

Biological Characterization of the New Invasive Brine Shrimp *Artemia franciscana* in Tunisia: Sabkhet Halk El-Menzel

Hachem Ben Naceur, Amel Ben Rejeb Jenhani and Mohamed Salah Romdhane

Abstract—Endemic *Artemia franciscana* populations can be found throughout the American continent and also as an introduced specie in several country all over the world, such as in the Mediterranean region where *Artemia franciscana* was identified as an invasive specie replacing native *Artemia parthenogenetica* and *Artemia salina*. In the present study, the characterization of the new invasive *Artemia franciscana* reported from Sabkhet Halk El-Menzel (Tunisia) was done based on the cysts biometry, nauplii instar-I length, Adult sexual dimorphism and fatty acid profile. The mean value of the diameter of non-decapsulated and decapsulated cysts, chorion thickness and naupliar length is 235.8, 226.3, 4.75 and 426.8 μm , respectively. Sexual dimorphism for adults specimen showed that maximal distance between compound eyes, diameter for compound eyes, length of first antenna and the abdomen length compared to the total body length ratio, are the most important variables for males and females discrimination with a total contribution of 62.39 %. The analysis of fatty acid methyl esters profile of decapsulated cysts resulted in low levels of linolenic acid (LLA, C18:3n-3) and high levels of eicosapentaenoic acid (EPA, C20:5n-3) with 3.11 and 11.10 %, respectively. Low quantity of docosahexaenoic acid (DHA, 22:6n-3) was also observed with 0.17 $\text{mg}\cdot\text{g}^{-1}$ dry weight.

Keywords—Invasive *Artemia franciscana*, biometry, sexual dimorphism, fatty acid, Tunisia.

I. INTRODUCTION

THE brine shrimp *Artemia* (Branchiopoda, Anostraca) is highly adapted to living in the hypersaline waters of inland salt lakes, coastal lagoons, and the evaporation ponds, channels and reservoirs of salterns. Since the initial record of 80 *Artemia* sites by Abonyi in 1915, the number has steadily increased, and the branchiopod crustacean *Artemia* has been reported in more than 600 coastal locations and inland waters around the world [1]. The genus *Artemia*

comprises a group of seven bisexual species and a variety of parthenogenetic strains of diverse ploidy. In the Mediterranean basin a bisexual species *A. salina* (formerly *A. tunisiana*) occurs together with a large number of parthenogenetic populations, one diploid and another tetraploid [2]. The other Old World bisexual species are distributed in Asia, with *A. urmiana* [3] from Iran, *A. sinica* [4] from P.R. China, *A. tibetiana* [5-6] from Tibet, and *Artemia* sp. from Kazakhstan [7]. The bisexual *A. persimilis* [8] (Argentina and Chile) and *A. franciscana* [9] (North, Central and South America) are endemic to the New World.

The brine shrimp, *Artemia* sp. is used in aquaculture as a live prey in any of its life history stages, but the nauplii stage are the most utilized food for crustacean and fish larvae [10]. Annual *Artemia* cysts consumption by aquaculture hatcheries increased dramatically from 60 metric tones in 1980 [11] to about 2000 metric tones in 1994 [12]. Presently, the maximum sustainable volume of harvestable cysts in the GSL is restricted to 9000 metric tons in 2000-2001 (wet weight) [13], and production in the GSL alone is not sufficient to supply the ever expanding aquaculture industry. This situation has led to a more exhaustive study of *Artemia* biogeography, to the world-wide prospecting of sources of cysts

The origin of *Artemia* populations can be natural through cyst dispersal by water birds or wind, or artificial through inoculation of cysts in salt works [1-14]. The first recorded deliberate introductions of *Artemia franciscana* were those carried out on a Pacific Island and in Brazil in the 1970s [1]. Moreover, Van Stappen [1] reported that *Artemia franciscana* may replace other species, such as *A. salina* which is known to occur on the African continent from Tunisia to Southern Africa. Amat et al. [2] reported that the American brine shrimp *A. franciscana* is an invasive species rapidly expanding its range in hypersaline ecosystems in the Mediterranean region since the 1980s and replacing the native *Artemia parthenogenetica* and *Artemia salina*. This event was initially stated in Portugal [2] and later in France [15], in Egypt [16], in Italy [17] and in Spain and Morocco [2-18-19].

The aim of this research was the characterization of the new introduced invasive *Artemia franciscana* reported from Sabkhet Halk El-Menzel in Tunisia based on their cysts biometry, nauplii instar-I length, adult sexual dimorphism and fatty acid profile.

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II. MATERIALS AND METHODS

A. Site description

Sabkhet Halk El-Menzel (HM) is situated 25 km north-west of Sousse city (Fig 1). It is located at an altitude of 0 to 50 m above sea level. The total surface area range between 19.5 km² (periodic area: during winter and dry season) and 13 km² (permanent area: during summer and dry season). The maximum length and width of the lake are about 12 and 2 km, respectively. The average and maximum depths are reported to be about 0.5-0.6 m and 1 m, respectively. Sabkhet Halk El-Menzel is divided into a north (periodic area) and south (permanent area) arms, separated by the road connecting the city of Hergla to Sidi Bou-Ali. The road has a gap of about 100 m, which allows a limited exchange of water between the two parts of the salt lake. The most important source of water feeding the lake is the Essod Wadi (wadi is an Arabic term traditionally referring to a dry riverbed that contains water only during the rainy season). The depth, salinity, and temperature vary with season, affected by precipitation and evaporation rate. Sabkhet Halk El-Menzel is one of the most important sites for waterbirds in Tunisia. In fact, with water available for most of the year, in addition to overwintering species (such as *Platalea leucorodia*, *Tadorna tadorna* and *Anas acuta*) the site attracts many passing migrants (such as *Limosa limosa*, *Calidris minuta* and *Numenius arquata*). Moreover, during the winter, many Greater Flamingo (*Phoenicopterus* sp.) were observed in Sabkhet Halk El-Menzel. Furthermore, Sabkhet Halk El-Menzel provides in its southeast part the main marine aquaculture (fish farm) in Tunisia. This private farm for the intensive production of sea bass and sea bream, includes a hatchery and use commercial *Artemia* cysts for feeding larval stage.

Ben Naceur et al. [23] prepared last check list of distribution of *Artemia* in Tunisia and signalled the presence of this branchiopod in 21 sites and don't record *Artemia* in Sabkhet Halk El-Menzel. However, in June 2009, the presence of *Artemia* in this site was reported for the first time. Based on the morphometrical and SEM observation, we identified *Artemia* from Sabkhet Halk EL-Menzel such as the invasive *Artemia franciscana* (unpublished data).

B. Biometrics of cysts and nauplii

Cysts were sampled on the sabkha banks and treated following the protocol described by Sorgeloos et al. [14]. The diameter of the hydrated and decapsulated cysts (n=100) was measured under a microscope equipped with a calibrated micrometer, according to Vanhaecke and Sorgeloos [20]. The chorion thickness was also measured [20]. To analysis the biometrical characteristics of nauplii instar-I, cysts were hatched in natural seawater (32 ± 1 psu) at 28 ± 1 °C, pH remain above 8, under continuous illumination (2000 lux) and aeration. The length of nauplii instar-I (n=100) was measured under a microscope equipped with a calibrated micrometer.

C. Adult's dimorphism

Sexual dimorphism of adults *Artemia* from HM was done using cultured specimens. Cysts were hatched according to the method described by Sorgeloos et al. [14].

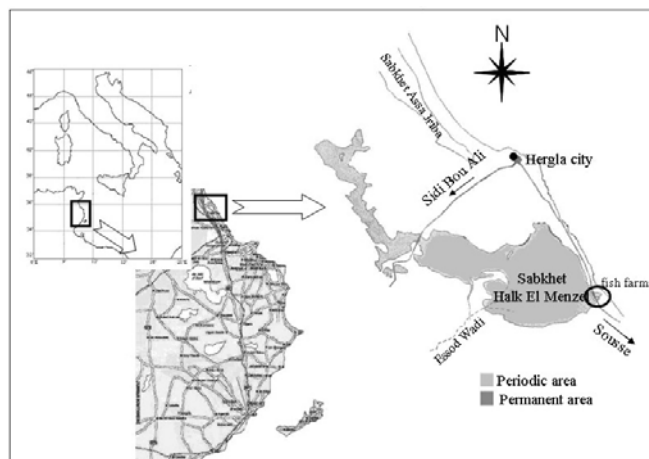


Fig. 1 Location of Sabkhet Halk El Menzel

Nauplii obtained were transferred to 2 litres cyliindroconical plastic tubes, with 90 ± 10 g.l⁻¹ filtered and autoclaved sea water plus crude sea salt [2]. The temperature was maintained at 24°C under 16 h light/8 h dark photoperiod. The animals were fed on the unicellular algae *Chlorella* sp. The medium was completely renewed twice a week with fresh microalgal culture. The animals were examined morphologically when most of them reached the adult stage (i.e. when offspring production observed). The following morphological parameters were examined on at least 20 individuals per sex: total length (*tl*), abdominal length (*al*), width of third abdominal segment (*wts*), length of furca (*lf*), number of setae inserted on left branch of the furca (*nlf*), number of setae inserted on right branch of the furca (*nrf*), width of head (*wh*), diameter for compound eyes (*dy*), maximal distance between compound eyes (*dby*) and length of first antenna (*la*), the abdomen length compared to the total body length ratio (*ra*, %) was also calculated for both sexes [2]. The animals were anaesthetized with few droplets of water saturated with chloroform and measured under a dissecting microscope provided with a calibrated micrometer eye piece.

Sexual dimorphism, between male and female individuals, was investigated by Principal Components Analysis using XLSTAT-Pro 7.5. computer program.

D. Fatty acids profile

Prior to lipid extraction, cyst samples were hydrated in distilled water under strong aeration until cysts were observed to be completely spherical. They were then decapsulated with sodium hypochlorite [14].

Lipid extractions and fatty acid analyses for decapsulated cysts were carried out as in Navarro et al. [21-22]. Total lipids were extracted and stored in chloroform/methanol (ratio 2/1 v/v) with 0.01% butylated hydroxytoluene (BHT) (Sigma Chemical) as an antioxidant. Lipid aliquots were transmethylated overnight after the addition of nonadecaenoic fatty acid (19:0) (99% pure; Sigma Chemical) as an internal standard. Fatty acid methyl esters (FAMES) were extracted with hexane/diethyl ether (ratio 1/1 v/v) and purified by thin-layer chromatography (silica gel G 60, Merck) using hexane/diethyl ether/acetic acid (ratio 85/15/1.5 by vol.) as the

solvent system. Analyses of FAMES were performed with a Fisons Instruments GC 8000 (Thermo Electron) gas chromatograph equipped with a fused silica 30 m×0.25 mm open tubular column (tracer, TR-WAX, film thickness: 0.25 µm; Teknokroma) and a cold on-column injection system, using helium as carrier and a 50 to 220°C thermal gradient. Peaks were recorded on a personal computer using the software Chrom-Card for Windows (Fisons CE Instruments), and were identified by comparison with known standards.

III. RESULTS

A. Biometrics of cysts and nauplii

The diameter of hydrated non-decapsulated and decapsulated cysts, the chorion thickness and the instar-I naupliar length of *Artemia* population harvested from Sabkhet Halk El-Menzel (HM) are summarized in Table I. Fig 2 shows the size frequency distribution of non-decapsulated cysts and nauplii length. The mean value of the diameter of non-decapsulated cysts is 235.8 µm, while for the decapsulated cysts it is 226.3µm. Chorion thickness mean values was 4.75 µm, and naupliar length was measured from 336 to 488 µm, with mean values of 426.8 µm.

TABLE I
 BIOMETRICS OF CYSTS AND NAUPLII OF THE INVASIVE *ARTEMIA* FROM SABKHET HALK EL-MENZEL.

	Mean (SD)	Range
Diameter of hydrated non-decapsulated cysts (µm)	235.8 (10.8)	205.3-254.2
Diameter of hydrated decapsulated cysts (µm)	226.3 (11.6)	195.6-244.4
Chorion thickness (µm)	4.75	-
Nauplii instar-I length (µm)	426.8 (32.8)	336.0-488.0

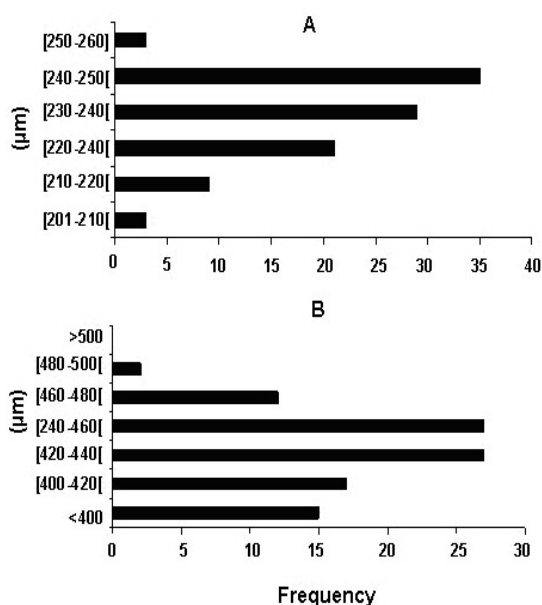


Fig. 2 Size frequency distribution, A: hydrated non-decapsulated cysts; B: nauplii.

B. Adult's dimorphism

Table II shows the loadings of the morphometrical parameters used to determine the sexual dimorphism for adult *Artemia* from HM. Principal Components Analysis (PCA) revealed two main directions of variation, with axis 1 explaining 35.47 % and axis 2 explaining an additional 25.71 % of the total variance. Relatively to the first component (PCA axis 1), maximal distance between compound eyes (*dby*), diameter for compound eyes (*dy*), length of first antenna (*la*) and the abdomen length compared to the total body length ratio (*ra*, %) are the most important variables for males and females discrimination with a total contribution of 62.39 %. Whereas, for the second component (PCA axis 2), length of furca (*lf*), width of head (*wh*), width of third abdominal segment (*wts*), number of setae inserted on left branch of the furca (*nlf*) and number of setae inserted on right branch of the furca (*nrf*) are the most important variables with a total contribution of 74.69 %.

The ACP divided adult specimens in 2 evident groups (Fig 3). Variables were most correlate with factor 1 (horizontal axis) than with factor 2 (vertical axis). Positive loadings of maximal distance between compound eyes (*dby*), diameter for compound eyes (*dy*) and length of first antenna (*la*) were observed relatively to the principal component 1, whereas the abdomen length compared to the total body length ratio (*ra*, %) show negative loadings.

C. Fatty acids profile

Table III shows the fatty acid composition of the invasive *Artemia franciscana* decapsulated cysts collected from Sabkhet Halk El-Menzel. The predominant fatty acids were the palmitic acid (C16:0), palmitoleic acid (C16:1n-7), oleic acid (C18:1n-9) and *cis*-vaccenic acid (C18:1n-7), with 12.92, 13.3, 12.34 and 13.79 mg.g⁻¹ dry weight. Moreover, results exhibited high levels of eicosapentaenoic acid (EPA, C20:5n-3) but low levels of linolenic acid (LLA, C18:3n-3) with 11.10 and 3.11 %, respectively. Docosahexaenoic acid (DHA, 22:6n-3) was found in less quantity with 0.17 mg.g⁻¹ dry weight. Concerning arachidonic acid (ARA, 20:4n-6) decapsulated cysts from HM exhibit a low quantity with 1.73 mg.g⁻¹ dry weight.

IV. DISCUSSION

The presence of *A. franciscana* in the Sabkhet Halk El Menzel might possibly be explained by a new introduction of the species to this site (albeit that, of course, we may have missed *Artemia* specimen in our previous sampling). However, the time and the origin of the introduction of this invasive species in HM are completely unknown, and could have two explications: natural introduction via waterbirds or by human impact.

Focusing on non-decapsulated cysts, the comparison between this result and other Tunisian *Artemia salina* population showed that cysts harvested from HM are similar to cysts harvested from Sfax saltworks (235.4 µm, [24]), but smaller than cysts from Sabkhet Sijoumi (260.9 µm, [25]) and from Sabkhet El Adhibet (258.1-263.7 µm, [26]).

TABLE II
 CONTRIBUTION AND PORTION OF EACH MORPHOMETRICAL PARAMETERS USED TO THE SEXUAL DIMORPHISM OF ADULTS
 ARTEMIA FROM SABKHET HALK EL-MENZEL IN THE TWO FIRST COMPONENTS.

	Variables contribution (%)		Component	
	F1	F2	F1	F2
Total length	8.690	8.509	-0.582	0.491
Abdominal length	6.445	5.929	-0.501	0.410
Width of 3 th abdominal segment	2.609	14.850	-0.319	0.648
Length of furca	2.581	19.466	0.317	0.742
Number of setae inserted on left branch of the furca	7.882	13.497	0.555	0.618
Number of setae inserted on right branch of the furca	9.388	12.338	0.605	0.591
Width of head	0.009	14.545	0.019	0.641
Diameter for compound eyes	17.791	1.842	0.833	0.228
Maximal distance between compound eyes	20.192	0.023	0.888	-0.026
Length of first antenna	14.093	0.806	0.742	-0.151
Abdomen length / total body length ratio (<i>ra</i> , %)	10.320	8.195	-0.635	0.481
Total variance explained				
% of variance	35.469	25.712		
Cumulative %	35.469	61.181		

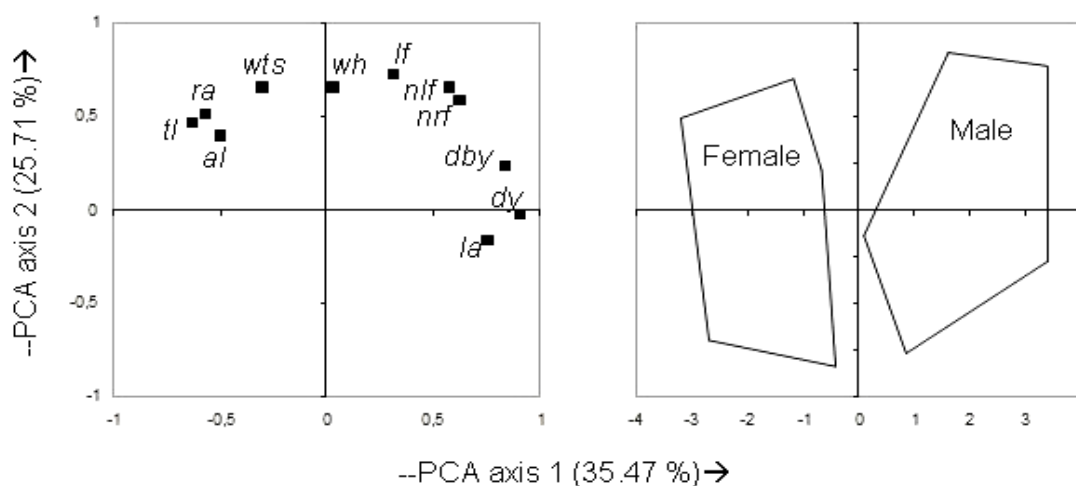


Fig. 3 Results obtained from the application of PCA to compare the morphological traits of adult males and females of the invasive *Artemia franciscana* specimens from Sabkhet Halk El-Menzel. Total length (*tl*), abdominal length (*al*), width of third abdominal segment (*wts*), length of furca (*lf*), number of setae inserted on left branch of the furca (*nlf*), number of setae inserted on right branch of the furca (*nrf*), width of head (*wh*), diameter for compound eyes (*dy*), maximal distance between compound eyes (*dby*), length of first antenna (*la*) and the abdomen length compared to the total body length ratio (*ra*, %).

TABLE III
 FATTY ACID METHYL ESTER ANALYSIS OF THE INVASIVE ARTEMIA DECAPSULATED CYSTS FROM SABKHET HALK EL-MENZEL

FAME	Area (%)	mg.g ⁻¹ dry weight	FAME	Area (%)	mg.g ⁻¹ dry weight
C14	1.69	1.28	C20	0.12	0.09
C14:1n-5	0.25	0.20	C20:1n-11	0.00	0.00
C15	0.67	1.06	C20:1n-9	0.45	0.37
C15:1	0.50	0.86	C20:1n-7	0.22	0.30
C16	16.34	12.92	C20:2n-6	0.60	0.17
C16:1n-7	16.20	13.30	C20:3n-6	0.16	0.17
C16:2	0.67	0.56	C20:4n-6	1.50	1.73
C17	0.68	1.08	C20:3n-3	0.28	0.70
C16:3	1.04	1.76	C20:4n-3	0.39	0.20
C16:4	0.17	0.16	C20:5n-3	11.10	12.20
C18	5.59	4.85	C22:5n-3	0.00	0.00
C18:1n-9	11.28	12.34	C22:6n-3	0.10	0.17
C18:1n-7	10.87	13.79	Somme	88.63	86.95
C18:2n-6	3.17	3.42	Total n-3	16.07	15.64
C18:3n-6	0.37	0.21	Total n-6	5.94	5.69
C18:3n-3	3.11	2.26	n-3/n-6	2.7	2.75
C18:4n-3	1.09	0.80	16:0/16:1	1.01	0.97

However, the comparison between HM and other *Artemia franciscana* cysts, demonstrated that HM cysts were smaller than those harvested from Bahia de Ohuira and Salinas of Hidalgo, Mexico (266.3 and 292 μm respectively, [27]), from Pozos Colorados, Colombian Caribbean (252.9 μm , [28]) and from Great Salt Lake, Utah (244.2-252.5 μm , [14]). Furthermore, cysts from HM were most similar to those from Tayrona and Kangaru Colombian Caribbean (233.4 and 236.8 μm respectively, [28]) and from San Pablo Bay, USA (235.6 μm , [14]), but bigger than cysts from San Francisco Bay, USA (223.9-228.7 μm , [14]) and 201.0 μm [28]. Based on the cysts classification reported by Vanhaecke and Sorgeloos [20], HM cysts (235.8 μm) belong to the small egg class.

The length of instar-I nauplii from HM is similar to those of *Artemia salina* from Chott Marouane 'Algeria' (428.7 μm , [29]) and Sfax 'Tunisia' (422.2 μm , [24]), and of *Artemia franciscana* from Kangaru 'Colombian Caribbean' (426.1 μm , [28]) and from San Francisco Bay, USA (428 μm , [14]). On the other hand, naupliar length from HM show a smallest length that Great Salt Lake *Artemia franciscana* nauplii (486 μm , [14]) which are one of the main sources of brine shrimp cysts for use in aquaculture. This feature makes the population from HM very attractive for commercial use.

In *Artemia*, morphological traits have been the basis to describe species and strains, although controversy exists on the way of selecting and using the suitable morphological traits as well as on the degree of their genetic or environmental determination [30]. External sexual dimorphism in *Artemia* is seen in the larger size and modified shape of the male antennae. The male has a penis on each side of the body, whereas the female has one median ovisac. Body size comparison of male and female belong to each bisexual *Artemia* species generally show a size sexually dimorphic which female individuals have larger body than males [31]. A size difference between sexes can be interpreted as a mating advantage because, so far as the *Artemia* breeding system is concerned, the female carries the male during copulation. For this reason the female needs a large body for this mating procedure and for surviving the mating process [32]. Zhou et al. [33] have shown that the overall percentage of correctly classified cases of females was a little higher than the male in *A. sinica* and *A. tibetiana* from China. The result of Camargo et al. [34] show that the classification based on male characters provides better group membership than females of *A. franciscana* populations from the Colombian Caribbean. Asem and Rastegar-Pouyani [35] shows that morphological differentiation between male is higher than female samples of *A. urmiana*. In our case, PCA showed that the main morphological differences between males and females of adults *Artemia* from HM were the maximal distance between compound eyes (*db*), diameter for compound eyes (*dy*), length of first antenna (*la*) and the abdomen length compared to the total body length ratio (*ra*, %).

The biochemical composition of *Artemia* sp., in particular the fatty acid profiles of cysts (dehydrant dormant eggs) and nauplii, has been extensively studied because of the use of nauplii as live food in aquaculture [11]. Several studies have shown the important role of essential fatty acids (EFA), such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic

acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20: 4n - 6) in larval fish nutrition [36-37]. It is known that the fatty acid composition of *Artemia* nauplii can vary among strains and also from one batch to another within the same strain [38]. This led Watanabe et al. [39] to classify *Artemia* into two groups: freshwater type *Artemia*, with n-3 unsaturated fatty acids such as linolenic acid (18:3n-3) but lacking eicosapentaenoic acid (20:5n-3), producing good survival and growth among freshwater animals, and marine-type *Artemia* whose lipids contain 20:5n-3 thereby making them suitable for feeding marine animals. Based in this classification *Artemia* from HM can be considered as marine-type *Artemia* thus suitable for feeding marine animals. The comparison of the most common fatty acids between FAME of decapsulated cysts from HM and those from GSL [13] revealed that cysts from HM present a higher quantity on palmitoleic acid and eicosapentaenoic acid, whereas cysts from GSL were much higher on oleic acid, linoleic acid and linolenic acid.

The difference in the fatty acids profile established, in this work, between the invasive *Artemia franciscana* from HM and those reported in the bibliography, can be considered as an adaptation of this specie in their new environment. Most invasive aquatic species are characterized by adaptations to the new environment, leading to rapid population growth supported by high fecundities [40-2]. The usefulness of lipid as an index of potential reproductive capacity was confirmed by Holm and Shapiro [41] in freshwater cladoceran populations. However, the studies of the variation in the fatty acid profiles have only focused on the nutritional condition of the animals, while their relationship with the population's reproductive performance is still unknown and requires further investigation [42]. Several authors reported that fatty acid composition of *Artemia* is considered to be more environmentally than genetically determined, and that *Artemia* adults and the cysts they produce strongly reflect the fatty acid profile of their diet [11-43]. In fact, some fatty acids, or groups of them, are preferentially associated with certain classes of algae, and can therefore be useful as biomarkers for these algal classes whose presence (diversity) and growth may in turn vary depending on the characteristics of the medium in which they live [44]. However, Navarro and Amat [45] brought attention to a possible genotypic influence on the fatty acid profile of *Artemia*, given the presence and proportion of some fatty acids in cysts irrespective of dietary levels available to parental populations.

On the other hand, in view of the importance of the DHA and ARA in aquaculture, it's essentially to signal that, such as for the commercial cysts from GSL, *Artemia* from HM must be enriched by these two fatty acids before their use in aquaculture.

V. CONCLUSION

The study of the invasive *Artemia franciscana* from Sabkhet Halk El-Menzel showed that the size of cysts and nauplii are among the smallest for *Artemia* sp. populations. Sexual dimorphism showed that the main morphological differences between males and females were observed for the maximal distance between compound eyes (*db*), diameter for

compound eyes (*dy*), length of first antenna (*la*) and the abdomen length compared to the total body length ratio (*ra*, %). Based on the high level in EPA and the low quantity in LLA, *Artemia* from HM can be considered as marine-type *Artemia*.

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