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Effects of microalgal diet changes on resting egg surface morphology in *Artemia franciscana* Kellogg, 1906 from Chile

by

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Abstract

Resting egg surface morphological variations usually occur among different *Artemia* species; however, in *Artemia franciscana* Kellogg, 1906 from Chile, which is a typical dweller of brackish and hypersaline inland lakes and coastal lagoons in America, the extent and the origin of these morphological variations are largely unknown. This study analyses the effects of two microalgal diets, *Dunaliella tertiolecta* Butcher, 1959, and *Tetraselmis suecica* (Kylin) Butcher, 1959, on the resting egg surface morphology of nine *A. franciscana* populations from northern and central Chile, including two reference populations. The scanning electron microscopy (SEM) was used to document the effect of microalgal dietary change on the resting egg surface morphology. Nine resting egg surface morphotypes across populations were observed, with the smooth (48.3%), rough (13.7%), and humped (9.3%) surfaces being the most common. The principal component (PCs) analysis revealed that the smooth (PC1) and rough (PC2) morphotypes explain a large proportion of surface morphotype variance (59.78%). The microalgal dietary change significantly modified the frequency of resting egg surface morphotypes in all populations ($p < 0.00001$). These results support a great variation in the resting egg surface morphotypes in *A. franciscana* populations from Chile, variability that may be affected by changes in the microalgal diet.

Key words: *Artemia*, resting eggs, surface morphology, microalgal diet, SEM

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1. Introduction

Brine shrimp *Artemia* is a small branchiopod crustacean characterised by a high tolerance to extreme salinity levels (e.g. 300 g · L^{-1}) (Amat et al., 1994; Asem et al., 2012; Browne & MacDonald, 1982; Clarke, 1924; Quiroz et al., 2015; Redón et al., 2021; Ruíz et al., 2007; Zúñiga et al., 1999) and the ability to survive in extreme conditions of water temperature and chemical composition (MacRae, 2016; Tanguay et al., 2004). This extraordinary adaptation is related to its oviparous reproductive mode, which allows females to produce resting eggs or embryos in a diapause phase, also called cysts, within an egg encapsulated by the chorion (Clegg, 2005; Jackson & Clegg, 1996), a shell that protects the embryos from the harsh environment for long periods.

Surface topology studies of various structures, such as organs or reproductive cells, with scanning electron microscopy (SEM) have demonstrated the technology as a powerful tool with wide application in taxonomy, species identification, and morphological research because SEM allows a detailed description of the structure due to its yield of high-resolution images (Ul-Hamid, 2018). In *Artemia*, the SEM technology has been useful for species separation of bisexual species based on the frontal knobs analysis (Mura & Brecciaroli, 2004), to identify invasive *Artemia* species in saltworks and coastal saltlands from Europe (Mura et al., 2006; Scalone & Rabet, 2013), and to recognise the two species from the New World based on distal retractile penis structures (Torrentera & Belk, 2002).

Since the 1970s, a number of studies using SEM have examined the chorion structure of *Artemia* resting eggs (Asem & Sun, 2014; Gilchrist, 1978; Mazzini, 1978; Munuswamy, 1988; Mura, 1986; Ramasubramanian & Munuswamy, 1993; Rosowski et al., 1997; Shepard & Hill, 2001; Spotte & Anderson, 1988; Sugumar & Munuswamy, 2006; Wang & Sun, 2007). These studies also include characterisations of the ultrastructure of resting eggshells by transmission electron microscope (Li et al., 2024; Sugumar & Munuswamy, 2006; Wang & Sun, 2007). In the case of *Artemia franciscana* Kellogg, 1906 from Chile a species native to the American continent, analysis of the resting egg surface morphology by using SEM has indicated that the topology of resting eggs is generally constant because most populations distributed in North America have a smooth surface morphotypes (Rosowski et al., 1997; Shepard & Hill, 2001; Spotte & Anderson, 1988). However, recent SEM analysis of several populations of other *Artemia* populations or species have shown that resting egg surface morphology is a highly variable character in the genus at the intraspecific or interspecific levels. For instance, the resting egg surface textures of *Artemia monica* Verrill, 1869 and some Indian *Artemia* populations vary from smooth to rough, and they are adorned with button-shaped patterns (Shepard & Hill, 2001; Sugumar & Munuswamy, 2006). In *Artemia salina* (Linnaeus 1758) at least three surface morphotypes have been recorded (Gilchrist, 1978; Mazzini, 1978; Morris & Afzelius, 1967; Munuswamy, 1988). Examinations of *Artemia* populations from China revealed a similar pattern because six resting egg surface morphotypes, including smooth, wart-like, rough, and a surface with density-spaced, pore-like fossulae, were found (Wang & Sun, 2007). Furthermore, 15 distinct surface patterns were found in populations of *Artemia* parthenogenetic lineages in China; the two most common morphotypes were rough and with widely spaced tubercles (Asem & Sun, 2014). In addition, Vesnina et al. (2024) also found a large number of resting egg surface morphotypes in four *Artemia* parthenogenetic lineages from Western Siberia, totalling 11, with one or two more common than all others, specifically, the rough and tubercle surfaces that appear to be characteristic of the parthenogenetic lineages. To date, few populations of *A. franciscana* have been studied using SEM (Rosowski et al., 1997; Shepard & Hill, 2001; Spotte & Anderson, 1988). Therefore, it is likely that the resting egg morphotype variability reported for this species might be underestimated. Therefore, further analyses of *A. franciscana* from new localities are required, especially from populations not yet investigated, such as those distributed in South America, to clarify the extent of resting egg surface variability in the species.

The origin of resting egg surface morphology variation is largely unknown in *Artemia*. However, it has been observed that the resting egg surface morphotypes are related to the strain's origin, as Sugumar and Munuswamy (2006) observed for Indian strains. Given the distinctive resting egg surface morphology observed in some populations from China, it has been suggested that this character may be a useful tool for identifying certain *Artemia* strains (Asem & Sun, 2014). Since the resting egg size and chorion thickness are highly variable characters among populations, the resting egg surface morphology variability could be related to environmental factors of the *Artemia* habitats, such as salinity, temperature, food, and photoperiod (Amat et al., 2004; Castro et al., 2006; Esteve & El Masri, 2007; Hontoria, 1990; Vanhaecke & Sorgeloos, 1980). However, no experimental evidence of the role of these factors on the modification of the resting egg surface morphology has been provided. Nevertheless, there is evidence of the possible role of genetic factors because experimental inactivation of genes involved in the resting egg chorion formation in *Artemia* can change the resting egg surface morphology (Dai et al., 2011; Liu et al., 2009).

This study assess the effects of two microalgal diets on the resting egg surface morphology of nine *Artemia* populations from northern and central Chile, including two reference populations. We used SEM technology to examine the resting egg surface morphology to obtain a detailed characterisation of the chorion surface structure aimed at evaluating the effect of different microalgal diets. Samples of each population were obtained from experimental crosses carried out under controlled laboratory conditions to reduce the likelihood of experimental variability. The outcomes of this study provide, for the first time, morphological characterisations of the resting egg chorion from several *A. franciscana* populations in Chile to understand the origin of the intraspecific variability in the country.

2. Materials and methods

2.1. Studied populations

Nine *A. franciscana* populations from temporary and permanent coastal salt pools, salterns and inland saline lagoons of central and northern Chile (23°–34° S latitude), were examined (Table 1 and Fig. 1). Previous studies indicate that these habitats present a wide salinity variation ranging from 97 ppt to 301 ppt (Quiroz et al., 2015; Zúñiga et al., 1999). Live specimens were collected in 2011 and transported to the Laboratorio de Genética, Acuicultura y Biodiversidad at the Universidad de Los Lagos. The collected individuals were subsequently acclimated in 20-L capacity aquaria filled with artificial seawater at 35 ppt salinity (Kinne, 1970) and fed two microalgal diet treatments: (1) *Dunaliella tertiolecta* Butcher, 1959 (DUN) and (2) *Tetraselmis suecica* (Kylin) Butcher, 1959 (TETRA). We chose these microalgal species because they are considered among the best diets for *Artemia* culture (Balachandar & Rajaram, 2019). Two reference populations were included in the study. These comprised the Laguna Hidalgo population (LHI) from La Pampa, Argentina, which represents *Artemia persimilis* Piccinelli & Prosdocimi, 1968 (provided by Francisco Amat), and the San Francisco Bay population (SFB 1364) from the USA, which corresponds to *A. franciscana,* which was obtained from the *Artemia* Reference Centre (University of Ghent, Belgium).

2.2. Broodstock and culture conditions

In 2014, a broodstock for each collected Chilean *Artemia* population was established, taking adult

Table 1

Artemia population used in this study

Country	Locality	Code	Region	Geographical coordinates	Habitat type	Salinity (ppt)
Chile	De la Piedra (saline lagoon)	LPI	Antofagasta	23°05'S, 68°21'W	Inland	97[e]
	Cejar (saline lagoon)	CEJ	Antofagasta	23°03'S, 68°13'W	Inland	45[b]–292[c]
	Tebenquiche (saline lagoon)	TEB	Antofagasta	23°08'S, 68°15'W	Inland	180[c]–320[f]
	Burro Muerto (saline lagoon)	BMU	Antofagasta	23°26'S, 67°19'W	Inland	60[f]
	Barros Negros (saline lagoon)	BNE	Antofagasta	23°36'S, 68°15'W	Inland	132[f]–200[f]
	Copiapó (saline pool)	COP	Atacama	27°17'S, 70°56'W	Coastal	–
	El Convento (saltern)	CON	Valparaíso	33°48'S, 71°42'W	Coastal	320[f]
	Pichilemu-Cahuil (saltern)	PCH	Libertador General Bernardo O'Higgins	34°30'S, 71°59'W	Coastal	60[d]–252[e]
	Boyeruca (saltern)	BOY	Maule	34°41'S, 72°03'W	Coastal	110[g]
USA	San Francisco Bay 1364 (saltern)	SFB	California	37°32'N, 122°13'W	Coastal	318.2[a]
Argentina	Laguna Hidalgo (saline lagoon)	LHI	La Pampa	37°10'S, 63°32'W	Inland	300[d]

[a] Clarke (1924).
[b] Amat et al. (1994).
[c] Zúñiga et al. (1999).
[d] Ruíz et al. (2007).
[e] Quiroz et al. (2015).
[f] Redón et al. (2021).
[g] This work.

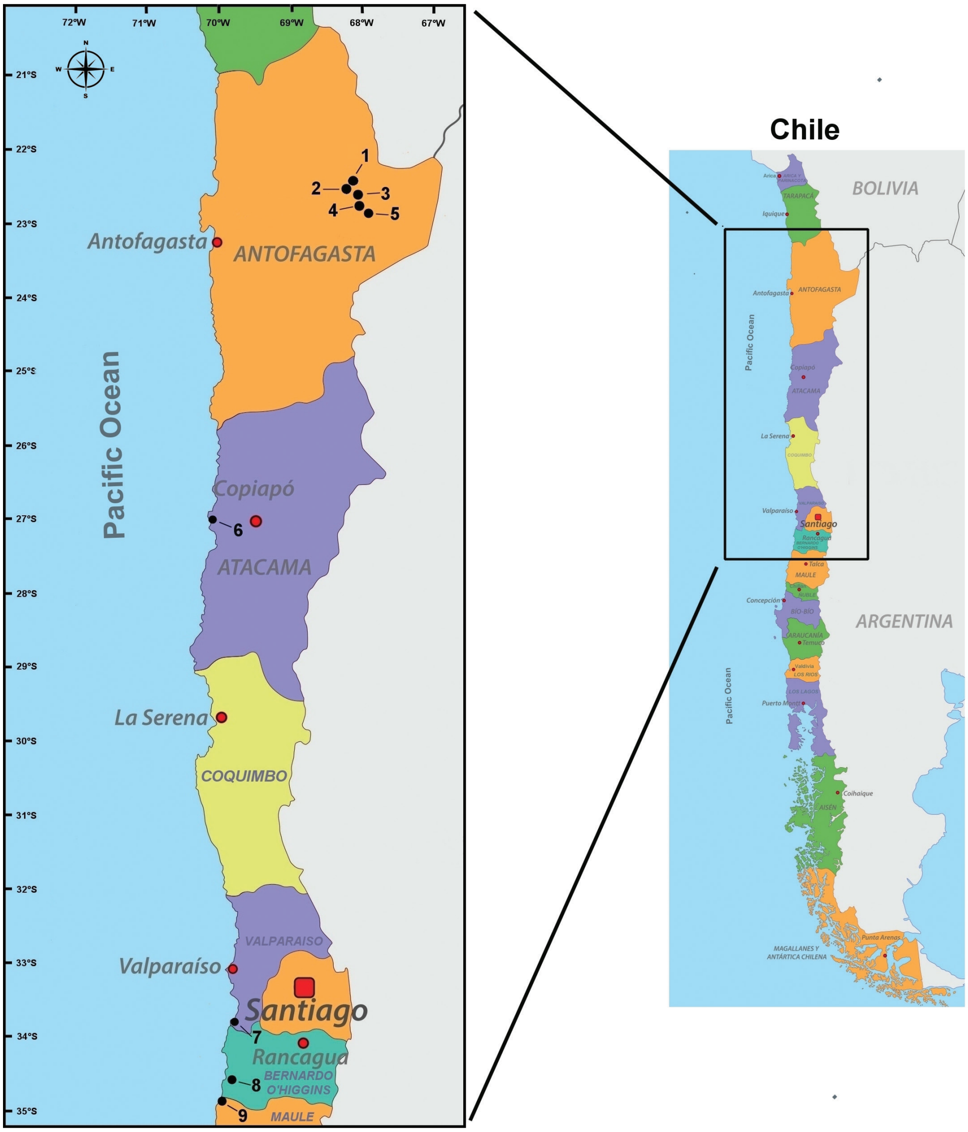

Figure 1

Map of collection sites of *Artemia franciscana* populations from central and northern Chile. Collection sites (numbers in boldface type) were as follows: (1) De la Piedra saline lagoon, (2) Cejar saline lagoon, (3) Tebenquiche saline lagoon, (4) Burro Muerto saline lagoon, (5) Barros Negros saline lagoon, (6) Copiapó saline pool, (7) El Convento saltern, (8) Pichilemu-Cahuil saltern, and (9) Boyeruca saltern.

individuals to be subsequently cultivated in separate aquaria (50 pairs per population). During the larval stage, the offspring from each population were cultured in two distinct aquaria, each containing one litre of seawater at 75 ppt salinity. The brine shrimp in one aquarium were fed DUN, and the brine shrimp in the other aquarium were fed TETRA. The individuals were cultured under these conditions until they expressed sexual dimorphism to proceed with the selection of males and females to establish the experimental crosses.

2.3. Experimental crosses and conditions

From 8 to 34 experimental crosses per population by mating one male with one female were established and cultured in individual flasks containing 100 ml of artificial seawater at 75 ppt salinity. The experimental pairs were fed daily for 45 days with *Dunaliella tertiolecta* (DUN) or *Tetraselmis suecica* (TETRA) at a concentration of 10^6 cells per flask (Gajardo et al., 2001). The flasks were kept under a constant photoperiod of continuous light (24L:00D), at room temperature ranging from 23°C to 26°C, and without artificial aeration.

2.4. Resting egg surface morphology determination

Brown resting eggs obtained from the *Artemia* crosses were collected and prepared using SEM by the following steps: (1) hydration in distilled water for 2 hr at room temperature; (2) washing in a 0.5 M formic acid ammonium salt solution for 30 min to remove surface salts; (3) immersion in 10% formalin for 30 min to remove microorganisms and debris attached to the chorion surfaces; (4) fixing in a 2.5% glutaraldehyde solution prepared in sodium cacodylate trihydrate (sodium phosphate) at pH 7.4 for 12 hr and subsequently washed in distilled water following Sivagnanam et al. (2013); and (5) drying at 30°C for 24 hr. A total of 10 and 47 visually clean and undamaged resting eggs per population were chosen for imaging. The resting eggs were coated with a 3 nm thick conductive gold-palladium layer for SEM with a ZEISS EVO 15 electronic microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at the Laboratorio de Microscopia Avanzada of the Universidad de Los Lagos. Photographs were taken at 800× magnification to capture the entire resting egg and again at 4000× magnification to enhance the resolution of the area of interest (Dai et al., 2011).

2.5. Statistical analysis

Fisher's exact test was applied to compare frequencies of resting egg surface morphotypes from individuals fed DUN and TETRA diets in each and the overall population. We chose Fisher's exact test because it is recommended for small sample sizes and therefore small cell frequencies are present in 2 × 2 contingency tables (Heumann et al., 2016). Fisher's exact test significance was determined by a simulated *p*-value by Monte Carlo simulation using 10 000 replications. Fisher's exact test for total resting egg surface morphotypes between diets was also performed. Confidence intervals (CIs) for the relative frequency of the resting egg surface morphotypes were calculated, with a 95% CI for the overall population according to the procedure of Clopper and Pearson (1934).

Principal component analysis (PCA) was performed using the absolute frequencies of the resting egg surface morphotypes of the studied populations. This multivariate method enabled us to identify a subset of variables defined by the principal components (PCs) by reducing the dimension of the data matrix obtained through linear combinations (Härdle & Simar, 2015). The eigenvectors and eigenvalues were derived from the PCA correlation matrix, which allowed the original variables to be reduced to a small number of components that captured most of the total variance. Then, a biplot was constructed to visualise the morphological pattern outcome. All statistical analyses were performed using R Studio Team (2017) version 4.3.1.

3. Results

In the overall population, nine resting egg surface morphotypes were identified using SEM (Fig. 2): Type I: smooth surface without decorations; Type II: smooth surface with sparsely distributed wart-like knobs; Type III: smooth surface with grouped fibres forming sparsely distributed conglomerates; Type IV: spongy surface; Type V: rough surface; Type VI: smooth surface with shallow depressions; Type VII: surface with evenly distributed humps; Type VIII: surface with evenly distributed, small granules; and Type IX: surface with small, dense, pore-like pits. The most common surface morphotypes in the overall population were Type I smooth (45.6%, 95% CI = 41.7–49.4), Type V rough (17.3%, 95% CI = 14.5–20.3), and Type VII humped (11.1%, 95% CI = 8.8–13.7) (Table 2). The same three dominant morphotypes were observed for the populations fed DUN and TETRA. The overall Chilean population showed a similar pattern of smooth (48.3%, 95% CI = 44.0–52.5), rough (13.7, 95% CI = 10.9–16.8), and humped (9.3%, 95% CI = 7.0–12.0) morphotypes. The overall population presented from 1 to 4 and from 1 to 5 surface morphotypes for DUN and TETRA diets, respectively, while the Chilean *Artemia* populations showed between 1 and 4 morphotypes with both diets (Fig. 3). Notably, the morphotype Type III (Fig. 2C) was rare because it was only present in two Chilean populations, namely TEB and LPI. The SFB population showed from 2 to 4 morphotypes, among which Type V morphotype was highly frequent with the DUN (22/28 resting eggs) and TETRA (20/36 resting eggs) diets. The LHI population showed from 2 to 3 morphotypes, and the Type I morphotype was highly frequent with both diets (DUN = 12/19 resting eggs and TETRA = 24/46 resting eggs).

There were significant differences in the frequency of resting egg surface morphotypes between diets in the overall populations according to Fisher's exact test ($p < 0.00001$) (Table 3). The same significance level was observed for each of the nine populations, indicating that diet affected the proportion of each resting egg surface morphotype across the studied populations. However, across different populations, the total resting egg surface

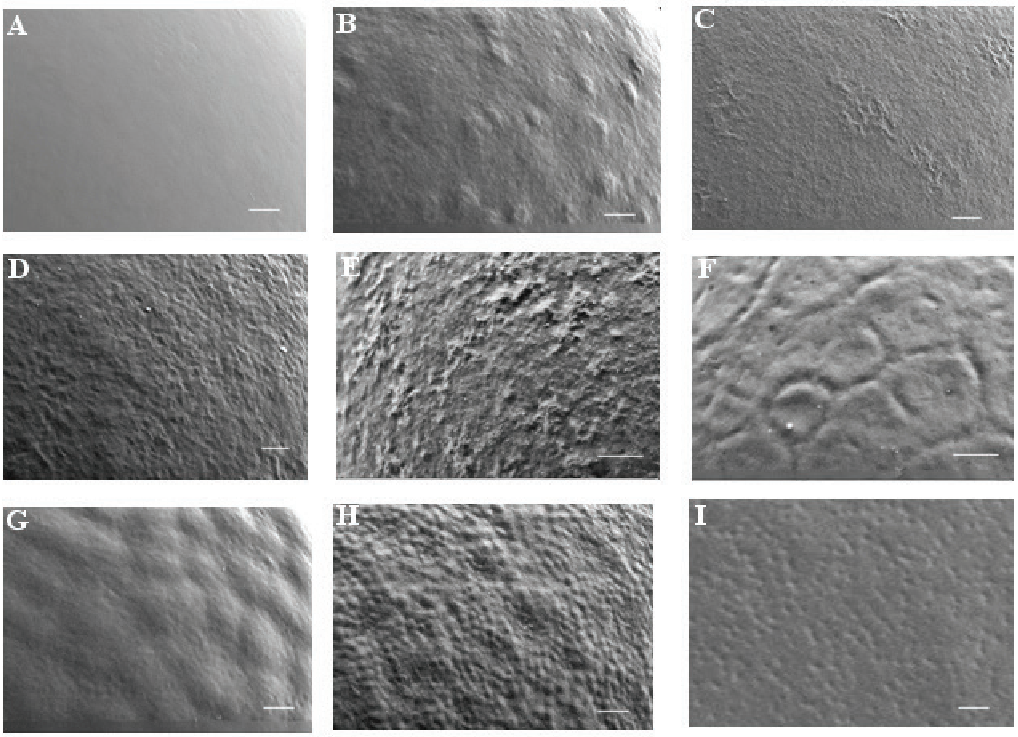

Figure 2

Resting egg surface morphotypes recorded using SEM analysis in *Artemia franciscana* populations from Chile. **(A)** Type I (smooth surface without decorations); **(B)** Type II (smooth surface with sparsely distributed wart-like knobs); **(C)** Type III (smooth surface with grouped fibres forming sparsely distributed conglomerates); **(D)** Type IV (spongy surface); **(E)** Type V (rough surface); **(F)** Type VI (smooth surface with shallow depressions); **(G)** Type VII (surface with evenly distributed humps); **(H)** Type VIII (surface with evenly distributed, small granules); and **(I)** Type IX (surface with small, densely distributed pore-like pits). SEM images at 4000× magnification. Scale bar scale in **A**, **B**, **C**, **D**, **G**, **H**, and **I** = **5** μm; scale bar in **E** and **F** = **10** μm. SEM, scanning electron microscopy.

morphotypes between diets did not result in statistically significant differences in this parameter ($p > 0.05$).

The PCA indicated that the first two components accounted for 59.78% of the variation; therefore, they were particularly important in the separation of the clusters (Table 4). PC1 was strongly influenced by the smooth morphotype (Type I), which accounted for 41.61% of the variation, and with a high weight of 0.889. However, PC2 was most influenced by the rough morphotype (Type V), with a weight of 0.765, which accounted for 18.18% of the variance. The humped morphotype (Type VII) contributed negatively to this component with a weight of −0.462.

The biplot showed that the first two PCs contributed to the formation of four distinct clusters (Fig. 4). The first cluster, which accounted for 79.11% of the overall variation, was related to the smooth morphotype (Type I) and grouped four populations fed TETRA (CON-TETRA, CEJ-TETRA, LPI-TETRA, and PCH-TETRA) along with three populations fed DUN (BNE-DUN, LPI-DUN, and PCH-DUN). The second cluster, which included two populations fed DUN (BOY-DUN and CON-DUN) as well as one population fed with DUN and TETRA (SFB-DUN and SFB-TETRA), was associated with the rough morphotype (Type V) and accounted for 58.64% of the overall variation. The third cluster, which included two populations fed TETRA (BOY-TETRA and COP-TETRA) in addition to three populations fed DUN (BMU-DUN, TEB-DUN, and COP-DUN), was associated with the humped morphotype (Type VII) and accounted for 21.37% of the overall variation. There was no association of the fourth cluster with any particular morphotype. Thus, the resting egg surface morphotypes I, V, and VII captured most of the total variance of the analysed *Artemia* populations.

Table 2

Absolute and relative frequencies of resting egg surface morphotypes of *Artemia* populations fed with two microalgal diets

Populations	Resting egg surface morphotype classes										Sample size	Total morphotypes (range)
	Diet	Type I	Type II	Type III	Type IV	Type V	Type VI	Type VII	Type VIII	Type IX		
LPI	DUN	25	0	0	4	0	0	0	0	0	29	2
	TETRA	25	0	6	0	0	0	0	3	0	34	3
CEJ	DUN	9	11	0	0	4	0	2	0	0	26	4
	TETRA	31	0	0	0	0	0	0	0	0	31	1
TEB	DUN	3	0	15	2	0	0	0	0	0	20	3
	TETRA	20	0	0	7	11	0	0	2	1	41	5
BMU	DUN	6	4	0	0	8	0	18	0	0	36	4
	TETRA	10	4	0	0	1	0	5	0	0	20	4
BNE	DUN	24	0	0	0	0	0	0	0	1	25	2
	TETRA	19	0	0	0	0	22	0	0	0	41	2
COP	DUN	0	0	0	0	0	0	22	0	0	22	1
	TETRA	9	0	0	27	0	0	0	0	0	36	2
CON	DUN	0	0	0	0	25	0	0	0	0	25	1
	TETRA	29	0	0	0	14	0	0	0	0	43	2
PCH	DUN	27	17	0	0	0	0	3	0	0	47	3
	TETRA	25	6	0	0	0	0	1	0	0	32	3
BOY	DUN	0	0	0	0	12	17	0	0	0	29	2
	TETRA	2	0	0	8	0	0	0	0	0	10	2
SFB	DUN	6	0	0	0	22	0	0	0	0	28	2
	TETRA	2	7	0	0	20	0	7	0	0	36	4
LHI	DUN	12	0	0	0	0	0	0	7	0	19	2
	TETRA	24	0	0	5	0	0	17	0	0	46	3
Chilean populations	DUN	94	32	15	6	49	17	45	0	1	259	8 (1–4)
	TETRA	170	10	6	42	26	22	6	5	1	288	9 (1–4)
	DUN + TETRA	264	42	21	48	75	39	51	5	2	547	9 (1–4)
	%	48.3	7.7	3.8	8.8	13.7	7.1	9.3	0.9	0.4		
	95% CI	44.0–52.5	5.5–10.2	2.3–5.8	6.5–11.4	10.9–16.8	5.1–9.6	7.0–12.0	0.2–2.1	0.0–1.3		
Overall	DUN	112	32	15	6	71	17	45	7	1	306	9 (1–4)
	TETRA	196	17	6	47	46	22	30	5	1	370	9 (1–5)
	DUN + TETRA	308	49	21	53	117	39	75	12	2	676	9 (1–5)
	%	45.6	7.2	3.1	7.8	17.3	5.8	11.1	1.8	0.3		
	95% CI	41.7–49.4	5.4–9.4	1.9–4.7	5.9–10.1	14.5–20.3	4.1–7.8	8.8–13.7	0.9–3.0	0.0–1.0		

Population codes are presented in Table 1. DUN = *D. tertiolecta*, TETRA = *T. suecica.*
Type I: smooth surface without decorations.
Type II: smooth surface with sparsely distributed wart-like knobs.
Type III: smooth surface with grouped fibres forming sparsely distributed conglomerates.
Type IV: spongy surface.
Type V: rugged surface.
Type VI: smooth surface with shallow depressions.
Type VII: surface with evenly distributed humps.
Type VIII: surface with evenly distributed small granules.
Type IX: surface with small, densely distributed pore-like pits.
CI, confidence interval.

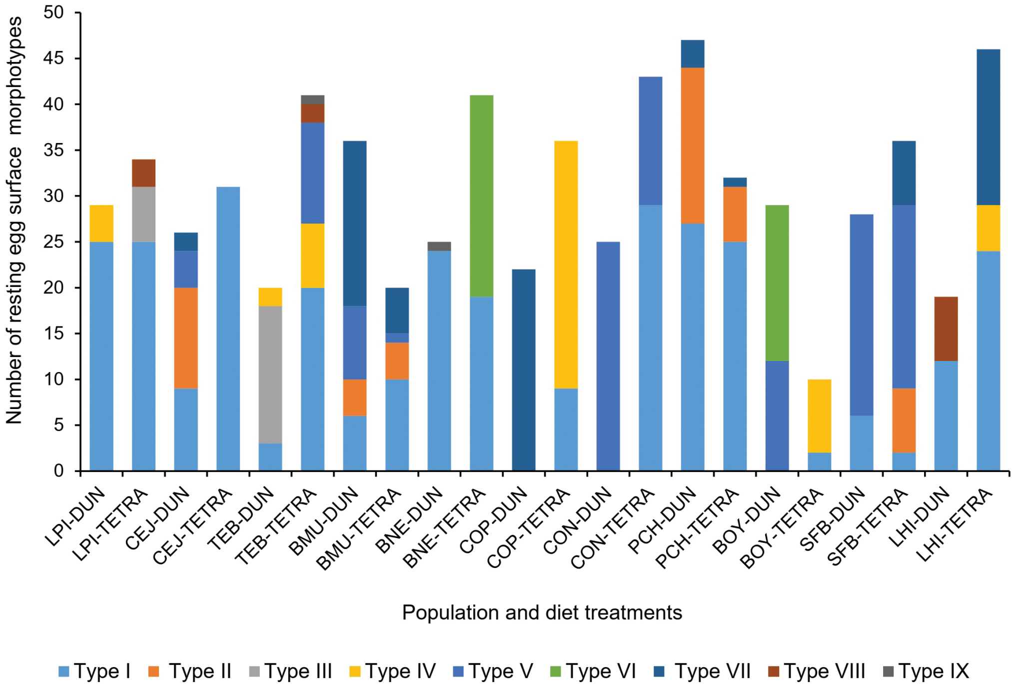

Figure 3

Absolute frequency of resting egg surface morphotypes observed by population and diet treatments. *Dunaliella tertiolecta* diet denoted as DUN; *Tetraselmis suecica* diet denoted as TETRA. Population codes are defined in Table 1.

Table 3

Fisher's exact test for the difference in the absolute frequencies of resting egg surface morphotypes in *Artemia* populations fed with two microalgal diets

Population	Between surface morphotype classes	Between total surface morphotypes
	p	*p*
LPI	0.0001^{**}	1.0000^{NS}
CEJ	0.0001^{**}	0.2941^{NS}
TEB	0.0001^{**}	0.6342^{NS}
BMU	0.0001^{**}	1.0000^{NS}
BNE	0.0001^{**}	1.0000^{NS}
COP	0.0001^{**}	1.0000^{NS}
CON	0.0001^{**}	1.0000^{NS}
PCH	0.0001^{**}	1.0000^{NS}
BOY	0.0050^{*}	1.0000^{NS}
SFB	0.0001^{**}	0.6199^{NS}
LHI	0.0001^{**}	1.0000^{NS}
Overall	0.0001^{**}	1.0000^{NS}

$^{*}p < 0.01$.
$^{**}p < 0.00001$.
NS, non-significant difference.

Table 4

Varimax normalised PCA of resting egg surface morphotypes in Chilean *Artemia* populations, including three reference populations

Morphotype	PC			
	PC1	PC2	PC3	PC4
Type I	**0.889**	0.309	−0.157	0.085
Type II	0.041	0.008	−0.247	0.012
Type III	−0.011	−0.077	0.101	−0.034
Type IV	−0.001	−0.300	0.536	0.540
Type V	−0.434	**0.765**	−0.172	0.258
Type VI	−0.044	0.086	0.290	−0.795
Type VII	−0.127	−**0.462**	−0.707	−0.007
Type VIII	0.017	−0.001	0.026	0.001
Type IX	0.005	0.006	0.003	0.007
Eigenvalues	133.134	58.158	44.761	35.502
Proportion of explained variance (%)	41.608	18.176	13.989	11.095
Cumulative proportion of variance (%)	41.608	59.784	73.773	84.869

Bold indicates variables having the largest weights.
PC, principal component; PCA, principal component analysis.

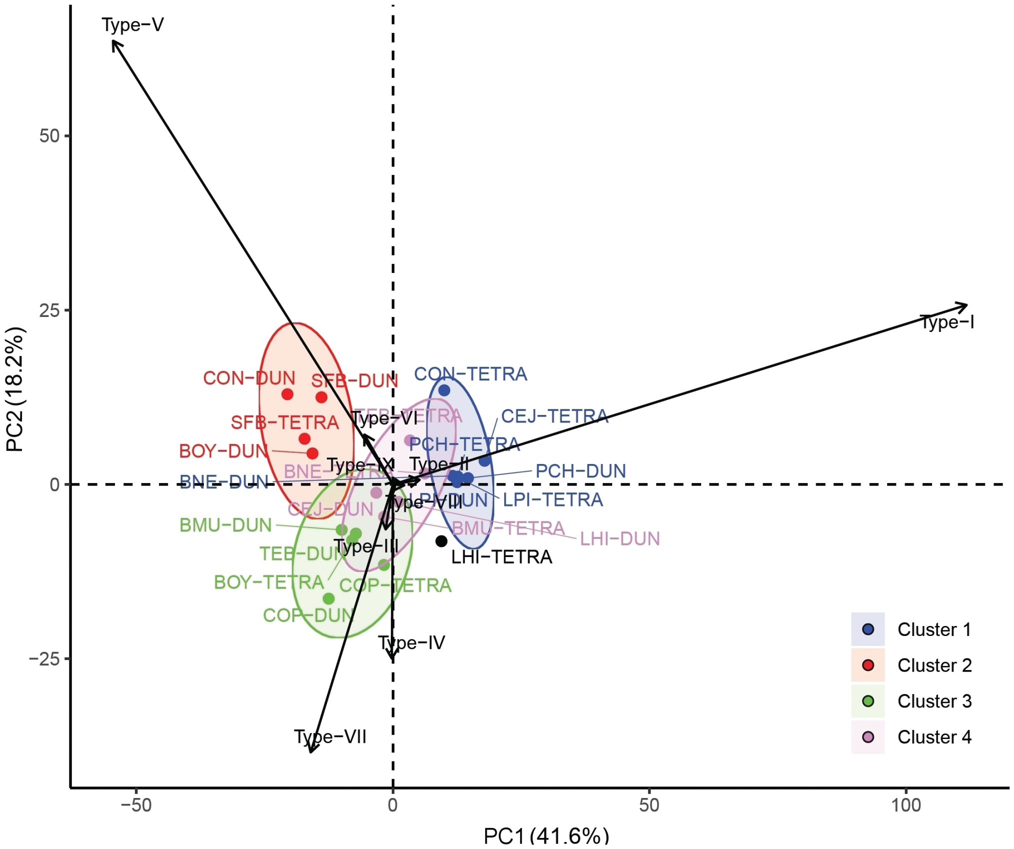

Figure 4

Biplot of PCA of resting egg surface morphotypes. Population and diet treatments are indicated. *Dunaliella tertiolecta* diet denoted as DUN; *Tetraselmis suecica* diet denoted as TETRA. Population codes are defined in Table 1. PCA, principal component analysis.

4. Discussion

Positive effect of food type on reproductive parameters has been observed in *A. franciscana* but in terms of the number of resting eggs produced per female in the Great Salt Lake population (Belovsky et al., 2025). Similar observations in a population of *A. franciscana* from Chile in terms of the percentage of resting egg per female have been also reported (Parraguez, 2022). This study shows that *A. franciscana* populations from Chile fed with different microalgal diets can produce resting egg surface morphology variations because the morphotypes displayed a different distribution in each population depending on the provided diet. Thus, our results provide support in favour of the food type as a relevant factor in the modification of the resting egg surface morphology in *A. franciscana* from Chile.

There are little data on the factors that may affect the resting egg surface morphology in *Artemia*. However, our results concur with molecular genetic studies supporting the occurrence of change in the resting egg surface morphology of *Artemia*, for example, from smooth to rough, but associated with the expression of genes involved in the resting egg chorion formation, such as the shell gland-specific gene I (*SGEG*) based on RNA interference analysis (Dai et al., 2011; Liu et al., 2009). Parraguez (2022) has observed that the *SGEG* gene can change its expression in *A. franciscana* in response to the same microalgal diet used in this study. Therefore, it is likely that this gene may be involved in the variation in resting egg surface morphotypes reported in this study. However, since a number of genes are involved in the resting egg shell formation of *Artemia* (Dai et al., 2011; Liu et al., 2009; Parraguez, 2022), more thorough studies will be required to better understand its involvement in the origin of variations of the resting egg surface morphotypes.

A large proportion of the surface morphotype variance observed in this study can be explained by the smooth and the rough morphotypes, according to the PCA. In fact, the most common morphotype found was the smooth morphotype devoid of surface ornamentation (i.e. Type I). This result is consistent with other resting egg surface characterisations of *A. franciscana* populations from the Great Salt Lake, San Francisco Bay, Utah and California, where this morphotype is highly prevalent (Rosowski et al., 1997; Shepard & Hill, 2001; Spotte & Anderson, 1988). However, because regardless diets up to nine resting egg surface morphotypes were observed, we can verify that in the analysed populations the resting egg surface morphology is highly variable. Therefore, this result led us to conclude that this character is more polytypic than monotypic in *A. franciscana* from Chile, which contrasts with previous investigations that support less variation of the resting egg surface topology in *A. franciscana* from North America (Rosowski et al., 1997; Shepard & Hill, 2001; Spotte & Anderson, 1988). In addition, the strong resting egg surface morphology variation revealed in this study is concordant with other SEM studies carried out on *Artemia* species or populations from Old World. For instance, in *A. salina* from India, at least three resting egg surface morphotypes were recorded (Gilchrist, 1978; Mazzini, 1978; Morris & Afzelius, 1967; Munuswamy, 1988) and in *Artemia* populations from China, six resting egg surface morphotypes were identified (Wang & Sun, 2007). The same results are reported in *Artemia* parthenogenetic lineages from China and Western Siberia, in which there were 15 and 11 distinct resting egg surface patterns, respectively (Asem & Sun, 2014; Vesnina et al., 2024). However, in contrast to the smooth morphotype commonly observed in *A. franciscana* populations in the parthenogenetic lineages, the rough and tubercle surfaces are the more frequently observed morphotypes (Asem & Sun, 2014; Vesnina et al., 2024; Wang & Sun, 2007).

Comparing our results with other SEM studies on *Artemia* resting eggs, the rough morphotype Type V of this study is similar to morphotype Type IV found by Wang and Sun (2007) and morphotype Type VI described by Asem and Sun (2014), both of which were seen in populations from China. All of these morphotypes have a dense arrangement of tiny tubercles. Similarly, Asem and Sun (2014) described morphotype Type XIII, which is similar to morphotype Type VII in our study, which is distinguished by a humped pattern. Furthermore, the uncommon morphotype Type IX, which is defined by densely arranged pore-like fossulae, is comparable to morphotype Type V described by Asem and Sun (2014), which is also characterised by pore-like fossulae. These results suggest that some *Artemia* resting egg surface morphotypes are conserved across populations from different countries. In contrast, the morphotype Type III, which is distinguished by a conglomerate of fibres and was observed in only two populations (i.e., LPI and TEB), has not been reported in *Artemia* populations from other geographical areas, which suggest that this morphotype is unique to *A. franciscana* from Chile.

In the applied field, *A. franciscana* resting egg surface morphology could be a useful trait to track origin because some morphotypes were exclusive to Chile. In fact, two Chilean populations showed a distinctive resting egg surface morphotype, although at low frequency and not reported in other studies. Asem and Sun (2014) had proposed a similar application for identifying *Artemia* strains because some populations from China also present a distinctive resting egg surface morphology. In addition, this character can complement resting egg quality parameters, such as resting egg size and viability, which are commonly considered as valuable indicators in the context of *Artemia* resting egg quality as a supply of live feed for the rearing of marine fish and crustaceans of importance in aquaculture (Browne & Wanigasekera, 2000; Dhont & Sorgeloos, 2002; Sorgeloos et al., 1986). Further SEM analysis of other *A. franciscana* populations from Chile will be required to improve the knowledge on the extent of the resting egg surface morphology variation reported in this study.

5. Conclusions

The analysis of the resting egg surface morphology of nine *A. franciscana* populations from northern and central Chile by using SEM technology revealed the presence of nine resting egg surface morphotypes, with the smooth (48.3%), rough (13.7%), and humped (9.3%) surfaces being the most common. The use of two different microalgal diets (*D. tertiolecta* and *T. suecica*) showed a large effect on the modification of the resting egg surface morphology frequency in these populations. These results indicate that the resting egg surface morphotypes are highly variable at the intraspecific level in *A. franciscana* from Chile and that such variation may be modified experimentally by using specific microalgal diets.

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