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RESEARCH ARTICLE

Does shell shape variation play a role in conservation of the long-lived freshwater bivalve *Diplodon chilensis* (Bivalvia, Hyriidae)?

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Abstract

Freshwater bivalves of the order Unionoida display an uncommon phenotypic plasticity with high interpopulation and intrapopulation morphological variability, which could be advantageous for coping with habitat modifications. However, unionoids have suffered a marked population decline in different parts of the world in the last decades. A decline in some populations of the South American long-lived freshwater mussel *Diplodon chilensis* as a consequence of habitat deterioration has recently been recorded. Ontogenetic allometry and shape variation in shells of *D. chilensis* from 2 different sites, Paimun lake and Chimehuin river, North Patagonia, Argentina, have been studied. For these purposes, geometric morphometric methods were used. Shell shape shows differences between sites, which the shells from Chimehuin river show less intrapopulation variability; are more elongated, with the anterior part extended upwards and the posterior part downwards; and show a steeper anterior curvature at the umbo compared to those from Paimún lake. These characteristics make shell shape more streamlined to withstand river current. Furthermore, the extended posterior-ventral part in river shells coincides with higher foot weight that would improve anchoring to the river rocky–sandy substrate. River shells present a bounded eco-morphotype whereas the higher variability of lake shells includes the "river eco-morphotype." Growth is allometric throughout life in both sites and is not sex-dependent. The success of river repopulation programmes using mussels from lake populations may be increased by transplanting selected individuals that show "river eco-morphotype."

KEYWORDS

Diplodon chilensis, geometric morphometric, lake, ontogeny, Patagonia, river

1 | INTRODUCTION

The freshwater mussel *Diplodon chilensis*, order Unionoida and family Hyriidae, is one of the most abundant benthic species both in lentic and lotic Patagonian environments. Its distribution ranges from

32°52′S to 45°51′S in southern Argentina and from 30°28′S to 46°37′S in Chile (Bonetto, 1973; Castellanos, 1959; Semenas, Ortubay, & Úbeda, 1994). The life cycle of this species is characterized by a reproductive phase in spring–summer and a faster shell growth period during winter (Semenas & Brugni, 2002; Soldati, Jacob,

Schone, Bianchi, & Hajduk, 2009). As in most unionoid mussels, the life cycle of *D. chilensis* involves parasitic larvae, which can attach to the gills of native as well as invasive fishes (Ortubay, Semenas, Úbeda, Quagiotto, & Viozzi, 1994; Semenas & Brugni, 2002; Viozzi & Brugni, 2001). This flexibility is thought to be one reason for the success (in terms of abundance) of *D. chilensis* in this region compared to other unionoid mussels (Skinner, Young, & Hastie, 2003; Young & Williams, 1984a, 1984b).

The major life phases of freshwater mussels, parasitic larvae, juvenile, and adult, involve different feeding modes (Watters, Hoggarth, & Stansbery, 2009). During the first years, juvenile mussels can live completely buried 10 cm deep, feeding on detritus and interstitial algae and bacteria, whereas adults expose the posterior edge and the pseudosiphons to feed in gravel, sandy, or muddy substrates (Lara & Parada, 1988; Lara & Parada, 2008; Österling, 2006). Consequently, adult individuals are more exposed to predators and environmental changes than juveniles. Previous experimental results suggest that adult *D. chilensis* mainly feeds on phytobenthos including bacteria and less frequently on phytoplankton (Lara, Contreras, & Encina, 2002). It has also been shown that population density and growth rate of this species correlate with substrate organic matter content and with the concomitant variations in phytobenthic community (Lara et al., 2002; Lara & Parada, 1988; Rocchetta et al., 2014).

The freshwater mussels of the order Unionoida are long-lived bivalves with slow growth; however, environmental conditions can cause important differences in life expectancy and growth rate between populations of the same species (Anthony, Kesler, Downing, & Downing, 2001; Cummings & Bogan, 2006; Watters et al., 2009). For *D. chilensis*, Rocchetta et al. (2014) have reported that differences on growth rate among three neighbour populations from North Patagonia (Argentina) are related to organic matter quality and quantity. In addition, environmental conditions (e.g., temperature, depth, and water flux) as well as ecological factors (e.g., population density, predation, and quality and quantity of food) may influence behavioural, physiological and morphological features related to the phenotypic plasticity of this species (Parada & Peredo, 2005; Peredo, Jara-Seguel, Parada, & Palma-Rojas, 2003), as has been reported for other unionoid mussels (Watters et al., 2009).

Within the frame of the alarming population decline of freshwater mussels reported worldwide (Carella, Villari, & De Vico, 2016), recent works report a decline in *D. chilensis* populations in Chilean and Argentinean lakes as a consequence of habitat deterioration (Rocchetta et al., 2014; Valdovinos & Pedreros, 2007), which may affect the biodiversity of limnic communities as the efficient filter feeding activity of this species plays a crucial role in oligotrophic maintenance (Parada, Peredo, Cárdenas, Valdebenito, & Peredo, 2008; Soto & Mena, 1999). Nevertheless, river populations of this species have been the most affected, showing an alarming depletion in Chilean rivers (Parada & Peredo, 2005; Parada, Peredo-Parada, Quevedo, & Peredo, 2009).

Conservation efforts, including repopulation management, through translocation of individuals from lakes to rivers, have been conducted in Chile (Parada & Peredo, 2005; Peredo, Parada, Valdebenito, & Peredo, 2005, 2006). These works were based on reproductive and ecological studies aimed at identifying the best health and habitat conditions for relocation, in order to increase percentage survival. However, further knowledge of relevant features of this species' biology, such as the relationship between shell morphology and environmental variables (Morais, Rufino, Reis, Dias, & Sousa, 2013), is needed to improve management strategies and postrelocation survival.

Recently, geometric morphometric analysis has been increasingly employed in bivalve studies with different objectives: recognition of phenotypic fishery stocks (Márquez, Robledo, Escati Peñaloza, & Van der Molen, 2010; Palmer, Pons, & Linde, 2004; Rufino et al., 2012), paleoenvironmental and paleoclimate reconstruction (Aguirre, Perez, & Sirch, 2006; Aguirre, Richiano, Álvarez, & Farinati, 2016; Gordillo, Márquez, Cárdenas, & Zubimendia, 2011), and analysis of evolutionary processes (Schneider, Fürsich, Schulz-Mirbach, & Werner, 2010; Sherratt, Alejandrino, Kraemer, Serb, & Adams, 2016; Tang & Pantel, 2005). In addition, geometric morphometrics have been used to study ontogenetic shape changes (Márquez, Amoroso, Gowland Sainz, & Van der, 2010) and to evaluate the invasive success of introduced freshwater species (Sousa et al., 2007). Among geometric morphometrics techniques, landmarks together with semilandmarks have been successfully applied recently in bivalve studies (Leyva-Valencia et al., 2012; Morais et al., 2013).

In order to improve management strategies for repopulation programmes, this paper aims to (a) analyse the ontogenetic allometry of *D. chilensis* obtained from two different locations, Paimun lake and Chimehuin river, and (2) compare shell shape variation within and between these two populations by using geometric morphometric analysis.

2 | MATERIALS AND METHODS

2.1 | Sample collection and study area

Shells were sampled in two neighbouring areas of the same basin, Paimún lake (39°44′48″ S 71°31′30″W) and Chimehuin river (39°54′57″S 71°6′23″W), North Patagonia, Argentina, in 2012. Both the lake and the river are characterized as oligotrophic water bodies of glacial origin (Figure 1) and are connected through the Huechulafquen lake. There is almost no anthropic activity in the study area, because the lake is located within the Lanin National Park. Seasonal environmental characteristics at the two sampling sites were obtained by combining literature and our data. Mean temperature, conductivity, dissolved oxygen, and pH were measured *in situ* during winter and summer by using a multiparameter analyser (Hanna HI 9828). Water samples were collected in triplicate from the river and the lake to analyse chlorophyll a concentration following Lichtenthaler (1987) in filtered samples through glass fibre filters (Whatman GFF, 0.45-mm pore). Total phosphorous and nitrogen concentration were measured after acid persulfate digestion at 120 °C for 55 min (HACH Method 8048 and 8192, respectively). Bivalves were collected by hand by SCUBA diving at a depth of 1 m in the river and 8 m in the lake. Tissue dissection was carried out, and fresh foot weight was obtained. Sex was recorded by analysing gonads with a light microscope. Shells were washed, dried, and used for the following analyses.
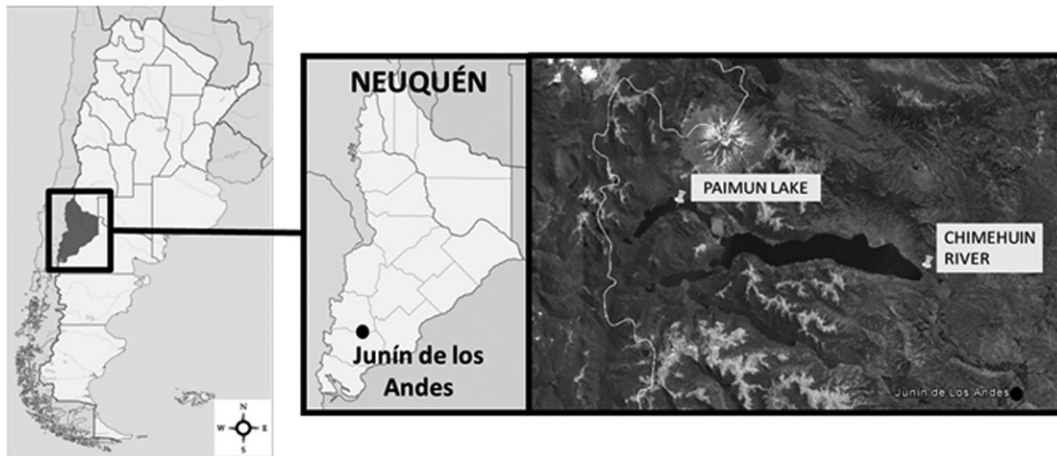
FIGURE 1 Location of sampling sites, Paimun lake and Chimehuin river, Neuquén Province, Argentina

2.2 | Individual age estimation

Internal shell growth bands of 76 right valves of *D. chilensis* from each site were counted as described by Rocchetta et al. (2014). Briefly, after washing and drying, each valve was embedded in epoxy resin (GLUTEC Wiko Epoxy Metal 05) and sectioned along the axis of maximum height growth with a circular table saw (PROXXON FKS/E No. 28070). The cross-sections were polished on lapidary wheels (BUEHLER alpha 2 Speed Grinder-Polisher) using grits of 600, 1,200, 2,500, and 4,000 grade. Dark and light patterns were observed in the polished shells under a stereo microscope (Olympus SZX 12). The number of dark lines was counted and taken as annual marks and used as age estimator. This annually formed pattern in *D. chilensis* was previously validated by stable oxygen and carbon isotopes ratio analysis (Soldati et al., 2009).

2.3 | Morphometric analysis

Geometric morphometrics were used to capture the Cartesian coordinates of a two dimensional configuration of 10 landmarks and 30 semilandmarks. Details of the framework of geometric morphometrics using landmarks can be found in Zelditch, Swiderski, Sheets, and Fink (2004). Landmarks and semilandmarks information were combined from points and curves for a more comprehensive quantification and analysis of shell shape (Gunz & Mitteroecker, 2013). The landmarks were defined as (a) anterior insertion point of the pseudocardinal tooth, (b) posterior insertion point of the pseudocardinal tooth, (c) insertion point of the upper lateral tooth, (d) insertion point of the lower lateral tooth, (e) intersection point between the pallial line and the posterior adductor muscle, (f) posterior point of the pallial line, (g) intersection point between the pallial line and the posterior adductor muscle, (h) intersection point between the contour line and the straight linking 3 and 1, (i) intersection point between the contour line and the straight linking 1 and 7, and (j) intersection point between the contour line and the straight linking 4 and 5. The 30 semilandmarks were located along the contour line, 5 between landmarks 10 and 8, 8 between landmarks 8 and 9, and 17 between 9 and 10 (Figure 2).

To avoid rotation, translation, and scale effects, the landmark coordinates were superimposed by Procrustes analysis (Rohlf & Slice,
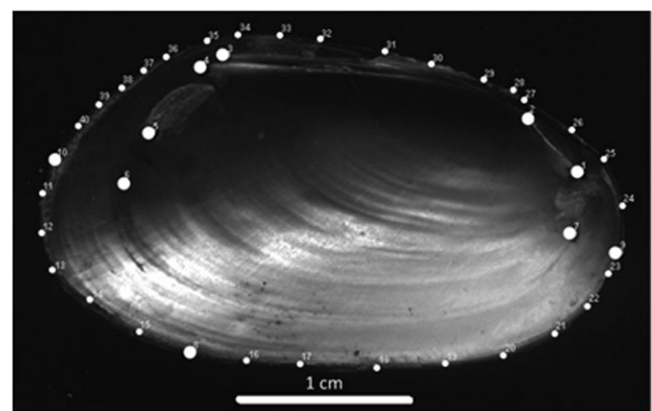

FIGURE 2 Position of the 10 landmarks (big dots) and 30 semilandmarks (small dots) on the left valve of *Diplodon chilensis*

1990) using the TPSRelw software (Rohlf, 2004). An algorithm included in this programme was also used, which allows relaxing the semilandmarks along the contour tangents, to minimize the bending energy until they fit a reference configuration (Bookstein, 1997). The aligned Procrustes coordinates were then used as shape variables, and centroid size (CS) was used as proxy of shell size to perform multivariate statistical analyses. CS was computed as the square root of the sum of the square distances from all landmarks to their centroid. Comparison of foot weight between sites was carried out by estimating the ratios between foot weight (g) and CS (cm) in mussels of the same age range.

2.4 | Statistical analysis

Differences among sites (Paimun lake and Chimehuin river) and seasons (summer and winter) in environmental variables were analysed using two-way analysis of variance followed by a Bonferroni's post hoc test. To assess and control putative ontogenetic and allometric effects, the pooled within-sites multivariate regression of shape (aligned Procrustes coordinates were used as dependent variables) on ln-age and ln-centroid size (independent variables) was computed (Bookstein, 1991; Klingenberg, 2011). To evaluate the independence

between shape, and age and size variables, we carried out a permutation test with 10,000 rounds (Bookstein, 1991; Zelditch et al., 2004).

To study the magnitude and direction of shell shape variation, a principal component analysis of the variance–covariance matrix (Zelditch et al., 2004) was performed, and males and females were identified. In order to visualize and test the separation of shell shapes between Chimehuin river and Paimún lake, a linear discriminant analysis was performed, using leave-one-out cross validation procedure to estimate the reliability of the discrimination. Shape differences along the discriminant axis were described using a wireframe graph. The statistical significance of pairwise differences in mean shapes was assessed with permutation tests using Mahalanobis distance as statistic test (1,000 permutations per test). Differences between populations in foot weight/CS ratio were analysed by t test.

3 | RESULTS

Table 1 summarizes environmental characteristics from Paimún lake and Chimehuin river, showing their oligotrophic condition. The comparison between sites and season showed higher conductivity values at Chimehuin river than Paimun lake ($p_{interaction} > 0.05$; $p_{site} = 0.004$; $p_{season} > 0.05$). Mean dissolved oxygen during winter at Paimun lake was the highest recorded ($p_{interaction} = 0.008$) coincident with the peak in chlorophyll a ($p_{interaction} < 0.0001$). Total phosphate

was significantly higher in samples collected from Chimehuin river than those from Paimun lake during summer; however, this relationship was opposite during winter ($p_{interaction} = 0.02$).

The age of the collected shells ranged from 1 to 118 years for Paimún lake and from 2 to 59 years for Chimehuin river. No significant differences between sexes in shell shape were found (p value = 0.087 analysing 55 females and 54 males). The multivariate regression of shape versus ln-transformed age was significant ($p < .001$); however, age was not the main source of morphological variation ($r^2 = 0.058$; Figure 3). Despite the regression of shape versus size explained only 6% of the variability ($p < 0.001$), its residuals were used as a new size-independent shell shape variable for subsequent analyses.

The first three principal components (PCs) of shell shape variation in lake and river explained 84% of the total variation. PC1 (69% of total variation) showed a wider phenotype for the shape of shells collected from Paimún lake compared to those collected from Chimehuin river, which showed a bounded phenotype (Figure 4). The geometrical interpretation of the negative extremes of PC1 was associated with an enlargement of the shell's dorso-ventral axis, showing the anterior part upward and posterior part downward and steeper anterior curvature at the umbo. The positive extremes of PC1 showed the opposite. The other PCs were not taken into account because the values for specimens from the river and lake were mostly overlapped.

The mean Chimehuin river shell shape was different from that of Paimun lake (T-square: 1,234.9, p value for permutation tests

TABLE 1 Summary of environmental characteristics at the two sampling sites, Paimun lake and Chimehuin river, in summer and winter

	Paimun lake		Chimehuin river		
Variable	Summer	Winter	Summer	Winter	Reference
Temperature (°C)	13.49 ± 0.14[A]	5.59 ± 0.04[B]	11.35 ± 0.18[C]	6.28 ± 0.01[D]	This paper
Conductivity (μS/cm)	39.33 ± 0.58[A]	38.00 ± 0.35[A]	42.67 ± 1.15[B]	42.67 ± 3.21[B]	This paper
Disolved oxygen (mg/L)	9.13 ± 0.42[A]	12.43 ± 0.06[B]	9.23 ± 0.06[A]	9.93 ± 0.23[A]	This paper
pH	6.61 ± 0.04[A]	6.51 ± 0.01[A]	6.17 ± 0.23[A]	6.62 ± 0.07[A]	This paper
Chlorophyll a (μg/L)	1.79 ± 0.67[A]	4.42 ± 0.14[B]	1.02 ± 0.1[C]	0.41 ± 0.03[D]	This paper
Total N (mg/L)	0.15 ± 0.06[A]	0.19 ± 0.02[A]	0.18 ± 0.04[A]	0.10 ± 0.01[A]	This paper
Total P (mg/L)	0.06 ± 0.02[A]	0.24 ± 0.09[B]	0.21 ± 0.07[B]	0.16 ± 0.01[C]	This paper
Subtrate	Muddy–sandy		Rocky–sandy (main course) and muddy (margins)		This paper
Surface	16 km^2		2,649 km^2		1
Origin	Postglacial lake		Huechulafquen lake		1
Annual flow rate	—		70 m^3/seg		1

Differences between sites and season were analysed by two-way analysis of variance. Different letters indicate significant differences with $p < .05$ ($n = 3$). Results are expressed as mean ± SD. 1, Quiros & Drago, 1985; total N = total nitrogen; total P = total phosphate.
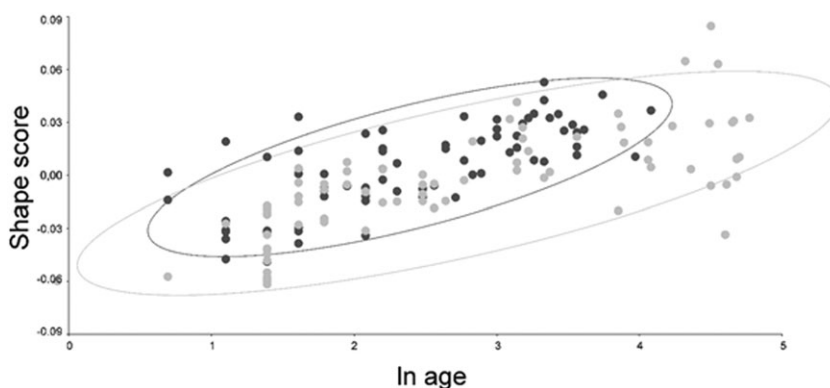

FIGURE 3 Shape scores as a function of ln-transformed age of *Diplodon chilensis* from Paimun lake (grey dots) and Chimehuin river (black dots) population
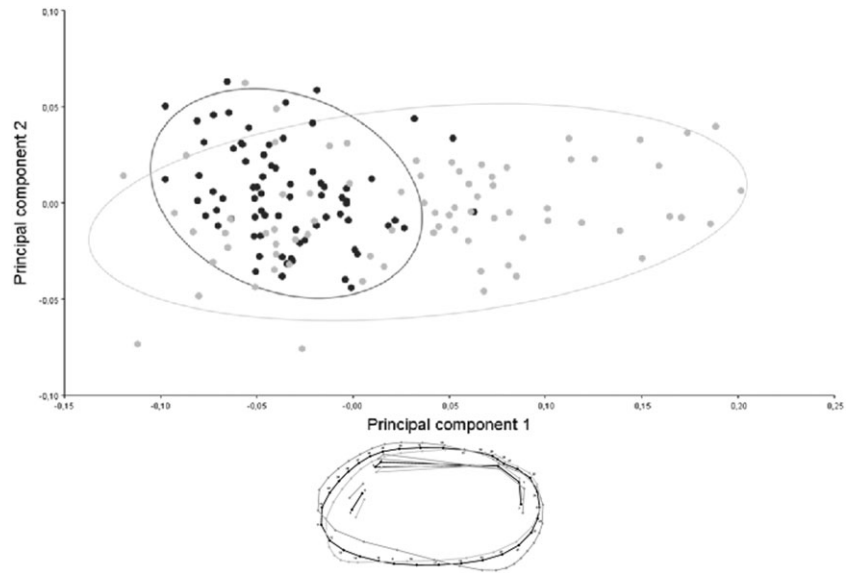
FIGURE 4 Analyses of shell shape variation along the first two principal components axes of *Diplodon chilensis* from Paimun lake (grey dots) and Chimehuin river (black dots) population. The diagram at bottom shows the principal component 1 (PC1) extreme morphotypes configurations (black line: consensus, grey line: positive extreme, light-grey line: negative extreme)

<0.0001). The discriminant analysis between sites showed that the mean shell shape calculated for Chimehuin river was more elongated, with the anterior area upward and the posterior area downward, steeper curvature at the umbo, shorter pseudocardinal tooth, and less pointed posterior area than the mean shell shape obtained for Paimún lake (Figure 5). Results for the percentage of individuals that were correctly classified and Mahalanobis distances, from both discriminant function and cross validation, are shown in Table 2. Besides, the average foot weight/CS of bivalves from Chimehuin river is higher than that of Paimún lake bivalves (0.023 ± 0.002, n = 47 for Chimehuin river versus 0.014 ± 0.002, n = 33 for Paimún lake, p = 0.005).

4 | DISCUSSION

This study shows that shell morphology in *D. chilensis* is not mainly associated with ontogenetic factors neither with sex; however, there are differences in shape between the two sites analysed. Chimehuin
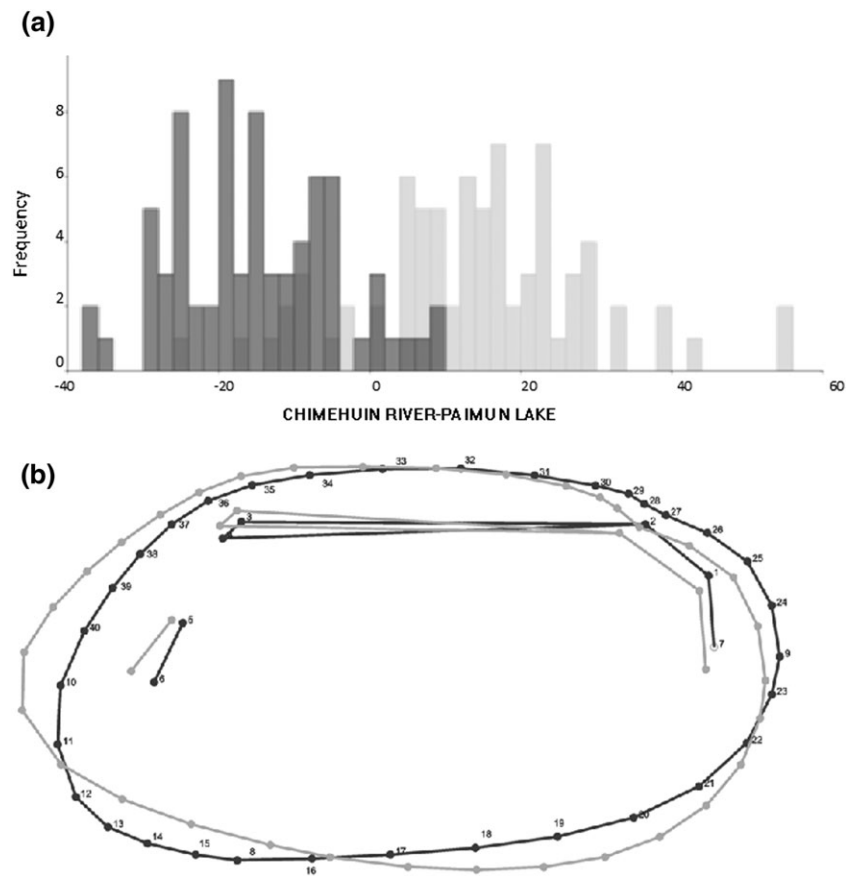

FIGURE 5 (a) Frequency distribution of the first canonical function scores for Paimun lake (grey) and Chimehuin river (black) populations by cross validation analysis. (b) Mean shape of *Diplodon chilensis* (lake and river) after cross validation analysis (scale factor = 2)

TABLE 2 Classification matrix with the discriminant and jacknife cross-validated classification, differences between means, and Mahalanobis distances of each site

Site	Chimehuin river	Paimun lake	Total	Percentage correct	T-square	D²
Discriminant function					<0.0001	5.74
Chimehuin river	76	0	76	100		
Paimun lake	1	75	76	98.68		
Cross validation						
Chimehuin river	68	8	76	89.47		
Paimun lake	11	65	76	85.52		

river population's shell shape presents a more bounded eco-phenotype whereas the Paimún lake population shows a wider variability in shell shape that includes the "river eco-phenotype."

Despite the clear differences in life style between juveniles and adults regarding burial ability, feeding habits and food availability, *D. chilensis* shell shape remains constant over lifetime both in the river and in the lake population. In contrast, shell shape changes related to changing behavioural patterns throughout life have been reported for several species (Demayo, Cabacaba, & Torres, 2012; Guarneri et al., 2014; Márquez, Amoroso, Gowland Sainz, & Van der Molen, 2010).

Shell shape differences between Paimún lake and Chimehuin river populations seem to be related to the environmental conditions of the sampling sites. Several environmental variables showed differences between sites with seasonal differences, mainly in Paimun lake. The sites are rather close (33 km) and are hydrographically connected. Hence, genetic flow between river and lake populations is likely to occur through glochidium larvae attached to the gills of fish, which move between both sites (Semenas & Brugni, 2002; Semenas et al., 1994; Walker, Byrne, Hickey, & Roper, 2001). Accordingly, studies on different *D. chilensis* populations in Chilean Patagonia propose a high genetic flux between water systems, even with important geographical barriers between them, demonstrating that there was a unique population with the same genetic variability (Fuentealba, Figueroa, González, & Palma, 2010). However, the fact that the alozymes studied by these authors have very low genetic polymorphism casts some doubts about significance of the results obtained. On the other hand, phenotypic plasticity has been previously reported for *D. chilensis* collected from lotic and lentic environments. This was attributed mainly to changes in physicochemical parameters such as regional winds, temperature, pH, dissolved oxygen, and current (Parada & Peredo, 1994). Thus, the fact that the individuals collected from Chimehuin river are restricted to a shell shape eco-morphotype could be attributed to phenotypic plasticity rather than to genetic differences. Chimehuin river is characterized by a rocky–sandy bottom in the main course, with muddy banks in low-energy areas at its margins. Within the studied area of this river, most adult bivalves live in the main course, sheltered from the current behind rocks. The elongated anterior area upward and the steeper anterior umbonal curvature observed in this study may enhance anchoring ability, thus increasing stability under the dynamic river condition. Watters et al. (2009) suggested that the prominent umbo found in river bivalves could result in a more streamlined body. In concordance with the results observed in *D. chilensis*, a steeper umbonal curvature has also been observed in other mussels such as *Unio delphinus* from river and estuarine environments and related to a better burrowing capacity (Morais et al., 2013).

Lara and Parada (2009) have reported that *D. chilensis* usually adopts vertical, horizontal, or oblique position in sandy or muddy substrates; whereas in gravel-sandy bottoms, it is rarely found in vertical position. The depression in the dorsal posterior region of river bivalves observed in our study suggests a reduction of water current resistance in that shell region that always remains exposed for feeding and respiratory functions. The wider ventral region of these shells could also contribute to a better anchoring to the substrate. This advantage would be complemented by the higher foot weight found in Chimehuin river bivalves compared to those of Paimún lake. Zieritz and Aldridge (2009) have reported similar characteristics in the dorsal posterior region for three different unionoid species collected from the river Thames and its marinas and have related these characteristics to hydrological conditions. In accordance, several authors suggest that hydrological conditions and substrate composition are the main factors that can induce shell shape modifications (Demayo et al., 2012; Guarneri et al., 2014; Hornbach, Kurth, & Hove, 2010). Moreover, according to Morais et al. (2013), water velocity is the main hydrological variable that influences shell morphology in unionoid mussels.

In addition, Lara and Moreno (1995) have proposed that sandy–rocky substrates can provide greater refuge and food abundance between rocks than sandy substrates, reducing predation on *D. chilensis* by, for example, *Aegla* sp. But bivalves living in Chimehuin river and possibly in rivers and streams of the same region with similar sandy–rocky bottoms cannot be completely buried and are, thus, more exposed to other predators. The dorsal posterior depression of Chimehuin river shells could help to reduce the exposed area.

Intrapopulation analysis shows higher shell shape morphological variability in Paimún lake bivalves than in the Chimehuin river ones, which show a more bounded phenotype. Accordingly, growth studies on *D. chilensis* in a nearby area (Nahuel Huapi National Park, North Patagonia), have shown higher variability of annual shell growth increment in young individuals from a lake population compared to those collected from a river population (Soldati et al., 2009). Besides, environmental variables further differed seasonality at Paimun lake that would be consequence of stratification and mixture cycles. This bounded phenotype in river populations would result in lower capacity to respond to environmental changes or habitat modification.

Peredo et al. (2005) have reported that the relocation of *D. chilensis* from a lake to restore depleted river populations resulted in 24% survival after 18 years, the period in which adult bivalves have completely adapted from the lentic to the lotic environment. According

to this, lake populations can be considered to be a suitable source for repopulation programmes (Lara & Parada, 2008; Parada & Peredo, 2005; Peredo et al., 2005, 2006). The results obtained in *D. chilensis* indicate that future repopulation programmes could be improved by selecting lake individuals that closely resemble the "river eco-phenotype," which would increase post-transplantation survival rate. Because shell shape in this species is not affected by age neither by sex, this procedure would involve little additional effort. Because population genetics of *D. chilensis* has been scarcely studied so far, future studies in this subject would be helpful to improve repopulation management practices.

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