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Mixture of parthenogenetic and zygogenetic brine shrimp *Artemia* (Branchiopoda: Anostraca) in commercial cyst lots from Great Salt Lake, UT, USA

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Abstract

The brine shrimp *Artemia* is one of the most studied animals in the world. A large part of the knowledge of this crustacean is based on cysts harvested from two main sources; the Great Salt Lake, UT (GSL), and the San Francisco Bay salterns, CA (SFB), USA. *Artemia* populations from these habitats are recognized to belong to a single zygogenetic species, *Artemia franciscana* Kellogg, 1906. However, the GSL *Artemia* has been in doubt for more than a century about the existence of parthenogenetic reproduction. By using morphological, reproductive, and molecular analyses, we report that commercial GSL cyst lots contained two different brine shrimp species; a parthenogenetic (60%) and a zygogenetic (*A. franciscana*) (40%). From this finding, at least three hypotheses can be drawn. The parthenogenetic *Artemia* is native of GSL, or it was introduced to GSL, or foreign parthenogenetic cysts were mixed with *A. franciscana* cysts and canned for commercial distribution. Researchers using brine shrimp cysts from GSL should therefore pay careful attention to the correct identity of the species under study. The potential of an easy and unnoticed introduction of parthenogenetic *Artemia* into America is discussed.

Keywords: Parthenogenesis; Co-occurrence; New world; Exotic; Autochthonous; 16S rRNA mtDNA

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1. Introduction

Brine shrimp *Artemia* can reproduce sexually (zygogenetically), i.e. there are separate sexes and an obligated mating between a female and a male is needed to have offspring, or parthenogenetically, where females do not mate with males and their offspring consist almost exclusively of females and, on rare occasions, males (Charniaux-Cotton, 1960).

In Europe, Asia, Africa, and Australia, both zygogenetic and parthenogenetic *Artemia* species occur, whereas in the New World only zygogenetic forms are recognized (Criel and MacRae, 2002). However, the *Artemia* population from the Great Salt Lake (GSL), UT, USA, has been in doubt for more than a century about the existence of parthenogenetic reproduction. The first suggestion was made at the end of the 19th century (Packard, 1883; von Siebold, 1883). In the beginning of the 20th century, it was reported that GSL brine shrimp females could reproduce uniparentally as well as biparentally (Jensen, 1918). However, in the early 1960s, individually isolated virgin females were tested for parthenogenesis and offspring were not observed (Bowen, 1962). In 1990, it was again suggested that there was a switch from bisexuality to parthenogenetic reproduction during the year in the GSL (Cuellar, 1990). Currently, the GSL brine shrimp is recognized as the zygogenetic *Artemia franciscana* Kellogg, 1906, and old discrepancies have not yet been clarified.

Here, we report commercial GSL cyst lots contained a mixture of two different brine shrimp species; a parthenogenetic and the zygogenetic *A. franciscana*.

2. Materials and methods

2.1. Brine shrimp cyst sources

We used commercial cysts from two recognized brands. Cans of brand 1 indicated they contained genuine cysts harvested from GSL, UT, USA, and cans of brand 2 indicated they contained genuine cysts harvested from SFB. Both brands showed a 75–80% cyst hatching-rate/24 h at 27 °C.

2.2. First trial using GSL brine shrimp cysts

In the first trial, cysts of brand 1 (0.03 g; 4 replicates) were placed for hatching in cylindrical-transparent plastic containers (8-cm long, 4-cm diameter) with 150-μm mesh at both ends. Each container was placed inside a 600-ml glass beaker with aerated sea water at 38 g/l salinity. Glass beakers were placed inside a water bath at 27 ± 0.2 °C for 24 h. Two 40-Watt fluorescent lamps were fitted above the water bath to provide continuous artificial light to the cysts. From each of the four incubation replicates, 100 swimming nauplii were randomly collected and transferred to a 4-l plastic container with aerated sea water at 38 g/l salinity kept in a water bath at 27 ± 0.5 °C. Nauplii were grown for 12 days to reach adult stage, feeding them twice a day with a mixture of *Chaetoceros* sp. and *Isochrisis* sp. microalgae maintaining a concentration of 200–400 × 10^3 cells/ml. A 100%

water exchange was made every 3 days by gently passing *Artemia* through a 200-μm mesh net, cleaning the container, refilling again with fresh marine water and microalgae at 27 °C, and then returning *Artemia* again to the container. Salinity and pH were maintained at 38 g/l and 8.1. On day 12, survival was obtained for each replicate. Surviving *Artemia* were anesthetized with seawater-sparkling mineral water (1:1), fixed in 70% ethanol, and finally deposited in the Collection of Crustacea at CIBNOR. Individuals were sexed by phenotypic sexual dimorphism; males were identified with the observation of the large second antennae and penes, females by the presence of the brood pouch. Gender ratios were analysed by a Chi-square test.

2.3. Second trial using GSL brine shrimp cysts

In a second trial, cysts of brand 1 (0.1 g) were incubated and swimming nauplii were transferred to a 40-l rectangular bathtub. Physical, chemical, and feeding conditions were the same as described in Section 2.2.

2.3.1. Separation and isolation of brine shrimp phenotypes

From day 6 to day 7 of the second trial, *Artemia* phenotypes identified by external morphology and colour were separated and isolated as follows: (1) 15 virgin bright-red females, (2) 15 virgin reddish females, (3) 15 virgin bright-red females together with 15 mature pale reddish males, (4) 15 virgin reddish females together with 15 mature pale reddish males, (5) 10 virgin reddish females together with 5 mature bright-red males, with groups 1 to 5 maintained in a 40-l bathtub, (6) 15 virgin bright-red females were individually isolated in 4-l containers, and (7) 15 virgin reddish females were individually isolated in 4-l containers.

2.3.2. Progeny of female phenotypes

Progeny from both types of females were raised with our standardized culture conditions and adults were sexed and identified morphologically.

2.3.3. Histology of gonads

Gonads of individuals of the four phenotypes were studied using histological preparations (haematoxylin and eosin) (Bell and Lightner, 1988). Slides were examined under an Olympus and a Zeiss light compound microscopes.

2.3.4. Molecular analysis

A molecular analysis of individuals of the four phenotypes was made. Total DNA was extracted using 5% Chelex. A fragment of the large subunit of mitochondrial ribosome (16S rRNA) DNA was amplified using primers 16Sar and 16Sbr (Palumbi et al., 1991). Sequences were obtained by using an ABI prism 310 Genetic Analyzer.

2.4. Third trial using SFB brine shrimp cysts

Artemia cysts of brand 2 (SFB) were incubated and cultured using the same conditions as described in Section 2.3.

3. Results

3.1. First trial using GSL brine shrimp cysts

3.1.1. The gender ratios

The gender ratios were significantly skewed to females (average: 94% females and 6% males) with the four replicates not being significantly different and having an overall survival of 82%.

3.1.2. Colour of phenotypes

From day 1 to about day 8, all individuals were reddish, however from about day 9 and onwards, most of the females were bright-red (Fig. 1, top left), whereas some females remained reddish (Fig. 1, top right), and males looked mostly pale (Fig. 1, bottom right). From about day 9 and onwards, males started to couple preferentially with reddish females, and by about day 12, most of reddish females had embryos inside the brood pouch, whereas the bright-red females looked thinner and immature. Additionally two distinguishable bright-red males were observed, both of which were sexually active shown by their chasing and clasping both types of females (Fig. 1. bottom left).

3.1.3. Morphology of phenotypes

Observations of each phenotype using a stereomicroscope revealed remarkable morphological differences between females and between males, e.g. bright-red females have longer antennules (first antennae) (Fig. 2, top), abdomens, and cercopods than the reddish females; bright-red males have a semi rectangular distal joint of claspers (second antennae), while pale males have the claspers with a typical triangular distal joint (Fig. 2, bottom). Detailed comparative description of their morphology will be published elsewhere.

3.2. Second trial using GSL brine shrimp cysts

3.2.1. Proportions of phenotypes

From a random sample of 1000 individuals, four individuals were bright-red males (0.4%), 620 were bright-red females (62%), 182 were reddish females (18%), and 194 were pale males (19%). Reddish females and pale males had a sex ratio not significantly different from 1:1 ($X^2 = 0.38$).

3.2.2. The greenish colour phenotype

At about 25 days, we observed that some reddish females and pale males turned greenish. This phenotype did not seem to affect the formation of couples because greenish females coupled with pale males and greenish couples were also formed. However, the green colour vanished gradually over 2 weeks.

3.2.3. Type of reproduction of phenotypes

Virgin bright-red females separated in a bathtub and isolated in 4-l containers reproduced several times uniparentally. From about day 15, bright-red females initially

Fig. 1. Colour of *Artemia* individuals obtained from commercial Great Salt Lake cysts. Parthenogenetic bright-red female (top left) and bright-red male (bottom left), and zygogenetic reddish female (top right) and pale male (bottom right). Scale bar = 5 mm.

gave birth to nauplii and later some of them began to produce cysts. Virgin reddish females separated in a bathtub and isolated in 4-l containers never reproduced uniparentally; females just emptied their brood pouches of oocytes or nonviable eggs. In contrast, virgin reddish females together with pale males formed couples and reproduced sexually. They initially gave birth to nauplii and afterwards began to produce cysts.

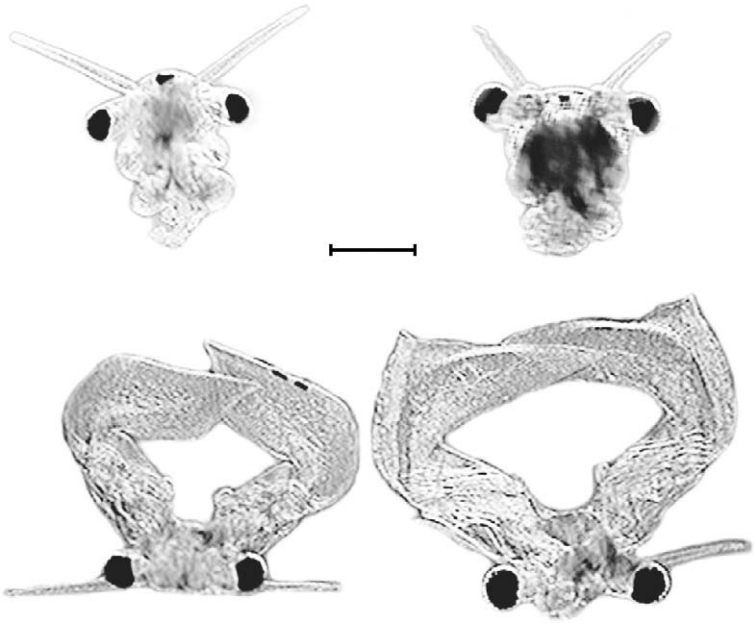

Fig. 2. Head morphology of *Artemia* individuals obtained from commercial Great Salt Lake cysts. Parthenogenetic bright-red female (top left) and bright-red male (bottom left), and zygogenetic reddish female (top right) and pale male (bottom right). Scale bar = 1 mm.

3.2.4. Progeny of female phenotypes

Group 1: progeny from 15 virgin bright-red females ($n = 1000$) consisted of females morphologically identical to dams, along with three bright-red males.

Group 2: no progeny from 15 virgin reddish females was observed.

Group 3: progeny from 15 virgin bright-red females kept with 15 mature pale reddish males ($n = 1000$) consisted of females identical to dams, plus four bright-red males.

Group 4: progeny from 15 virgin reddish females kept with 15 mature pale reddish males ($n = 1000$) consisted of females (507) and males (493) morphologically identical to the progenitors and the gender ratio was not significantly different from 1:1 ($X^2 = 0.19$).

Group 5: no progeny from 10 virgin reddish females kept with five mature bright-red males was observed.

Group 6: progeny from 15 virgin bright-red females individually isolated ($n = 40 - 120$) consisted of females morphologically identical to each dam, and from one dam, a bright-red male out of 111 females was observed.

Group 7: no progeny from 15 virgin reddish females individually isolated was observed.

3.2.5. Histology of gonads of phenotypes

Light microscopy of gonads revealed neither a histological difference in ovary between the two types of females ($n = 12$ each phenotype) nor in the testes between the two types of males ($n = 6$ each phenotype).

3.2.6. Molecular genetics of phenotypes

Genetic identity analysis of phenotypes revealed the same base sequence for reddish females and pale males with 487 base pairs (GenBank accession number: AY327247) and the same base sequence for bright-red females and bright-red males with 488 base pairs (GenBank accession number: AY327248). These two sequences differed from each other by 13%. Blast search indicated that the reddish female and pale male sequence was identical to the sequence of the New World *Artemia franciscana*, South San Francisco Bay (Accession number: AF202740) and southern end of the GSL (Accession number: AF202741). The bright-red female and male sequence was identical to the sequence of parthenogenetic Old World *Artemia* from Gulf of Kutch, India (Accession number: AF202761), and 97% to 99% similar to other Old World parthenogenetic populations from India, Australia, and France.

3.3. Third trial using SFB brine shrimp

Artemia individuals of brand 2 (SFB) were identical in morphology and colour to reddish females and pale males from GSL. In both progenitors and offspring ($n = 1000$ adult individuals; 518 females and 482 males, and 1000 adult individuals from the first generation; 479 females and 521 males), the gender ratio was not significantly different from 1:1 ($X^2 = 1.29$ and $X^2 = 1.76$).

4. Discussion

Any offspring from gonochoric progenitors is expected to have nearly half females and half males, just as the SFB brand showed. In our first trial using the GSL brand, it was observed that the gender ratios were significantly skewed to 94% females. This result prompted us to analyse adult brine shrimp in more detail. Thus, a simple sex ratio analysis can be a preliminary test to reveal a mixture of parthenogenetic and zygogenetic brine shrimp cysts.

The colour variation in the brine shrimp may depend on the combination of type of diet and dissolved oxygen available in the water (Bowen et al., 1969). The colour of alga-fed animals under standard conditions helped us, in great extent, to identify and separate the phenotypes. These phenotypes fully agree with Packard's (1883) description of individuals at GSL.

After we found the morphological differences between coloured phenotypes, our first thought was that bright-red females and red males could be intersexes, however, light microscopy of gonads dismissed this notion.

After confirming the two types of reproduction and the analysis of progeny of experimental groups, our first conclusion was that there were two different species cooccurring in the commercial lots from GSL, a zygogenetic and a putative parthenogenetic. With the genetic identity analysis (mtDNA), there was no doubt that our zygogenetic species corresponds to the taxonomic species *A. franciscana* of SFB and GSL; however, it was surprising to see that the 16S fragment of our putative GSL parthenogenetic brine shrimp was identical to the Old World parthenogenetic brine shrimp reported from Gulf of Kutch, India.

The results of this study are in agreement with field and experimental observations made during more than a century on GSL brine shrimp (Packard, 1883; von Siebold, 1883; Jensen, 1918; Cuellar, 1990), i.e. the skewed gender ratios toward females, the colour variation, the mating behaviour, and the suggestion of bisexual and parthenogenetic reproduction.

From these results, at least three hypotheses can be drawn: the parthenogenetic *Artemia* is native of GSL, or it was introduced to GSL, or foreign parthenogenetic cysts were mixed with *A. franciscana* cysts and canned for commercial distribution. Given the obvious implications of this report on academic and ecological issues, an urgent research project at GSL is needed to test these hypotheses. If the second or third hypotheses are true, a serious ecological problem could exist by provoking an easy and unnoticed undesirable introduction of a parthenogenetic species. Triantaphyllidis et al. (1994) presented evidence of accidental introduction of *A. franciscana* to the Old World brine shrimp populations through the importation of brine shrimp cans from America, mainly from the GSL. The same scenario could occur in America because marine fish and shrimp larvae are fed mainly with brine shrimp from GSL and SFB. Meanwhile, until this is resolved, researchers using brine shrimp cysts from GSL should therefore pay careful attention to the correct identity of the species under study. We are sure that our study will help workers around the world easily identify each species.

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