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The phenotypic response of ME_2 (M. Embolon, Greece) *Artemia* clone to salinity and temperature

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The phenotypic expression of a single genotypic lineage (ME_2) isolated from the apomictic tetraploid *Artemia* population of M. Embolon (Greece) was investigated. For this reason twelve reproductive and life span characteristics were assayed in six salinity-temperature (S-T) combinations under laboratory conditions. The ME_2 clone performed best at 22°C and 40 g l^{-1}, the lowest salinity value reported for parthenogenetic *Artemia*. Salinity by temperature interaction proved to be significant (two-way ANOVA, $p<0.05$) for ten out of twelve characters studied. Discriminant function analysis gave an overall prediction of 99.21% over the six S-T combinations tested. Elevation of salinity had the major impact on most of the characters scored in this study. The comparison of the reproductive performance of ME_2 clone to that of ME_1 (previously isolated from the same *Artemia* population) revealed that co-existing clones in the M. Embolon population respond differently to the increase of salinity.

Key words: *Artemia*, parthenogenetic, salinity, temperature, reproductive characteristics.

INTRODUCTION

The genus *Artemia* comprises of a number of sexual species and a large number of obligatory parthenogenetic strains inhabiting saline and hypersaline coastal or inland lakes. All bisexual species are diploid while asexual populations may be diploid, polyploid or mixtures of different ploidies. The parthenogenetic forms, found only in the Old World, are grouped under the binomen *A. parthenogenetica*; the use of this name should be avoided because of the different ploidy levels and the existence of numerous clones in the same population (Abatzopoulos *et al*., 2002). Diploid asexual *Artemia* populations are mainly polyclonal due to automixis, i.e., exhibiting limited meiotic recombination. Polyploid parthenogens are apomictic (meiosis is totally suppressed) and therefore mainly monoclonal (for extensive reviews see Barigozzi, 1974; Browne & Bowen, 1991; Triantaphyllidis *et al*., 1998; Abatzopoulos *et al*., 2002).

Estimated environmental and genetic components of variance for life span and reproductive traits of *Artemia* have been investigated by several research teams (Browne *et al*., 1984; Wear & Haslett, 1986; Wear *et al*., 1986; Abatzopoulos *et al*., 1993; Triantaphyllidis *et al*., 1995; Browne & Wanigasekera, 2000; Browne *et al*., 2002; Abatzopoulos *et al*., 2003; Baxevanis *et al*., 2004). Most of these studies have focused on two of the most important abiotic parameters affecting the life history of hypersaline organisms: salinity and temperature.

Artemia has been proven to be a model organism, offering substantial advantages for investigating the effects of temperature and salinity on life span and reproductive characters (Browne *et al*., 1984; Triantaphyllidis *et al*., 1995; Barata *et al*., 1996; Browne & Wanigasekera, 2000; Abatzopoulos *et al*., 2003). Also, several researchers have studied the combined effects of temperature and salinity on survival and reproductive performance of *Artemia*, mainly in laboratory conditions (Vanhaecke *et al*., 1984; Wear & Haslett, 1986; Wear *et al*., 1986; Vanhaecke & Sorgeloos, 1989; Triantaphyllidis *et al*., 1995; Browne & Wanigasekera, 2000; Abatzopoulos *et al*., 2003).

Although monoclonal populations produced by apomictic parthenogenetic *Artemia* (i.e. lineages derived from a single mother) could be successfully used for studying phenotypic traits, this approach

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has been so far poorly explored (see Browne *et al*., 2002). Only recently, Abatzopoulos *et al*. (2003) have investigated i) the potentiality of using a single genetic lineage in order to visualize the effects of different environmental cues on a specific clone and ii) the effects of salinity and temperature on ten reproductive and life span characteristics of that *Artemia* clone from M. Embolon (Greece) and found differences between the phenotypic responses at each treatment.

In the present study we document the phenotypic expressions (twelve life history traits) of another single genotypic lineage from the same population (M. Embolon) exposed to variable levels of two major environmental components: salinity and temperature. Discriminant analysis was used to define possible grouping based on the different salinity and temperature regimes, while the PCR-RFLP technique was applied for the identification/verification of this specific clone.

MATERIALS AND METHODS

Clonal material

An *Artemia* clone (ME_2) was isolated from the parthenogenetic population of M. Embolon saltworks (Thessaloniki, Greece – *Artemia* Reference Center code no. 1420). This asexual population is tetraploid and apomictic (Abatzopoulos *et al*., 1986, 1987, 1993, 2003).

Culture conditions

Culture media of four different salinities, i.e., 40 g l^{-1}, 80 g l^{-1}, 120 g l^{-1} and 160 g l^{-1}, were prepared using Instant Ocean® (Synthetic Sea Salts 1998, Aquarium Systems). The salinity was measured with a temperature controlled ATAGO refractometer. The two experimental temperatures, i.e., 22 and 30°C, were maintained in thermostatically controlled water baths (±0.5°C). Stock cultures of ME_2 (1 individual / 4 ml of culture medium) were kept in 1 l cylindroconical glass jars maintained at 22°C. The salinity of the culture medium was 80 g l^{-1}. Animals were fed with 75% of yeast-based diet LANSY-PZ (INVE Aquaculture NV, Belgium) and 25% *Dunaliella tertiolecta* according to Triantaphyllidis *et al*. (1995). Approximately 50% of the culture medium was replaced by fresh culture medium every seven days in the mass or stock cultures and every four days for the cultures in the 50 ml plastic cylindroconical tubes.

Experimental design

After hatching, instar-I nauplii were transferred directly to eight different 1 l cylindroconical vials (four salinities, i.e., 40, 80, 120 and 160 g l^{-1} and two temperatures, i.e., 22 and 30°C) at an initial density of 2 nauplii/ml of culture medium according to the following procedure: the salinity in each flask was gradually increased to the desired level in such a way, that the animals were not stressed (e.g. 160 g l^{-1} was reached after 3 days). When the salinity reached the appropriate level in each cylindroconical tube, it was kept constant for the rest of the experiment by adding distilled water to compensate evaporation (Triantaphyllidis *et al*., 1995). From day 8 on, the density of the animals was maintained to one individual per 4 ml of culture medium. Mild aeration was applied from the bottom of the tubes which were covered with perforated Petri dishes to minimize evaporation. The photoperiod was 12 h light/12 h dark and was provided by fluorescent light tubes. When the animals showed signs of ovarian development, they were removed from the mass culture and placed individually in 50 ml plastic cylindroconical tubes containing 40 ml of 0.45 μm filtered synthetic medium. Approximately 30 females were examined for each treatment (S-T combinations). The tubes were examined every two days for offspring production or deaths. Reproductive and life span characteristics were determined according to Browne *et al*. (1984, 1988). It should be noted that no results were obtained from the treatments of 160 g l^{-1} at 22 and 30°C because of the high mortality of the individuals.

The reproductive and life span characteristics scored for both treatments were the following: number of nauplii, number of encysted embryos, number of broods, pre-reproductive period, reproductive period, post-reproductive period, total number of offspring, offspring per reproductive day, offspring per brood, percentage of encysted embryos, life span and days between broods.

mtDNA-RFLP analysis

DNA was extracted from 10 randomly chosen females per each treatment using the CTAB method described in Hillis *et al*. (1996). Part of the 16S rRNA gene was amplified using the universal primers L_{2510} and H_{3080}, described by Palumbi *et al*. (1996). PCR reactions were set up in a volume of 75 μl and composed of 45 pmoles of each primer, 0.15 mM of each dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM

$MgCl_2$, and 1.5 units of *Taq* DNA polymerase (Invitrogen®). Approximately 100 ng of total genomic DNA in Tris-EDTA (pH=8) was added to this. The following amplification cycle program was used: initial denaturation at 95˚C for 4 min, 33 cycles consisted of 94˚C for 1 min, 50˚C for 50 sec, 72˚C for 1 min. Four to seven μl of the PCR product were digested with 9 restriction endonucleases, electrophoretically separated in 1.5% agarose gel, stained with ethidium bromide, visualized and photographed under UV light. The following restriction enzymes were used: *Alu*I, *Bfa*I, *Dde*I, *Dpn*II, *Hae*III, *Msp*I, *Not*I, *Rsa*I, *Taq*^{a}I. For the molecular weight size standard, a 100 bp ladder (New England BioLabs®) was used.

Statistical analyses

Reproductive and life span characteristics were analyzed by two-way ANOVA, where variances are assumed to be homogeneous (Sokal & Rohlf, 1981). Normality and homogeneity of group variances were checked by Kolmogorov-Smirnov and Bartlett's tests. Some of variables were log- or square root-transformed to satisfy assumptions of normality and homogeneity (Triantaphyllidis *et al*., 1995). The 12 reproductive and life span variables determined in all individuals were used to establish the relationships among the different treatments applied on the same *Artemia* clone through discriminant analysis. The predefined groups were each treatment (i.e., A: culture at 40 g l^{-1} and 22˚C; B: culture at 80 g l^{-1} and 22˚C; C: culture at 120 g l^{-1} and 22˚C; E: culture at 40 g l^{-1} and 30˚C; F: culture at 80 g l^{-1} and 30˚C; and G: culture at 120 g l^{-1} and 30˚C). The rationale for using this approach is described in Kachigan (1986) and Triantaphyllidis *et al*. (1995). The analysis has been performed using a standard procedure where all selected variables were entered simultaneously into the model. Those variables that were redundant were removed by the analysis. STATISTICA 6.0 was used for the analyses (StatSoft Inc., 2001).

RESULTS

mtDNA-RFLP analysis

The size of the PCR-amplified mtDNA fragment was 535bp (Fig. 1). RFLP analysis was based on nine restriction enzymes (profile acquired after digestion of PCR-amplified 16S rDNA with *Dpn*II is given as an example in Fig. 2) and all tested animals produced the same composite haplotype (DBAAACABC). Restriction patterns are available upon request. These results support that the examined individuals belonged to the same clone and there was no contamination.

Reproductive and life span characteristics

Eight reproductive and four life span characteristics from the parthenogenetic clone ME_2 cultured at three different salinities and two different temperatures are summarized in Table 1. The results of the two-way ANOVA are presented in Table 2. In 10 out of 12 characteristics studied (i.e., number of nauplii, number of encysted embryos, number of broods, pre-reproductive period, post-reproductive period, total number of offspring, offspring per reproductive day, offspring per brood, percentage of encysted embryos and days between broods) salinity by temper-

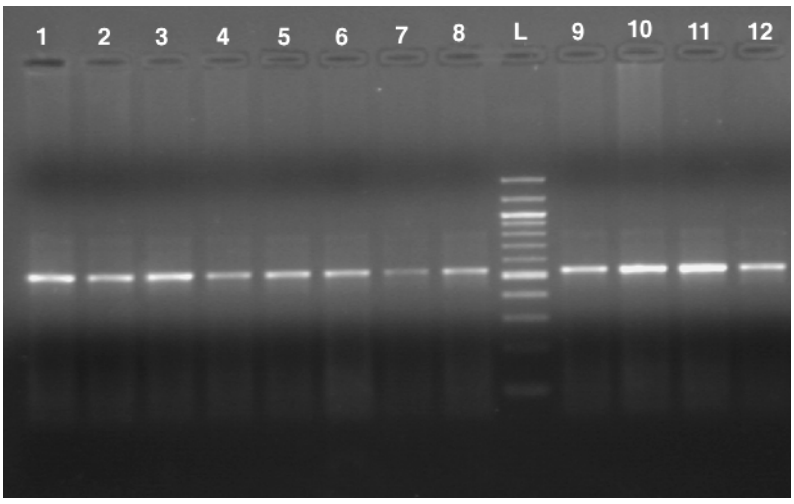

FIG. 1. PCR-amplified fragments of 16S rDNA. Lanes 1-2: individuals cultured at salinity 40 g l^{-1} and temperature 22˚C. Lanes 3-4: individuals cultured at salinity 80 g l^{-1} and temperature 22˚C. Lanes 5-6: individuals cultured at salinity 120 g l^{-1} and temperature 22˚C. Lanes 7-8: individuals cultured at salinity 40 g l^{-1} and temperature 30˚C. Lanes 9-10: individuals cultured at salinity 80 g l^{-1} and temperature 30˚C. Lanes 11-12: individuals cultured at salinity 120 g l^{-1} and temperature 30˚C. L: 100bp molecular weight ladder.

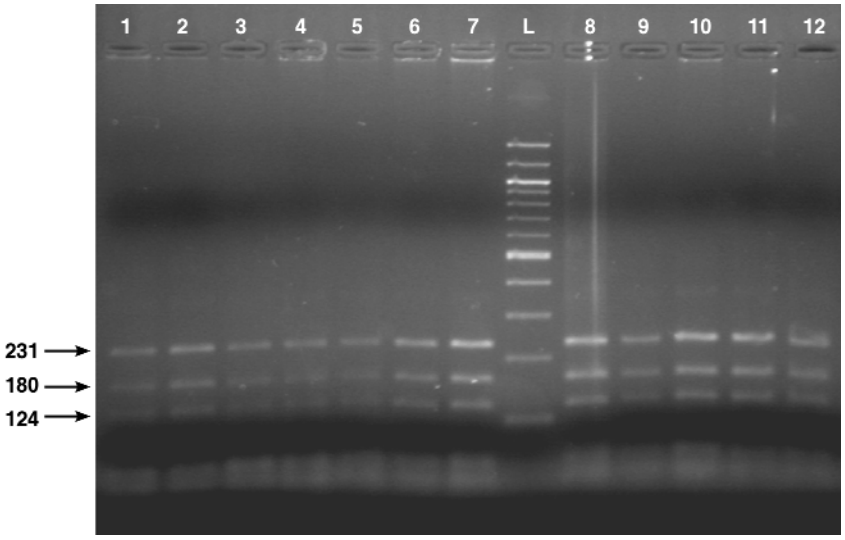

FIG. 2. Restriction profile after digestion of 16S rDNA with *Dpn*II. Lanes 1-2: individuals cultured at salinity 40 g l^{-1} and temperature 22°C. Lanes 3-4: individuals cultured at salinity 80 g l^{-1} and temperature 22°C. Lanes 5-6: individuals cultured at salinity 120 g l^{-1} and temperature 22°C. Lanes 7-8: individuals cultured at salinity 40 g l^{-1} and temperature 30°C. Lanes 9-10: individuals cultured at salinity 80 g l^{-1} and temperature 30°C. Lanes 11-12: individuals cultured at salinity 120 g l^{-1} and temperature 30°C. L: 100bp molecular weight ladder.

TABLE 1. Mean (±s.d.) of various reproductive and life span characteristics for the parthenogenetic *Artemia* clone ME_2 reared at three different salinities and two different temperatures. Significant ($p<0.05$) differences between combinations of temperatures and salinities were determined by Least Significant Difference tests. Mean values for each character sharing the same letter do not differ significantly

	Temperature					
	22°C			30°C		
	Salinity			*Salinity*		
Characteristics	40 g l^{-1}	80 g l^{-1}	120 g l^{-1}	40 g l^{-1}	80 g l^{-1}	120 g l^{-1}
Number of nauplii	637.20^{d}	371.50^{c}	93.20^{b}	24.25^{a}	18.50^{a}	17.75^{a}
	(85.70)	(45.66)	(8.36)	(5.18)	(3.86)	(2.04)
Number of encysted embryos	0.00^{a}	79.65^{d}	152.45^{e}	51.75^{c}	20.15^{b}	25.00^{b}
	(0.00)	(27.33)	(53.73)	(19.75)	(12.84)	(3.94)
Number of broods	7.80^{e}	7.15^{d}	4.95^{c}	2.65^{b}	1.75^{a}	1.38^{a}
	(0.83)	(0.67)	(0.67)	(0.49)	(0.44)	(0.50)
Pre-reproductive period*	34.15^{a}	34.55^{b}	37.75^{d}	34.35^{a}	35.65^{c}	40.06^{e}
	(0.37)	(0.51)	(0.44)	(0.33)	(0.49)	(0.27)
Reproductive period*	39.00^{d}	37.00^{d}	30.70^{c}	12.65^{b}	6.90^{a}	4.25^{a}
	(4.19)	(4.32)	(5.71)	(3.51)	(3.52)	(2.34)
Post-reproductive period*	2.85^{d}	1.40^{bc}	2.35^{cd}	0.30^{ab}	1.00^{ab}	0.06^{a}
	(2.16)	(2.01)	(2.48)	(0.66)	(1.65)	(0.25)
Total offspring	637.20^{e}	451.15^{d}	245.65^{c}	76.00^{b}	38.65^{a}	27.13^{a}
	(85.70)	(35.66)	(54.16)	(21.66)	(12.41)	(8.14)
Offspring per reproductive day	16.34^{c}	12.26^{b}	8.00^{a}	6.03^{a}	8.24^{a}	13.23^{b}
	(1.36)	(0.74)	(0.89)	(0.56)	(2.94)	(5.81)
Offspring per brood	81.63^{e}	63.24^{d}	49.49^{c}	28.18^{b}	21.50^{a}	19.38^{a}
	(5.99)	(2.90)	(4.98)	(3.78)	(4.55)	(3.28)
Percentage of encysted embryos	0.00^{a}	18.00^{b}	60.86^{d}	66.32^{d}	44.11^{c}	23.98^{b}
	(0.00)	(6.13)	(9.42)	(10.12)	(17.53)	(14.03)
Life span*	76.00^{d}	72.95^{c}	70.80^{c}	47.10^{b}	43.55^{b}	44.38^{a}
	(2.41)	(4.54)	(5.43)	(3.29)	(2.24)	(4.21)
Days between broods	4.45^{b}	4.08^{a}	6.87^{d}	6.33^{c}	6.90^{d}	8.00^{e}
	(0.35)	(0.21)	(0.39)	(0.52)	(0.64)	(0.89)

* In days

TABLE 2. Results of the two-way ANOVA analysis for the twelve reproductive and life span characteristics of *Artemia* individuals scored. ns: not significant

	Salinity		Temperature		Salinity × Temperature	
Character	*F*	p-level	*F*	p-level	*F*	p-level
Number of nauplii	432.81	<0.05	2104.98	<0.05	412.41	<0.05
Number of encysted embryos	31.56	<0.05	55.04	<0.05	74.75	<0.05
Number of broods	91.47	<0.05	1440.61	<0.05	20.42	<0.05
Pre-reproductive period	1338.00	<0.05	212.50	<0.05	71.82	<0.05
Reproductive period	35.02	<0.05	1173.42	<0.05	2.39	ns
Post-reproductive period	0.58	ns	28.15	<0.05	4.36	<0.05
Total offspring	214.57	<0.05	2134.38	<0.05	129.89	<0.05
Offspring per reproductive day	8.86	ns	265.12	<0.05	572.20	<0.05
Offspring per brood	205.57	<0.05	2547.48	<0.05	65.11	<0.05
Percentage of encysted embryos	4.07	<0.05	32.89	<0.05	88.51	<0.05
Life span	11.76	<0.05	1545.76	<0.05	1.58	ns
Days between broods	129.89	<0.05	353.34	<0.05	21.58	<0.05

TABLE 3. Discriminant analysis of the reproductive and life span characters of female *Artemia* for clone ME_2 reared at three different salinities (40, 80 and 120 g l^{-1}) and two temperatures (22 and 30°C). Standardized coefficients for canonical variables and predicted classifications are presented. 100 per cent prediction means that with the variables used, 100% discrimination of the seven groups can be obtained. For the abbreviations of the groups, see Fig. 3

Character	Root 1	Root 2	Root 3	Root 4	Root 5
Number of broods	3.879	-2.550	0.602	3.705	-2.489
Pre-reproductive period	-0.113	0.936	0.308	0.324	-0.126
Reproductive period	-1.869	3.172	-2.104	-2.576	2.436
Post-reproductive period	0.154	0.118	0.043	-0.276	0.164
Total offspring	-1.752	-0.080	1.684	-1.505	0.396
Offspring per reproductive day	-1.232	-0.014	-0.608	-0.326	-0.172
Offspring per brood	1.836	0.159	-0.452	0.207	-0.394
Percentage of encysted embryos	-1.949	-0.610	-0.324	-0.485	-0.979
Days between broods	-0.026	-0.036	0.379	-0.314	-0.104
Eigenvalues	126.519	23.234	4.529	1.624	0.192
Cum. Prop.	0.811	0.959	0.988	0.999	1.000
Predicted classifications					
40-22	100.00%				
80-22	100.00%				
120-22	100.00%				
40-30	95.00%				
80-30	100.00%				
120-30	100.00%				
Total	99.21%				

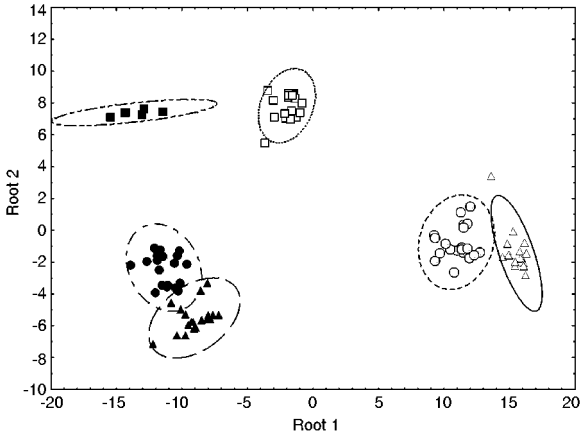

FIG. 3. Scatterplot resulting from the discriminant analyses (canonical scores) when using culture conditions as separating factor (△, 40-22: salinity 40 g l^{-1} and temperature 22°C; ○, 80-22: salinity 80 g l^{-1} and temperature 22°C; □, 120-22: salinity 120 g l^{-1} and temperature 22°C; ▲, 40-30: salinity 40 g l^{-1} and temperature 30°C; ●, 80-30: salinity 80 g l^{-1} and temperature 30°C; and ■, 120-30: salinity 120 g l^{-1} and temperature 30°C). Borderlines represent 95% confidence level.

ature interaction was significant ($p<0.05$) and the hypothesis that salinity effect is the same for the alternative temperatures can be rejected (the pattern of response to changes in salinity differed in the two temperatures) (Table 2). More specifically, salinity combined with any of the temperatures tested contributed a positive increment (synergism) to five characters (i.e., number of nauplii, number of broods, pre-reproductive period, total number of offspring and offspring per brood), while it had a negative effect (interference) on number of encysted embryos, post-reproductive period, offspring per reproductive day, percentage of encysted embryos and days between broods. Despite the fact that the reproductive period and life span were not significantly affected by S-T interaction ($p>0.05$), the two way ANOVA applied to these two variables revealed that they were significantly influenced by salinity and temperature, separately. The component of temperature had significant effects on all characters studied, while salinity significantly affected ten out of twelve characteristics (Table 2).

Individuals cultured at 40 g l^{-1} and 22°C were strictly ovoviviparous (producing only nauplii) and had the highest mean values for the number of nauplii, number of broods, reproductive period, post-reproductive period, total offspring, offspring per reproductive day, offspring per brood and life span, when compared to all other S-T combinations.

At 22°C, the percentage of encysted embryos and the number of encysted embryos increased proportionally to the elevation of salinity, while the same parameters were negatively affected by the increase of salinity at 30°C. The greatest number of encysted embryos was recorded at 120 g l^{-1} and 22°C.

Discriminant analysis, using as separating factor the six different treatments (i.e., S-T combinations), produced five roots (Table 3). Nine out of twelve reproductive and life span characteristics were used for the construction of the discrimination model; total life span, total number of encysted embryos and total number of nauplii were excluded as redundant because total life span was highly correlated with reproductive period, and total number of encysted embryos and nauplii were highly correlated with total offspring. The first two roots (out of the five produced by discriminant analysis) explained 95.90% of the total variability and they were used to plot the graph presented in Fig. 3. The responses of the ME_2 clone in the six different treatments were separately grouped, although an overlap occurred in treatments 40 g l^{-1}/30°C and 80 g l^{-1}/30°C. It is worth noting that the characters which mostly contributed in Root 1 were the number of broods and the percentage of encysted embryos, while for Root 2 the reproductive period and pre-reproductive period were responsible. According to the discriminant model, the percentage of correct predicted classifications performed by discriminant analysis was 99.21% (Table 3).

DISCUSSION

In this study we tried to define the response of an *Artemia* clone (ME_2) to two major environmental components (i.e., salinity and temperature) in terms of eight reproductive and four life span characteristics. This clone has been isolated from the M. Embolon population, which is tetraploid and apomictic (Abatzopoulos *et al*., 1986, 1993). Apomixis implies that a parthenogenetic clone has no other mechanism for genotypic change but mutation, which may induce genetic differentiation, although it is not likely to occur within the very short duration of the experimental procedure (Abatzopoulos *et al*., 2003).

Therefore, the genetic make-up of all offspring produced by a single female is identical (Barigozzi, 1974; Suomalainen *et al*., 1980; Lokki, 1983). Variations within the populations are assumed to be due to environmental sources only, since no genetic differences among individuals occur (total suppress of meiotic recombination). Allozyme analysis of the M. Embolon population revealed that the low level of observed genetic variation may be due to polyclonality and possible selective differences among clones (Abatzopoulos *et al*., 1993). There is considerable evidence that coexisting clones may not be ecologically equivalent and therefore they may be ecologically specialized (Young, 1983). In this study, the periodical checking of the ME_2 clone using mtDNA-RFLP analysis eliminated the possibility of contamination throughout the experiment.

Salinity and temperature had significant effects on the majority of the twelve characters studied in this investigation. The interaction of salinity and temperature affected significantly ($p<0.05$) ten of the twelve examined characters (see Table 1 and 2), with temperature having the major contribution to it (Table 2). The increase of both temperature and salinity had negative influence on the overall reproductive performance of clone ME_2 (all twelve characters studied were affected, see Table 1). The highest reproductive output expressed as total offspring per female occurred for 40 g l^{-1} and 22°C. Also, this was obvious when considering the offspring per reproductive day, the offspring per brood and number of broods, variables that were significantly ($p<0.05$) higher compared to those recorded for remaining S-T combinations (Table 1). It is well known that *Artemia* follows the r-strategy. Therefore, the most successful clonal response would be the production of a great number of offspring within the shortest possible period. In this sense, the combination of 40 g l^{-1} and 22°C must be considered as optimal for the ME_2 clone. This clone appeared to be very well adapted to the salinity of 40 g l^{-1}, which in fact has been considered unfavorable for other parthenogenetic populations (Triantaphyllidis *et al*., 1995) or for another clone (ME_1) isolated from the M. Embolon population and studied previously (Abatzopoulos *et al*., 2003).

It is apparent that the increase of salinity induced oviparity, which was expressed as significant ($p < 0.05$) increase in the percentage of encysted embryos (Table 1) when temperature was 22°C. This agrees with the results obtained for clone ME_1 (Abatzopoulos *et al*., 2003).

Both ME_1 (Abatzopoulos *et al*., 2003) and ME_2 (present study) clones performed best at 22°C; in contrast, the two clones exhibited their best reproductive performance at different salinities (i.e., 80 g l^{-1} for ME_1 clone and 40 g l^{-1} for ME_2). It is obvious that the partitioning effect is mainly due to salinity changes. The function of M. Embolon solar saltworks is based on the evaporation of sea water and progressive sedimentation of salts in consecutive salt ponds due to the gradual increase of salinity. Therefore, salinity is the prevailing environmental component and the major selective force sculpturing *Artemia* populations. In cladocerans (Hebert & Crease, 1980; Loaring & Hebert, 1981; Hebert *et al*., 1982; Ferrari & Hebert, 1982), rotifers (Snell, 1979, 1980) and in M. Embolon *Artemia* population (Abatzopoulos *et al*., 1993) there is good evidence that many clones coexist. One would expect that competitive exclusion would eliminate all but the fittest clone; however, this is not valid. In other words, uninterrupted changes in frequencies of coexisting clones take place. The above supports the idea that the only plausible source of stability is that of ecological specialization leading to frequency-dependent selection (Young, 1983). The evidence from our studies on different *Artemia* clones from the M. Embolon population can be in favour of coexistence and not of permanence.

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