex in specific stages (Gorokhova and Kyle, 2002), indicating high mitotic activity. Notably, the increase in DNA concentration from cysts to nauplii instar I was nearly 10-fold in the three studied parthenogenetic Artemia populations and this may be due to the polyploidy, compared to the triplication of the DNA concentration of the bisexual diploid population of A. franciscana (Cano et al., 1980). Consequently, the first naupliar stage of the tested parthenogenetic populations can be characterized as a stage of hyperplasia.

In nauplii instar II of the three Greek Artemia populations, the percentage content of carbohydrate and protein increased, whereas that of lipid reduced. Similarly, the total lipid (% dw) from nauplii instar I to nauplii instar II was reduced in A. franciscana (Hines et al., 1980) and a parthenogenetic Artemia population of Spain (Navarro et al., 1991). It seems that the lipid is consumed in this stage for energy purposes (Sorgeleso, 1980), while the respiratory substrate changes from carbohydrate to lipid as has been noticed in A. franciscana (Clegg and Conte, 1980). A similar increase in the percentage carbohydrate and protein content has been recorded previously in this developmental stage of A. franciscana (Hines et al., 1980). In addition, in nauplii instar II of the studied Artemia populations, the percentage content of RNA increased, while that of DNA remained constant, showing that this naupliar stage is a stage of cell growth due to protein synthesis (hypertrophy). McClean and Warner (1971) observed a higher increase in the percentage of RNA content in nauplii instar II from instar I of A. franciscana, which has a higher protein synthetic capacity of the cell. Despite the high RNA/DNA ratio estimated for nauplii instar II, in this study, their mean body dry weight was lower than that of nauplii instar I, evidently due to the absorption of yolk and, particularly, of the yolk lipid. The body weight of nauplii instar III, in this study, increased compared to that of nauplii instar II and was followed by an increase in DNA and a reduction in RNA/DNA ratio. It seems that nauplii instar III constitutes a stage of hyperplasia like nauplii instar I. Freeman (1986) referred that epidermal cell proliferation occurs in A. franciscana during this naupliar stage. However, the percentage content of lipid did not change, whereas that of carbohydrate and protein reduced, probably for the fulfillment of energy requirements.

The present study revealed an increase of the relative activity of 14 digestive enzymes in nauplii instar I from decapsulated cysts, especially as regards the alkaline phosphatase, β-galactosidase, β-glucosidase, acid phosphatase, alcaline aminopeptidases and esterase (C4). According to Rainier (1987) the activity of alkaline phosphatase increased 11 fold in nauplii instar I from cysts of A. franciscana, while this enzyme has been characterized by polymorphism and related to the specialization of plasma membrane of the cells of digestive and excretory-osmoregulatory organs, apart from the hydrolyzing the yolk. In addition, in nauplii instar I of A. franciscana the activity of acid hydrolases, like β-glucosidase, increased (Perona and Vallejo, 1985). Perona et al. (1988) suggested that the increase of acid phosphatase activity in nauplii instar I of A. franciscana was related to the degradation of about 25% of the yolk protein. The activity of alkaline proteases increased also after hatching of
A. franciscana (Pan et al., 1991), due to alkaline pH of the cell cytosol in nauplii instars I (Warner, 1987). From all proteases detected in this study, trypsin was an exception, which showed the least activity in nauplii instar III of the three studied Artemia populations, where the gut has become fully functional (Warner and Matheson, 1998). It has been shown that trypsin activity depends on the food levels and composition throughout the development of A. franciscana (Pan et al., 1991; Le Vay et al., 2001).

In nauplii instar II of this study, the relative activity of esterase (C4) and esterase lipase (C8) was at the highest levels. A strong increase in the level of esterases has been previously noticed in this stage of Artemia sp (Munilla-Moran et al., 1990). Among the naupliar stages studied, these enzymes revealed the lowest activity levels in nauplii instar I, where lipid appeared at the highest content. It has been shown (Le Vay et al., 2001) in crustacean larvae that the activity of enzymes is higher when the hydrolyzed substrate is at low levels. The activity of lipase (C14) was detected only in nauplii instars II and III, where the lipid was reduced compared to that in nauplii instar I.

Furthermore, in nauplii instar II, the relative activity of alkaline phosphatase, leucine aminopeptidase, valine aminopeptidase and cystine aminopeptidase increased significantly, in comparison to that in nauplii instar I, while that of acid phosphatase remained at the same high level. According to Warner (1987) the appearance of alkaline proteases in A. franciscana shortly after hatching and the significant increase of activity in nauplii instar II may signify an aspect of differentiation of cells lining the gut rather than involving in yolk metabolism. Perona et al. (1988) found that the 70% protein content was degraded at the end of this stage of A. franciscana and was associated with acid phosphatase activity. In this study, the activity levels of aminopeptidases followed those of protein, RNA and DNA/DNA ratio during development, showing maximum values in nauplii instar II. It seems that at this stage of hypertrophy, the degradation of yolk protein content was followed by a high protein synthetic capacity.

In addition, in nauplii instar II, the relative activity of acid hydrolases, like napththol-AS-BI-phosphohydrolase, β-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase, increased in comparison to that in nauplii instar I. Similarly, Perona and Vallejo (1985) found that in nauplii instar II of A. franciscana the activity of acid hydrolases was maximum and the yolk degradation was the most intense. The present findings of β-glucosidase and N-acetyl-β-glucosaminidase are in agreement with those previously reported for A. franciscana (Perona and Valillejo, 1985; Warner and Matheson, 1998). It has been noted (Warner and Matheson, 1998) that the activity of the chitinolytic enzyme N-acetyl-β-glucosaminidase along with that of cysteine protease – both well-known indicators of molting – showed the largest increase between the first and second nauplial molts of A. franciscana. Despite the highest activity levels of glycosidases in nauplii instar II, the highest carbohydrate content was observed. It seems that at this stage, the degradation of yolk carbohydrate was followed by an intense reconstruction of these biological molecules.

In nauplii instar III of the studied populations, the relative activity of the enzymes remained at levels similar to those in nauplii II or reduced. Only alkaline phosphatase increased per individual in nauplii instar III, following the increase of DNA that showed the highest levels at this stage of the studied Artemia populations. Raineri (1987) has also found an elevation of alkaline phosphatase in nauplii instar III of A. franciscana. The alkaline phosphatase seems to follow the increase of DNA, not only among the stages but among the populations as well. Artemia population of Messolongi, which had greater length (Moraiti-loannidou et al., 2007) and body weight than the Artemia populations of Milos and Polychinitos, exhibited also higher DNA levels and alkaline phosphatase activity per individual.

As generally accepted, the quantitative/qualitative stage-specific changes of enzymes can reflect selective gene expression in brine shrimp development (Raineri, 1987; Funke and Spindler, 1987; Warner and Matheson, 1998). The differences in the activity levels of most of the 14 digestive enzymes that were detected in this study appear to be attributed to stage-specific expression of the genes during the development of A. parthenogenetica of Messolongi, Polychinitos and Milos. In addition, the variations in the activity level of some enzymes among the three studied Artemia populations seem to be also genetically controlled and to be involved in the variations of biochemical composition. Specifically, protein content was related to leucine aminopeptidase activity, lipid content to esterase (C4) activity and carbohydrate content to β-galactosidase activity. These reverse relationships seem to be in accordance with previous findings (Le Vay et al., 2001), as well as those observed for lipid and esterase (C4) throughout the developmental stages. It is concluded that the differences in the biochemical composition and enzymatic activity estimated in this study should be taken into consideration for the exploitation of the three Greek Artemia populations for use in aquaculture. Since the production of Artemia cysts in the three Greek saltworks is not adequate for commercial purposes, human interventions in the environment of the saltworks will be necessary in order to get cultured Artemia populations, giving high priority to the one of Milos, to provide adequate amount of cysts to be used in aquaculture of sea bass and sea bream, species that are widely cultivated in Greece.

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References


