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Variation of the interphase heterochromatin in *Artemia* (Crustacea, Anostraca) of the Americas is related to changes in nuclear size and ionic composition of hipersaline habitats

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Abstract

The populations of *Artemia* (or brine shrimp) from the Americas exhibit a wide variation in the amount of interphase heterochromatin. There is interest in understanding how this variation affects different parameters, from the cellular to the organismal levels. This should help to clarify the ability of this organism to tolerate brine habitats regularly subject to strong abiotic changes. In this study, we assessed the amount of interphase heterochromatin per nucleus based on chromocenter number (N-CHR) and relative area of chromocenter (R-CHR) in two species of *Artemia*, *A. franciscana* (Kellog, 1906) (n=9 populations) and *A. persimilis* (Piccinelli and Prosdocimi, 1968) (n=3 populations), to investigate the effect on nuclear size (S-NUC). The relationship of the R-CHR parameter with the ionic composition (IC) of brine habitats was also analysed. Our results indicate a significant variation in the amount of heterochromatin both within and between species (ANOVA, $p<0.001$). The heterochromatin varied from 0.81 ± 1.17 to 12.58 ± 3.78 and from 0.19 ± 0.34% to 11.78 ± 3.71% across all populations, for N-CHR and R-CHR parameters, respectively. N-CHR showed less variation than R-CHR (variation index 15.5-fold vs. 62-fold). At least five populations showed a significant association ($p<0.05$) between R-CHR and S-NUC, either with negative (four populations, r= from -0.643 to -0.443), or positive (one population, r= 0.367) values.Within each species, there were no significant associations between both parameters ($p>0.05$). The R-CHR and IC parameters were associated significantly for the magnesium ion (r= 0.496, $p<0.05$) and also for the chloride, sodium and calcium ions (r = from -0.705 to -0.478, $p<0.05$). At species level, a significant association between both parameters was also found in *A. franciscana* populations, for the sulphate and calcium ions, in contrast to *A. persimilis*. These findings suggest that the amount of interphase heterochromatin modifies the nuclear size in *Artemia*. Our data also indicate that change in the amount of interphase heterochromatin is in line with the ionic composition of brines. This would be a species-specific phenomenon, whose occurrence may be involved in the ability of this organism to survive in these environments.

Keywords: Artemia, interphase heterochromatin, chromocenter, nuclear size, brine ionic composition.

Variação da heterocromatina interfásica em *Artemia* (Crustacea, Anostraca) das Américas está relacionada a mudanças no tamanho nuclear e composição iônica em habitats hipersalinos

Resumo

As populações de *Artemia* (ou camarão de salinas) das Américas apresentam uma grande variação na quantidade de heterocromatina interfásica. Há interesse em compreender como esta variação afeta diferentes parâmetros, desde o nível celular até os organismos. Isso deve ajudar a esclarecer a capacidade destes organismos tolerarem habitats extremos de água hipersalinas, que normalmente são submetidos a fortes mudanças abióticas. Neste estudo, avaliou-se a quantidade heterocromatina interfásica por núcleo através do número de cromocentros (N-CHR) e a área relativa de cromocentros (R-CHR) em duas espécies de *Artemia*, *A. franciscana* (Kellog, 1906) (n=9 populações) e *A. persimilis* (Piccinelli e Prosdocimi, 1968) (n=3 populações), para investigar o seu efeito no tamanho nuclear (S-NUC). Também foi analisada a relação de R-CHR com a composição iónica (CI) dos habitats hipersalinos. Nossos resultados indicam uma variação significativa na quantidade de heterocromatina dentro e entre espécies (ANOVA, $p<0,001$). Em todas as populações, a heterocromatina variou de 0,81 ± 1,17 para 12,58 ± 3,78 e de 0,19 ± 0,34% para 11,78 ± 3,71% para os parâmetros R-CHR e N-CHR, respectivamente. N-CHR apresentou menor variação do que R-CHR (amplitude de variação de 15,5 vezes vs. 62 vezes). Pelo menos cinco populações apresentaram uma associação significativa ($p<0,05$) entre R-CHR e S-NUC, seja com valores negativos (quatro populações, r = -0,643 a -0,443) ou positivo (uma população,

r = 0,367). Os parâmetros R-CHR e CI foram associados significativamente para o íon de magnésio (r = 0,496, p<0,05) e também para os íons cloreto, sódio e cálcio (r = -0,705 a -0,478, p<0,05). Ao nível de espécie, foi também encontrada uma associação significativa entre esses dois parâmetros em populações de *A. franciscana* para os íons de sulfato e de cálcio, em contraste com *A. persimilis*. Estes achados sugerem que a quantidade heterocromatina interfásica modifica o tamanho nuclear em *Artemia*. Os nossos dados também indicam que a mudança na quantidade de heterocromatina interfásica está associada com a composição iónica das salinas. Este seria um fenómeno específico da espécie, cuja ocorrência pode estar envolvida na capacidade deste organismo sobreviver em tais ambientes.

Palavras-chave: Artemia, heterocromatina interfásica, cromocentro, tamanho nuclear, composição iónica.

1. Introduction

Heterochromatin is a characteristic component of the eukaryotic nucleus which, as opposed to euchromatin, is highly compacted, non-coding and contains highly repetitive DNA sequences (Swanson et al., 1981; Brutlag, 1980). The heterochromatin in the interphase nucleus can be visualized as easily discernible heteropycnotic bodies, called chromocenters, which can vary in number and size. In the brine shrimp *Artemia*, a conspicuous inhabitant of hypersaline lakes and lagoons, the chromocenter number may have a diagnostic value as an indicator of species or populations (Badaracco et al., 1987; Abreu-Grobois and Beardmore, 1989; Colihueque and Gajardo, 1996; Papeschi et al., 2000; Gajardo et al., 2001; Torrentera and Abreu-Grobois, 2002; Papeschi et al., 2008). Previous studies indicate that the heterochromatin in *Artemia* varies in quantity and quality both within and among species (Gajardo et al., 2002). For instance, the New World *Artemia* species, *A. franciscana* and *A. persimilis*, have a significantly different chromocenter number (Badaracco et al., 1987; Abreu-Grobois and Beardmore, 1989; Colihueque and Gajardo, 1996; Papeschi et al., 2000; Gajardo et al., 2001; Torrentera and Abreu-Grobois, 2002; Lipko et al., 2004). While the former exhibits a high number of these structures (5-18 chromocenters) the latter has lower numbers (<5 chromocenters). Such variation in the amount of heterochromatin is also associated with the presence of repetitive *Alu*I sequences in the genome, with around a tenth less of these sequences being observed in *A. persimilis* in comparison with *A. franciscana* (Barigozzi et al., 1984; Badaracco et al., 1987). In addition, chromosome studies of these species have revealed an interspecific variation in the diploid chromosome number, consisting of 42 in *A. franciscana* and 44 in the case of *A. persimilis*.

The evidence available on plants and animals indicates that variation in the DNA content per genome, usually positively associated with the increase in the amount of heterochromatin (Rayburn et al., 1985; Kao et al., 2001; Bosco et al., 2007), may produce changes at the cellular (termed nucleotypic effect), or organismal level (Gregory and Hebert, 1999). In other words, the phenotype expression would not just depend on the interaction between genotype and environment, but also on the expression of the DNA quantity, irrespective of its informational content (Swanson et al., 1981; Hartl, 2000). For instance, at the cellular level, this modification may affect either the cell or nucleus size, or the duration of the cell cycle. These changes in DNA quantity have been related to variations in the biomass content, breeding season and even the physiological responses of organisms (Swanson et al., 1981; Hartl, 2000).

Artemia is a crustacean that can deploy numerous physiological adaptations; these enable it to tolerate abrupt abiotic changes in the brines inhabited by different species or populations that involve mainly changes in temperature, salinity, ionic concentration and dissolved oxygen. Such changes are driven by the high evaporation rate or the rain regime (Gajardo and Beardmore, 2012). Among the adaptations developed by *Artemia* to tolerate abiotic changes are a high osmoregulatory capacity, the efficient utilization of dissolved oxygen and the conditional switch in offspring quality between cyst (oviparous reproduction) and nauplii (viviparous reproduction) depending on unfavorable and favorable environmental conditions, respectively. Although most of these adaptive traits have a relatively well-known physiological and molecular basis (Gajardo and Beardmore, 2012), changes in heterochromatin could be another factor in the *Artemia* repertory adopted to withstand extreme conditions. This has been mentioned in other animal and plant studies, where some of their ecological features are associated with variation in the heterochromatin content (Walker et al., 1991; Ceccarelli et al., 1992, 2002).

In this study the interphase heterochromatin content in different American *Artemia* populations belonging to the *A. franciscana* and *A. persimilis* species were determined through the analysis of interphase nuclei from nauplii cells. This parameter was related to variations in nucleus size in order to explore the existence of nucleotypic changes. We also investigated the relationship of these heterochromatic changes with the ionic composition of the brines inhabited by these populations as a proxy of their adaptive nature.

2. Material and Methods

2.1. Populations studied

Twelve populations of *Artemia* from different locations in America were analysed: Salina la Colorada Chica (SCC, Argentina), Laguna Amarga-Torres del Paine (TPA, Chile), Laguna de Los Cisnes (CIS, Chile), San Francisco Bay-1258 (SFB, USA), Great Salt Lake (GSL, USA), Salar de Llamara (LLA, Chile), Chaxas (CHX, Chile), La Rinconada (RIN, Chile), Palo Colorado-Los Vilos (LVI, Chile), Salinas de Pichilemu (PCH, Chile), Río Grande (RGB, Brazil), and

Table 1. List of Artemia populations analysed in this study. Location, geographic coordinates and mean chromocenter number previously reported is shown.

Population	Country	Code	Species	Geographic coordinates	Mean chromocenter number reported[a]
1. Salina la Colorada Chica	Argentina	SCC	*A. persimilis*	38º22'46"S 63º25'43"W	1.0
2. Laguna Amarga-Torres del Paine	Chile	TPA	*A. persimilis*	50º58'32"S 72º44'57"W	17.7
3. Laguna de Los Cisnes	Chile	CIS	*A. persimilis*	53º10'35"S 70º19'41"W	7.0
4. San Francisco Bay-1258	USA	SFB	*A. franciscana*	37º32'53"N 122º13'41"W	16.8
5. Great Salt Lake	USA	GSL	*A. franciscana*	41º6'56"N 112º28'36"W	9.0†
6. Salar de Llamara	Chile	LLA	*A. franciscana*	21º21'00"S 69º35'56"W	13.8
7. Chaxas	Chile	CHX	*A. franciscana*	23º17'6"S 68º10'37"W	NA
8. La Rinconada	Chile	RIN	*A. franciscana*	23º26'21"S 70º30'20"W	9.5‡
9. Palo Colorado-Los Vilos	Chile	LVI	*A. franciscana*	32º4'27"S 71º29'38"W	9.9
10. Salinas de Pichilemu	Chile	PCH	*A. franciscana*	34º30'5"S 71º59'4"W	6.7
11. Río Grande	Brasil	RGB	*A. franciscana*	5º06'00"S 36º16'00"W	10.1
12. Macao	Brasil	MAC	*A. franciscana*	5º05'51"S 36º38'40"W	9.6

[a]According to Gajardo et al. (2001); †Papeschi et al. (2008); ‡Unpublished data from Laboratorio de Genética, Acuicultura and Biodiversidad, Universidad de Los Lagos.

Macao (MAC, Brazil) (as shown in Table 1). The populations from the United States (SFB and GSL) and Argentina (SCC), *A. franciscana* and *A. persimilis*, respectively, were used as reference species (Gajardo et al., 2001). According to previous studies (Colihueque and Gajardo, 1996; Papeschi et al., 2000; Gajardo et al., 2001), the chromocenter numbers of the remaining populations from Chile (n=7) and Brazil (n=2), are known to vary. The Chilean populations were obtained from laboratory cultures originating from live, wild animals.

2.2. Obtaining interphase nuclei

The interphase nuclei were obtained from nauplii by the squash method following the Colihueque and Gajardo (1996) protocol. Larvae were collected either from newly hatched cysts incubated in artificial seawater or from offspring of natural crosses of adults reared in the laboratory under standardised conditions of salinity (35%), temperature (~22 ºC) and light (~1000 lux). The chromocenters of the nuclei were stained using a fluorescent dye (Hoechst 33258) which displays a high affinity for interphase heterochromatic regions (Latt and Wohlled, 1975). The nuclei were photographed at 1000x using a 7 mpx digital camera mounted on an epifluorescence Nikon Labophot microscope. Before taking the photographs, excitation of the fluorochrome was undertaken with UV light through an appropriate filter (UV-2A, 330-380 nm). Five nuclei of each nauplii were photographed at random, totaling from 20 to 58 nuclei per population.

2.3. Heterochromatin quantification in the interphase nuclei

We use a computer-based image analysis to accurately determine the amount of interphase heterochromatin. This method permits increased objectivity, since it can register the totality of the heterochromatin distributed in the nucleus, regardless of its number, size, shape and associations. In this context, the interphase heterochromatin content was estimated using the IMAGEJ version 1.38 software (National Institute of Health, Bethesda, USA). The "count particles" function of the program was used to determine the following parameters: 1) chromocenter number per nucleus (N-CHR) and 2) relative area, as a percentage of the chromocenters per nucleus (R-CHR), representing a relative assessment of interphase heterochromatin. All the analyses were undertaken in grayscale, that fluctuates between 0 and 255 (where 0 = black and 255 = white), whereby a chromocenter was defined as any nuclear structure below the 150 threshold of the grayscale (intense black) and a size above 50 pixels. The relative area (RA) filled by chromocenters in the nucleus was calculated using the formula: RA = (AC/TA) x 100, where AC corresponds to

the area covered by the chromocenters in pixels, and TA was the total area of the nucleus in pixels. The nucleus size (S-NUC) was established using the formula for the area of a circumference $A = \pi r^2$, where A is the final area in μm^2 and r is the radius of the nucleus. The absolute diameter of the nucleus was determined using a reference scale incorporated into a micrometer ocular with 0.5 µm sensitivity. The scale bar was subsequently used to calibrate the actual diameter of each nucleus with the SIGMASCAN PRO 5.0 program (Systat software Inc., Chicago, USA), using the image size calibration function.

2.4. Ionic composition of brines

The ionic concentration of brines for SCC, TPA, CIS, SFB, GSL, LLA, RIN, LVI, and PCH populations (as shown in Table 2) was obtained from previous studies (Clarke, 1924; Adams, 1964; Stube et al., 1976; Post, 1980; Gomez-Silva et al., 1990; Amat et al., 1994; Schalamuk et al., 1999; Zúñiga et al.,1999; Campos et al., 1996; López et al., 1996; Ruíz et al., 2007; Jones et al., 2009; de Los Ríos and Soto, 2009; de Los Ríos and Salgado, 2012). Although such conditions are obviously particular to the year and season, it is assumed they represent the water composition of that particular site, depending on their marine (Athalassohaline) or inland (Thalasohaline) characteristics. In order to compare all sites, the values were represented in a Piper's diagram (Piper, 1944), using the DIAGRAMMES program, version 6.1 (Laboratoire d`Hydrogéologie, University of Avignon, Avignon, France). This method displays specific cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) and anions (HCO_3^{-}, CO_3^{2-}, Cl^{-}, SO_4^{2-}) as a percentage of the total cations and anions, respectively, in a trilinear diagram. Thus, chemically similar waters are grouped in the same position, as follows: a) waters containing sulphate and/or chloride, rich in calcium or magnesium; b) waters containing bicarbonate, rich in calcium or magnesium; c) waters containing chloride and/or sulphate, rich in sodium; and d) waters containing bicarbonated sodium. The percentage of each ion (as shown in Table 2) was calculated based on its value in meq/L, using the following formula: percentage of the ion X = ((∑ meq/L of total ions in water/meq/L ion X)*100.

2.5. Statistical analyses

The data obtained from the different populations was subject to a two-way analysis of variance (ANOVA), followed by a Tukey's multiple comparison test to carry out a post hoc pairwise comparison of means. Pearson's product-moment correlation analysis was applied to establish the following associations: 1) N-CHR vs. R-CHR; and 2) R-CHR vs. S-NUC. The correlations between R-CHR and percentage of a particular ion were established for the following ions: Cl^{-}, SO_4^{2-}, Na^{+}, K^{+}, Ca^{2+} y Mg^{2+}. The significance of correlations was calculated through a Student's t-test. The same statistical test was used to calculate differences between means. The STATISTICA program, version 5.1 (Statsoft, Inc., Tulsa, USA) was used to undertake these analyses.

3. Results

The interphase nuclei from nauplii cells, which display the chromocenters observed in the 12 populations studied, are shown in Figure 1. Quantification of these heterochromatic areas per nucleus indicated that the mean N-CHR values varied widely and significantly among populations (ANOVA, $F_{[11, 440]}=31.08$, $p<0.001$), from 0.81 ± 1.17 to 12.58 ± 3.78 (as shown in Table 3), with a variation index of 15.5 fold. Thus, there were populations whose means were low (SCC), medium (CHX, LLA, LVI, PCH) or high (RIN, CIS, RGB, MAC, SFB, GSL, TPA). In 30 out of 66 pairwise comparisons, differences in means were statistically significant (Tukey's test, $p<0.05$). The analysis of this parameter at species level also indicated significant variation among populations for *A. franciscana* (ANOVA, $F_{[8, 304]}=10.94$, $p<0.001$) and *A. persimilis* (ANOVA, $F_{[2,136]}=152.52$, $p<0.001$). With regard to the reference populations, the mean N-CHR in the SFB population (*A. franciscana*) was significantly higher than in the SCC population (*A. persimilis*) (10.54 ± 3.55 vs. 0.81 ± 1.17, Student's t-test, $p<0.05$). The mean N-CHR obtained in previous studies (as shown in Table 1) revealed less chromocenters (two to seven) in some populations, in contrast to the result found in this study, such as the LLA and LVI populations.

The mean R-CHR values also varied significantly among populations (ANOVA, $F_{[11,440]}=115.05$, $p<0.001$), from 0.19 ± 0.34% to 11.78 ± 3.71%, but with a higher level of variation (62 fold) than the N-CHR parameter. Within this distribution, populations presented mean values that were grouped into low (SCC), medium (CHX, LLA, LVI, RGB, PCH), or high (RIN, MAC, SFB, CIS, GSL, TPA) categories (as shown in Table 3). The pairwise comparison of means for the R-CHR parameter indicated that 50 out of 66 had significant differences (Tukey's test, $p<0.001$). The result of this parameter at species level also indicated significant variation among populations for *A. franciscana* (ANOVA, $F_{[8, 304]}= 49.74$, $p<0.001$) and *A. persimilis* (ANOVA, $F_{[2, 136]}=297.07$, $p<0.001$). In the case of the reference populations, mean R-CHR in the SFB population was significantly higher than in the SCC population (8.34 ± 3.32% vs. 0.19 ± 0.34%, Student's t-test, $p<0.05$). The mean N-CHR among *A. franciscana* and *A. persimilis* did not differ significantly (9.19±4.03 vs. 8.95±5.55, Student's t-test, $p>0.05$). However, the mean R-CHR was significantly lower in *A. franciscana* than in *A. persimilis* (4.59±3.21 vs. 8.31±5.76, Student's t-test, $p<0.05$).

The mean S-NUC values ranged from 155.43 ± 88.32 μm^2 to 372.32 ± 160.03 μm^2. The mean differences among populations were statistically significant (ANOVA, $F_{[11,440]}=19.67$, $p<0.001$), but only 23 out of 66 pairwise comparisons were significant (Tukey's test, $p<0.05$). At species level, this parameter also indicated significant variation among populations for *A. franciscana* (ANOVA, $F_{[8, 304]}=21.26$, $p<0.001$) and *A. persimilis* (ANOVA, $F_{[2, 136]}=5.53$, $p<0.01$). In addition, the S-NUC parameter,

Table 2. Ionic concentration and the transformation to percentages of brine waters inhabited by *Artemia* populations from the Americas according to previous studies.

Country	Population	Ionic composition												References
		Cl^-		SO_4^{2-}		Na^+		K^+		Ca^{2+}		Mg^{2+}		
		mg/L	%§	mg/L	%	mg/L	%	mg/L	%	mg/L	%	mg/L	%	
Argentina	SCC	174,000	94.69	13,020	5.24	108,340	94.32	1,110	0.57	500	0.50	2,800	4.61	Schalamuk et al. (1999)
Argentina	SCC	173,597	95.27	11,500	4.67	111,600	93.75	4,156	2.06	857	0.83	2,112	3.36	Ruíz et al. (2007)
Chile	TPA	14,600	46.30	22,900	53.70	27,300	84.01	1,390	2.52	20	0.07	2,300	13.40	Unpublished data‡
Chile	TPA	8,960	25.31	26,000	54.32	19,000	81.11	1,868	4.70	6	0.03	1,752	14.16	Unpublished data‡
Chile	TPA	9,650	25.78	26,600	52.55	21,900	81.79	1,930	4.25	6	0.03	1,971	13.93	Unpublished data‡
Chile	TPA	12,263	55.25	374	1.25	580	23.14	95	2.24	2	0.09	987	74.54	Campos et al. (1996)
Chile	TPA	12,264	57.54	330	1.15	5,330	54.57	35	0.21	55	0.65	2,300	44.57	Campos et al. (1996)
Chile	TPA	8,290	51.25	389	1.78	10,240	74.13	109	0.47	7	0.06	1,850	25.35	Campos et al. (1996)
Chile	TPA	9,466	46.73	673	2.46	25	0.70	106	1.78	0	0.00	1,816	37.51	Campos et al. (1996)
Chile	TPA	17,020	25.86	60,380	67.86	34,160	82.64	2,250	3.21	330	0.92	2,089	13.23	Zúñiga et al. (1999)
Chile	CIS	11,700	55.71	6,770	23.84	15,500	87.42	430	1.43	11	0.07	1,038	11.08	Unpublished data‡
Chile	CIS	12,900	59.06	6,780	22.96	16,300	89.06	324	1.04	7	0.05	952	9.85	Unpublished data‡
Chile	CIS	5,693	74.30	73	0.71	4,119	80.08	129	1.48	16	0.37	491	18.07	de Los Ríos and Soto (2009)
USA	SFB	179,200	89.14	29,530	10.86	NA	NA	NA	NA	NA	NA	NA	NA	Clarke (1924)
USA	GSL	111,100	90.85	14,850	8.98	NA	NA	NA	NA	NA	NA	NA	NA	Adams (1964)
USA	GSL	177,600	91.49	21,540	8.21	101,450	81.86	4,140	1.97	300	0.28	10,400	15.89	Stube et al. (1976)
USA	GSL	181,000	89.78	27,000	9.90	105,400	80.64	6,700	3.02	300	0.26	11,100	16.08	Post (1980)
USA	GSL	718,000	92.06	8,000	7.59	41,100	81.80	2,300	2.70	189	0.43	4,000	15.07	Jones et al. (2009)
Chile	LLA	134,722	76.35	56,256	23.58	107,042	95.18	3,094	1.62	332	0.34	1,700.4	2.86	López et al. (1996)
Chile	LLA	37,451	72.40	19,200	27.45	31,013	92.58	914	1.61	441	1.51	759.4	4.29	López et al. (1996)
Chile	LLA	29,302	74.18	13,632	25.52	23,513	91.93	737	1.70	836	3.76	352.8	2.61	López et al. (1996)
Chile	LLA	43,600	77.17	17,440	22.83	35,810	92.67	2,130	3.25	450	1.34	560	2.74	Zúñiga et al. (1999)
Chile	RIN	152,780	88.49	26,880	11.51	75,120	78.65	2,780	1.72	730	0.88	9,460	18.75	Gomez-Silva et al. (1990)
Chile	LVI	23,480	89.70	3,550	10.03	12,750	75.31	410	1.43	380	2.58	1,860	20.38	Amat et al. (1994)
Chile	LVI	53,300	90.23	7,580	9.49	32,480	77.48	1,320	1.86	1,260	3.46	3,810	17.21	Zúñiga et al. (1999)
Chile	LVI	53,300	90.10	7,600	9.50	32,500	77.46	1,300	1.83	1,300	3.56	3,800	17.15	de Los Ríos and Salgado (2012)
Chile	PCH	121,958	96.44	6,091	3.56	NA	NA	NA	NA	NA	NA	NA	NA	Unpublished data‡
Chile	PCH	54,780	90.23	7,690	9.37	31,380	78.29	1,100	1.62	1,210	3.47	3,520	16.62	Zúñiga et al. (1999)
Sea water	ANT†	54,840	90.20	7,710	9.38	29,850	77.38	1,090	1.67	1,220	3.64	3,530	17.32	Zúñiga et al. (1999)

§ The percentage of each ion was calculated based on its value in meq/L, using the following formula: percentage of the ion X = ((∑ meq/L of total ions in water/meq/L ion X)*100; † Data from Antofagasta (northern Chile) was included as reference of sea water; ‡ Data from Laboratorio de Genética, Acuicultura and Biodiversidad, Universidad de Los Lagos.

showed much less variation than the R-CHR parameter according to the variation index (2.39 vs. 62 fold). The mean S-NUC was significantly higher in *A. franciscana* than in *A. persimilis* (253.94±125.10 µm^2 vs. 205.76±90.48 µm^2, Student's t-test, $p<0.05$).

According to the regression analysis (as shown in Table 4), there was a positive and significant relationship between N-CHR and R-CHR in eight out of 12 populations ($r= 0.297$-0.792, Student's t-test, $p<0.05$,). All populations pooled exhibited a significant correlation between both

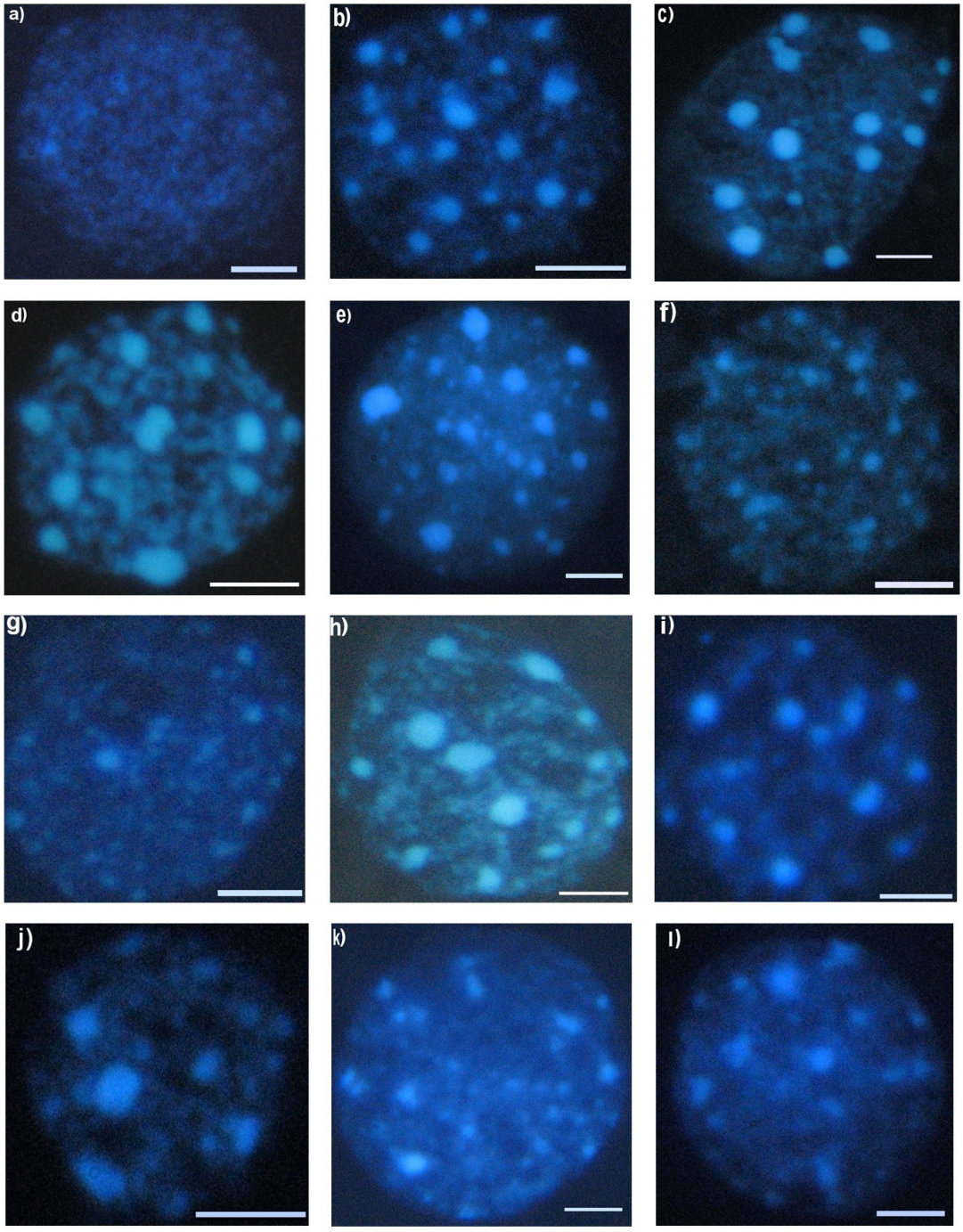

Figure 1. The interphase nuclei of nauplii cells, displaying the chromocenters stained by Hoechst 33258 fluorescent dye (bright bodies) observed in the 12 studied population of Artemia. Populations: (a) Salina la Colorada Chica (SCC), (b) Laguna Amarga-Torres del Paine (TPA), (c) Laguna de Los Cisnes (CIS), (d) San Francisco Bay-1258 (SFB), (e) Great Salt Lake (GSL), (f) Salar de Llamara (LLA), (g) Chaxas (CHX), (h) La Rinconada (RIN), (i) Palo Colorado-Los Vilos (LVI), (j) Salinas de Pichilemu (PCH), (k) Río Grande (RGB) and (l) and Macao (MAC). Bar represent 5 µm.

Table 3. Summary of the interphase heterochromatin content and nucleus size parameters (mean ± SD) in Artemia populations.

Population	Species	No. of nuclei analysed (No. of nauplii)	N-CHR (n)	R-CHR (%)	S-NUC (um^2)
SCC	*A. persimilis*	32(6)	0.81±1.17[a]	0.19±0.34[a]	212.98±84.05[a,b]
TPA	*A. persimilis*	58(7)	12.58±3.78[d]	11.78±3.71[e]	178.02±61.55[a]
CIS	*A. persimilis*	49(10)	9.95±3.06[c,d]	9.49±4.36[d,e]	233.87±112.55[a,b]
SFB	*A. franciscana*	26(5)	10.54±3.55[c,d]	8.34±3.32[c,d]	212.53±60.58[a,b]
GSL	*A. franciscana*	24(8)	11.71±3.67[d]	8.34±2.51[e]	300.18±57.96[b,c]
LLA	*A. franciscana*	38(4)	6.83±4.47[b]	1.89±0.91[b]	207.96±95.42[a,b]
CHX	*A. franciscana*	20(5)	5.3±2.36[a,b]	2.09±1.06[b]	155.43±88.32[a]
RIN	*A. franciscana*	41(8)	11.75± 3.14[d]	6.94±2.74[c]	372.32±160.03[e]
LVI	*A. franciscana*	38(5)	7.89±3.65[b,c]	2.11±1.44[b]	253.57±68.20[b]
PCH	*A. franciscana*	53(5)	8.35±2.82[b,c]	3.93±2.09[b]	172.54 ±42.07[a]
RGB	*A. franciscana*	33(4)	9.84±3.15[c,d]	2.87±1.46[b]	369.41±164.51[c,e]
MAC	*A. franciscana*	40(3)	10.12±4.64[c,d]	5.98±2.82[c]	237.62±191.52[a,b]
Pooled	*A. persimilis*	139(23)	8.95 ±5.55[x]	8.31 ±5.76[x]	205.76±90.48[x]
Pooled	*A. franciscana*	313(47)	9.19±4.03[x]	4.59±3.21[y]	253.94±125.10[y]
Pooled	Both species	452(70)	9.12 ±4.54	5.74 ±4.50	239.12 ±117.58
Variation index	Across populations		15.53	62.00	2.39

Population means in each column bearing different letters are significantly different from each other (Tukey's test, $p< 0.05$). Species means (pooled data) with different letters indicate significant differences (Student's t-test, $p< 0.05$). N-CHR= chromocenter number per nucleus, R-CHR= relative area in percentage of chromocenter per nucleus, S-NUC = nuclear size.

Table 4. Correlation values between heterochomatin content (N-CHR and R-CHR) and nucleus size (S-NUC) in Artemia populations.

Population	No. of nuclei analysed	N-CHR vs. R-CHR	R-CHR vs. S-NUC
SCC	32	0.792 (0.000)*	0.126 (0.478)
TPA	58	0.032 (0.757)	-0.202 (0.123)
CIS	49	0.465 (0.001)*	0.367 (0.009)*
SFB	26	0.335 (0.094)	-0.322 (0.107)
GSL	24	0.313 (0.000)*	-0.158 (0.282)
LLA	38	0.406 (0.011)*	-0.145 (0.364)
CHX	20	0.071 (0.755)	-0.643 (0.002)*
RIN	41	-0.063 (0.669)	-0.464 (0.002)*
LVI	38	0.639 (0.000)*	-0.100 (0.546)
PCH	53	0.609 (0.000)*	-0.540 (0.000)*
RGB	33	0.442 (0.009)*	-0.443 (0.009)*
MAC	40	0.297 (0.008)*	-0.235 (0.331)
A. persimilis	139	0.807 (0.000)*	-0.005 (0.947)
A. franciscana	313	0.525 (0.000)*	-0.009 (0.897)
Pooled	452	0.641 (0.000)*	-0.032 (0.407)

Values in brackets are p-values according to Student's t-test; *$p< 0.05$.

parameters (r= 0.641, Student's t-test, $p<0.05$). This pattern was also observed for populations of *A. franciscana* (r=0.525, Student's t-test, $p<0.05$) and *A. persimilis* (r=0.807, Student's t-test, $p<0.05$). The coefficient of determination ($r^2=0.41$) in the relationship of pooled data revealed that only 41% of R-CHR was determined by N-CHR, reflecting a low level of determination between both parameters. Likewise, there was a significant association between R-CHR and S-NUC in five out of 12 populations (Student's t-test, $p<0.05$), either negative, in four populations (CHX, r= -0.643; RIN, r= -0.464; RGB, r= -0.443; PCH, r= -0.540), or positive, in one population (CIS, r=0.367). All populations pooled showed a negative and not statistically significant association (r=-0.032, Student's t-test, $p>0.05$) between both parameters. A similar pattern was observed for populations of *A. franciscana* (r=-0.009, Student's t-test, $p>0.05$) and *A. persimilis* (r=-0.005, Student's t-test, $p>0.05$).

Piper's diagram (see Figure 2) grouped populations studied into two classes according to the predominant ions in each location: 1) waters containing sodium and/or chloride such as SCC, LLA, RIN, LVI, PCH, SFB and GSL populations; and 2) water with irregular ionic composition, represented by TPA and CIS populations, where the maximum percentage of SO_4^{2-} was relatively high (23-67%). With regard to the correlation between the ion concentration of each location and R-CHR (as shown in Table 5), the pooled data indicated a positive and significant association (Student's t-test, $p<0.05$) for the Mg^{2+} ion and negative and significant association (Student's t-test, $p<0.05$) for the Cl^-, Na^+ and Ca^{2+} ions. At species level, this analysis indicated that there were different associations for some ions, particularly, the SO_4^{2-} and Ca^{2+} ions displayed negative and significant correlations in *A. franciscana*, in contrast to *A. persimilis*.

4. Discussion

Analysis of the interphase heterochromatin in *Artemia* has been based mostly on counting the number of chromocenters using visual methods. Despite the taxonomic value attributed to this cytogenetic trait (Gajardo et al., 2001), some authors have stated that these studies have probably been subject to considerable experimental error (Papeschi et al., 2008). This situation may occur due to following sources of error: 1) the chromocenters tend to merge in the interphase nucleus, and thus the actual chromocenter number counted in different nuclei might differ; 2) the chromocenter counts are often carried out regardless of size, thus small chromocenters may be compared to large ones, despite the fact that the former may have less heterochomatin than the latter; this situation leads to a misinterpretation of the amount of heterochromatin present in the nucleus; and 3) some researchers may exclude the tiny chromocenters on the final count, based on a subjective decision. To address this problem, we included the determination of the relative amount of heterochromatin per nucleus in this study (i.e. R-CHR parameter), to ensure more reliable heterochromatin quantification in the nucleus. Indeed, large interpopulation differences were observed in the mean percentage of heterochromatin per nucleus, compared to the traditional method based on counting the number of chromocenters (62 fold vs. 15.53 fold), which confirms

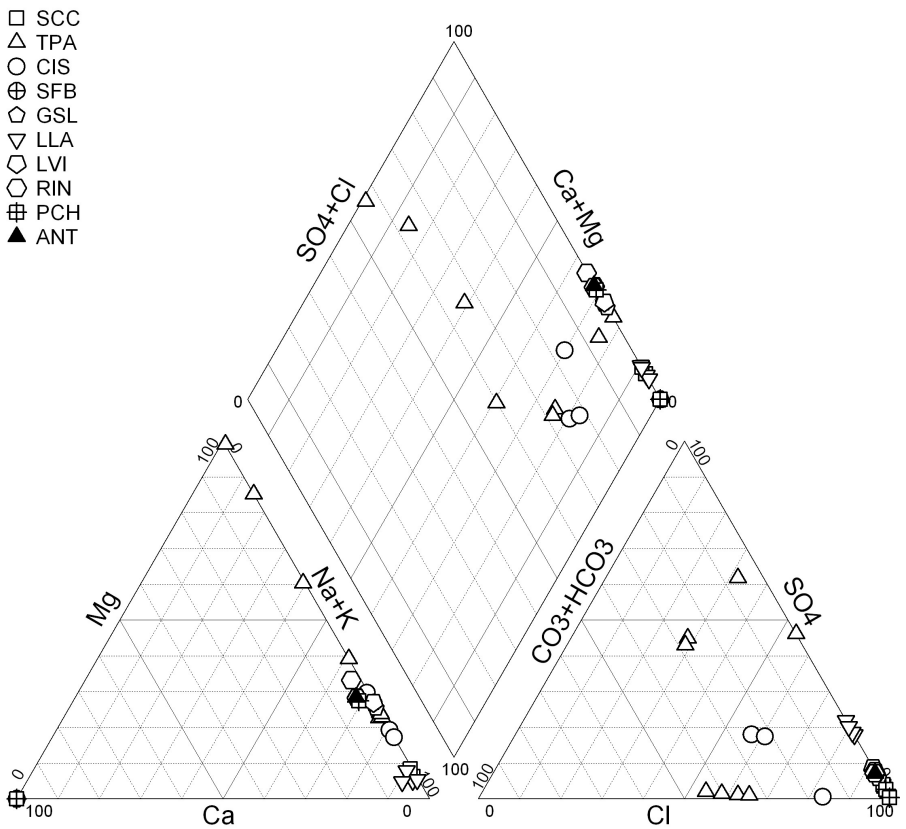

Figure 2. Piper's diagram displaying ionic concentration (%) of brine waters of nine American Artemia populations studied in this work. The populations were the following: Salina la Colorada Chica (SCC), Laguna Amarga Torres del Paine (TPA), Laguna de Los Cisnes (CIS), San Francisco Bay (SFB), Great Salt Lake (GSL), Salar de Llamara (LLA), Palo Colorado Los Vilos (LVI), La Rinconada (RIN) and Salinas de Pichilemu (PCH). Antofagasta (ANT) sample was included in the analysis as reference of sea water.

Table 5. Correlation values between ions concentration and heterochromatin content (R-CHR) in Artemia populations.

	Ions					
	Cl^-	SO_4^{2-}	Na^+	K^+	Ca^{2+}	Mg^{2+}
A. persimilis						
No. of samples	13	13	13	13	13	13
Correlation (r)	-0.845	0.350	-0.431	0.289	-0.476	0.419
	(0.000)**	(0.241)	(0.141)	(0.338)	(0.101)	(0.154)
A. franciscana						
No. of samples	15	15	12	12	12	12
Correlation (r)	-0.549	-0.546	-0.341	0.537	-0.653	0.443
	(0.034)*	(0.035)*	(0.278)	(0.072)	(0.021)*	(0.149)
Pooled						
Nº of samples	28	28	25	25	25	25
Correlation (r)	-0.705	0.276	-0.478	0.233	-0.640	0.496
	(0.000)**	(0.155)	(0.016)*	(0.263)	(0.001)**	(0.012)*

Values in brackets are p-values according to Student's t-test; *p< 0.05; **p< 0.001.

the robustness of this methodology. In other species, such as *Arabidopsis* (Soppe et al., 2002), a similar strategy has been adopted to improve the quality of this analysis.

The relationship between the percentage of interphase heterochromatin per nucleus and nuclear size showed no significant associations at species level. However, our results indicate that, in some cases, significant associations occur at a population level. For example, CHX, RIN, PCH and RGB populations exhibited a significant reduction in nuclear size associated with an increase in the amount of interphase heterochromatin, while the opposite was observed for the CIS population. This finding suggests the existence of a nucleotypic effect in *Artemia* from the Americas, mediated by variation in the amount of heterochromatin present in the nucleus. However, the effect would be specific to certain populations. It is important to note that the natural habitats of these population may vary widely throughout the year, for example, in temperature (18-29.8 ºC for RIN) (Gomez-Silva et al., 1990) and salinity (30-120 ppt for PCH) (Gajardo et al., 1998). Therefore, the associations observed between both parameters may reflect their adaptations to particular ecological conditions. On the other hand, the results showing reduction in nucleus size to be negatively correlated with heterochromatin content appears intriguing, although this effect has recently been demonstrated (Wang et al., 2013) in another organism, specifically in the *Arabidopsis* mutant Crowded Nucleus (*CRWN*). In the case of increasing nuclear size, the evidence available in animals and plants has revealed a positive correlation, although mainly with nuclear DNA content (Swanson et al., 1981; Jovtchev et al., 2006).

The strong and positive association between the content of interphase heterochromatin and magnesium concentration in the brine sites is a significant result. In other words, heterochromatin variation would induce changes that extend from the cell to the organism level, expressed as the differential ability to survive in brines with different ionic composition. Although this effect must be substantiated in further studies, especially relating the actual ion composition of the brine to the moment when samples of *Artemia* are obtained, this is a plausible working hypothesis worthy of future study. The two populations (TPA and CIS) considered in this study, located in Chilean Patagonia (below latitude 50º S) are atypical for *Artemia* standards (Clegg and Gajardo, 2009). According to data previously collected (Campos et al., 1996; Zuñiga et al., 1999; De Los Ríos and Soto, 2009), both hypersaline sites would differ from the traditional description of environments classified as Athassalohaline (inland waters) and Thassalohaline (marine waters). For instance, magnesium content (9.85-46.35%) is considerably higher (up to 2.5 times) than that of sea water. Despite the fact that the adaptive role of heterochromatin might still be considered controversial, evidence now available indicates that its role in the cellular function cannot be ignored, for example, this element is involved in the stabilization of the chromosome structure, chromosome segregation and gene silencing (Grewal and Jia, 2007). Furthermore, variation in heterochromatin content has been associated with the particular distribution of the species in response to divergent environments (Walker et al., 1991; Ceccarelli et al., 1992, 2002), or with the biomass level of the organisms (Edelman and Lin, 1996). Previous studies showing a latitudinal variation in chromocenter numbers in *A. franciscana* would be an indirect indication of such heterochromatin abilities (Gajardo et al., 2001). In fact, the *Artemia* used in this study, collected from Torres del Paine, present a larger relative heterochromatin content in the nucleus than that of the other populations analysed. Clarification of this paradox requires further analysis, but a preliminary hypothesis would be that particular environmental and water conditions may have effectively selected particular genomic and phenotypic features for this population. For instance, cyst size of this population, larger than that of other Chilean populations, appears to be associated with the particular ionic composition of the water in this site (Castro et al., 2006). In addition, analysis of the association between amount of interphase

heterochromatin and the ionic concentration of the brines revealed a species-specific negative association with the sulphate and calcium ions, only in the case of *A. franciscana*. Given that the brine habitats of populations of this species are mainly Thassalohaline waters, whose ionic composition is characterized as being rich in chloride and sodium and poor in sulphate and calcium ions (a result that was confirmed by the Piper's diagram), this association could be explained by the scarce presence of these ions in their natural habitats.

The result indicating interspecific or interpopulation differences in the relative amount of interphase heterochromatin found in *Artemia* is significant. The origin of this variation may have other causes, such as difference in genome size, or the particular evolutionary process of the karyotype. In the case of differences in genome size, greater amount of heterochromatin is expected in species with a large genome size than in those with a smaller genome size, a pattern that has been demonstrated in evolutionary closely related organisms (Rayburn et al., 1985; Kao et al., 2001; Bosco et al., 2007). Although this hypothesis is interesting, to date it is not possible to contrast *Artemia* species, since data is only available for the genome size of *A. franciscana*, reaching a value of 0.97 pg per haploid genome (De Vos et al., 2013). Differences in the evolutionary process of the karyotype could also be taken into account given that *A. franciscana* appears to be at more advanced stage of evolution than *A. persimilis* (Parraguez et al., 2009). Thus, it is expected that the former would accumulate more heterochromatin in the chromosomes than the latter. With the exception of the TPA and CIS populations of *A. persimilis*, which presented large amounts of interphase heterochromatin, most populations of both species studied followed this pattern. Hence in our case, this hypothesis seems to be consistent.

According to the evidence available in *Arabidopsis*, nuclear changes induced by heterochromatin cannot be ruled out, given the identification of various genes that specifically control such processes (Fransz et al., 2003; Wang et al., 2013). For example, in the *ddm1* mutant, it is observed that the heterochromatin content is significantly reduced in the nucleus (-30%) in comparison to the wild type. This is reflected at the cytological level in lower chromocenter numbers with small sizes. Likewise, in the *CRWN4* mutant the chromocenters are notoriously disorganized or dispersed when compared to those observed in a normal nucleus. Based on these cytological patterns, it is possible to classify the organization of the nucleus in *Arabidopsis* according to the appearance of chromocenters, either as adispersed phenotype (*CRWN4* mutant) or as acompact phenotype (wild type). It is important to note that the arrangement of chromocenters in the nucleus of some *Artemia* populations analysed in this study may match these phenotypes. For example, LLA, CHX and RGB populations would represent a dispersed chromocenter phenotype, while LVI population would correspond to a compact chromocenter phenotype (see Figure 1). Thus, we cannot discard the possibility that these phenotypes may reflect the existence of a particular organizational process of the heterochromatin in the nucleus, across different *Artemia* populations, whose control may depend on the action of specific genes. In addition, chemical and physical factors may also be involved in the packing status of the chromocenters in *Artemia* populations, given that natural populations of this organism may be subject to many abiotic stressors, such as changes in temperature, ionic composition and salinity (Gajardo and Beardmore, 2012). Although the participation of these factors has not generally been demonstrated in *Artemia*, experimental data collected in other organisms reveals their existence. For instance, in NIH 3T3 mouse cells treated with valproic acid produce decondensation in the chromatin structure of the nucleus, including the heterochromatin areas (Felisbino et al., 2014); while in Malpighian tubule cells of the blood-sucking insect, *Triatoma infestans*, heterochromatin decondensation occurs either after treatments with heavy metals (copper and mercury) or heat shocks (Mello et al., 1995, 2001). Moreover, heterochromatin decondensation after heat shock treatements in Malpighian tubule cells of a vector of Chagas' disease in Brazil, *Panstrongylus megistus*, infected and non-infected by *Trypanosoma cruzi*, has been also reported (Garcia et al., 2011). In our case we believe that such factors would not have affected the results obtained since the samples analysed are from laboratory cultures that were kept under standardised conditions of temperature and salinity. However, the chemical or physical factors that might be affecting the condensation of heterochromatin in the interphase nucleus in natural populations of *Artemia* emerge as an interesting topic for future studies.

Further studies on heterochromatin in *Artemia* aimed at providing more in depth information about its organization and structure may help to clarify the biological meaning of variation across populations. This type of analysis may contribute additional insight into the source of variation in the interphase heterochromatin, both at intraspecific and interspecific levels in *Artemia*.

5. Conclusion

The amount of interphase heterochomatin per nucleus that was estimated based on chromocenter number and relative area of chromocenter, in *A. franciscana* (n=9) and *A. persimilis* (n=3) populations from different locations of the Americas, varied significantly both within and between species. The relationship between relative area of chromocenter and nuclear size revealed a significant association, either negative or positive, in five out of twelve populations analysed. All populations pooled or categorised by species did not show a statistically significant association between both parameters. There was a significant association between relative chromocenter area and ionic concentrations of natural brines in the populations studied, with a positive correlation for magnesium and negative correlations for chloride, sodium and calcium. When populations were categorised by species, a negative and significant correlation with sulphate and calcium ions was found for *A. franciscana*. These findings suggest the

existence of a nucleotypic effect on nuclear size in some populations of *Artemia* from the Americas, mediated by the variation in the amount of interphase heterochromatin of the nucleus. Moreover, the association of this parameter with the ionic composition of natural brines suggests that it could be involved in the ability of this organism to survive in these environments, which are habitually subject to strong physicochemical changes.

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