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Genetic, morphological, and life history traits variation in freshwater snails from extremely high environments of the Andean Altiplano

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Abstract

Background: The isolated watersheds of the southwestern Andean Altiplano constitute a natural laboratory to study the evolutionary divergence of freshwater biota. Field observations showed that *Biomphalaria* snails from Parinacota, Colpa, and Caquena have different shell sizes. We performed morphometric analysis and common garden experiment to evaluate whether the observed shell variation has a genetic base and if this variation is manifested in other morphological characters and life history traits.

Results: Network analysis revealed that the snails of Parinacota form a lineage genetically distinct from Caquena and Colpa. The morphometric analysis of the shell showed that the Parinacota snails were larger than Caquena and Colpa, both in nature and laboratory conditions, but there was no evidence of difference in the shape of the shell when compared using multivariate analyses. The number of eggs per ovicapsule was the only life history trait that was significantly different between lineages, although this difference may be also attributed to size of the progenitor; the oviposition rate did not differ between lineages or localities, and the hatching size and growth rate differed only at the locality level, not lineages.

Conclusion: The results suggest that shell size of the snails has a genetic basis associated to the phenotype, while the expression and evolution of life history traits in extreme high environments are highly influenced by proximal causes.

Keywords: Planorbidae; *Biomphalaria*; Haplotype network; Shell morphology; Local adaptation; Chilean Altiplano

Background

The Neotropical Andean Altiplano is an intermountain depression that spans almost 400,000 km² and has a mean of 4,000 m altitude (Lavenu et al. 1984; Argollo and Mourguiart 2000; Risacher et al. 2003; Placzek et al. 2006). In the southwestern area, the landscape has suffered an intense volcanic and sedimentary activity since the Miocene to the present (Clappeston 1993; Wörner et al. 2000; Risacher et al. 2003), which has generated a number of closed endorheic basins whose central depression is occupied by a variety of hydrologic systems with different physicochemical properties (Chong 1988; Keller and Soto 1998; Márquez-García et al. 2010). During the Pleistocene, these systems were connected by a series of paleolakes (Lavenu et al. 1984; Fornari et al. 2001; Placzek et al. 2006)

that underwent successive cycles of expansions and regressions, which have been associated with the fractioning of the freshwater biota (Northcote 2000; Collado et al. 2011; Morales et al. 2011; Vila et al. 2011; Lüssen et al. 2003; Vila 2006) and the generation of ecosystems with a high degree of endemism (Veloso and Bustos-Obregón 1982; Rundel and Palma 2000; Vargas et al. 2004). Thus, this region constitutes a natural laboratory to study evolutionary divergence.

It has been suggested that the fragmentation of the habitat may reduce gene flow among populations, producing morphological and life history adaptations as a response to local environmental conditions (Pfenninger and Posada 2002). One way to evaluate how much of the observed variation among populations is due to differences between genotypes and how much is due to environmental influence is to perform 'common garden experiments', in which individuals from contrasting environments are raised under the same conditions (Conover and Schultz 1995). If the differences

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observed in nature persist in a common environment, they may be ascribed to genetic causes, while the environment must be the main cause of the variation if the differences disappear when the organisms are cultivated in the laboratory (Conover and Schultz 1995).

The freshwater snails of the genus *Biomphalaria* Preston, 1910 are found in a number of aquatic habitats, varying from shallow water to lentic environments or with weak currents (Barbosa and Barbosa 1994). Considering that its aquatic life cycle links them directly with the evolution of the hydrologic systems, these snails provide an ideal model to evaluate the variation in morphological and life history traits between populations spanned in fragmented habitats. Besides, these snails are hermaphrodite capable of selfing, although a preference for outbreeding has been reported in the group (Barbosa and Barbosa 1994). Furthermore, they have direct development and limited vagility increasing the possibility of divergence among populations since juvenile emerges as miniature adults in the habitat occupied by their parents.

Ecological researches in freshwater planorbids have shown that their population dynamics depends upon the physical geography of the region like terrestrial limits, soil composition, hydrography, and climate, as well as physical, chemical, and biological factors (Barbosa and Barbosa 1994). For example, in temperate and high-altitude tropical zones, low temperature interrupts or reduces reproduction in species of this group (Dazo et al. 1966). It has also been shown that the resistance to desiccation and reproductive capacity is variable within and between species (Barbosa and Olivier 1958; Olivier and Barbosa 1955a, b). Although high variability in reproductive characters such as clutch size or oviposition rate has been correlated with resource availability and environmental conditions, suggesting proximal causes, low variability among years in this kind of attribute may suggest a genetic-historical component associated with the phenotype.

In the southwestern Andean Altiplano (17° S to 22° S), the populations of *Biomphalaria* have discontinuous distribution associated with the fractionating of the landscape and the mosaic of habitats generated within and between basins (Valdovinos 2006; Collado et al. 2011). Observations by our group in the Chilean Altiplanic region of Arica and Parinacota showed that the snails from Parinacota in the Lauca basin are notoriously larger than those from Caquena and Colpa in the Caquena basin. In this paper, we use morphometric methods and performed common garden experiments to evaluate whether the morphological variation has a genetic basis and if these differences correlate with variation in life history traits. The material studied included snail samples from the Altiplano localities situated above 4,380 m altitude.

Methods

Sampling

Snails were collected from three hydrological systems of the southwestern Altiplano. We collected snails from the Parinacota wetland (18°11′ S, 69°15′ W, 4,399 m), located near the altiplanic town of Parinacota; this system has an extension of 27 km^2. The other watersheds sampled were Caquena (18°3′ S, 69°12′ W, 4,398 m) and Colpa wetlands (18°3′ S, 69°13′ W, 4,384 m), both located at 28 km northwest from Parinacota, in the Caquena basin (Figure 1). The approximate surface of Caquena and Colpa is 16 and 4 km^2 respectively. The snails were captured at least from three random sites within each locality with a small plastic net, by gently agitating aquatic plants.

Haplotype network

Previous molecular phylogenetic analyses suggest that *Biomphalaria* snails from the Caquena basin and those from the Lauca basin belong to different lineages (Collado et al. 2011). To further evaluate the lineage memberships of populations included in the present study, we analyzed published sequences of the cytochrome oxidase subunit 1 (COI) gene (GenBank: GU168060-GU168084, GU168087-GU168089, Collado et al. 2011). We incorporated 13 sequences from snails inhabiting localities situated in the Caquena basin, including one from the Colpa and three from the Caquena wetlands and fifteen snails more from localities situated in the Lauca basin (including three from the Parinacota wetland). The sequences were aligned by visual inspection in the BioEdit program (Hall 1999). The nucleotide frequency and percentage divergence between groups of sequences were estimated using the MEGA 3.0 program (The Biodesign Institute, Tempe, AZ, USA) (Kumar et al. 2004). The relationships among haplotypes were visualized with the median joining network implemented in the Network program (Bandelt et al. 1999). Diversity indices (k, number of haplotypes; S, number of polymorphic sites; H, haplotype diversity; π, nucleotide diversity) were estimated in the program DnaSP 4.0 (Rozas et al. 2003). To estimate deviations from the model of selective neutrality, we calculated Tajima's D and Fu and Li's F statistics.

Shell morphology

To evaluate the existence of differences in length shell among populations, we first perform an analysis of variance (ANOVA) for the three sites. All specimens were measured using an ocular micrometer accurate to 0.01 mm. We then performed a multivariate morphometric analyses comparing 11 shell measurements (Figure 2) using principal components analysis (PCA) and linear discriminant analysis (LDA). All variables were natural log-transformed prior to multivariate analyses.
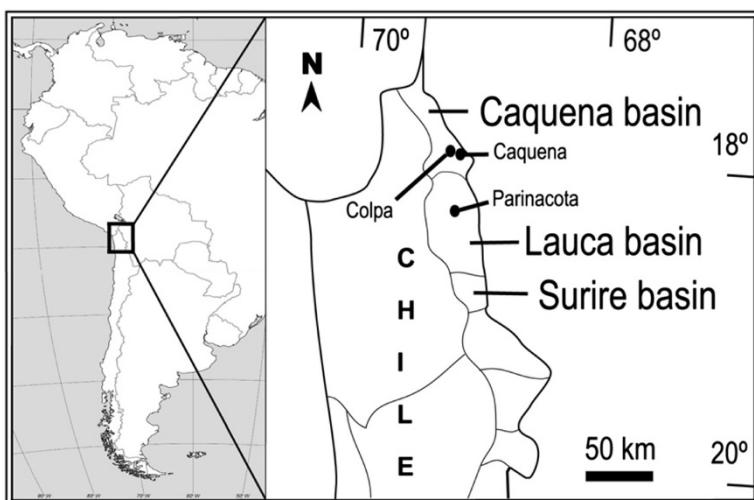
Figure 1 Location of the three collection sites in the southwestern Andean Altiplano.

Life history characters

Snails from the three localities were maintained in laboratory conditions in 1-l containers of water in a climate chamber at 20°C with a photoperiod of 12:12 light-dark and fed *ad libitum* with cooked lettuce. Twenty adult snails per locality were then placed individually in 200-ml flasks to be used as reproductive units. After 2 days, these parental (G_0) snails began to deposit ovicapsules in the walls of the flasks. We estimated the shell length, number of daily ovipositions, and number of embryos per ovicapsule for a period of 30 days. Later, the offpring (F_1) were separated from their progenitor and grouped by locality, date of birth, and experimental unit in groups of 15 to 20 individuals in 200-ml transparent containers and maintained in the same conditions as the G_0 individuals. We used 12 replicates for Colpa, 10 for Caquena, and 14 for Parinacota. We register the daily oviposition rate, number of embryos per ovicapsule, hatching size, and growth rate. Since not all individuals

began or finished reproduction simultaneously, we calculated the daily oviposition rate of parents dividing the number of ovicapsules deposited by the number of days that an individual was in the reproductive state. Snails that did not deposit ovicapsules were excluded from the analysis. The number of embryos per ovicapsule was estimated by direct counting. The hatching size of the offspring and the growth rate were measured with an ocular micrometer. To avoid pseudoreplication, we used the mean shell length per container in statistical analysis. To estimate the growth rate, we measured the shell length of the offspring weekly for 12 weeks.

To evaluate whether the lineage or locality explained better the variance of the life history traits, we compared the data using ANOVA. To evaluate the relation between number of eggs per ovicapsule and size of the progenitor we performed an analysis of covariance (ANCOVA), using the size of the progenitor as covariate. Statistical analyses were performed in Statistica v 6.0 (StatSoft Inc 2004).
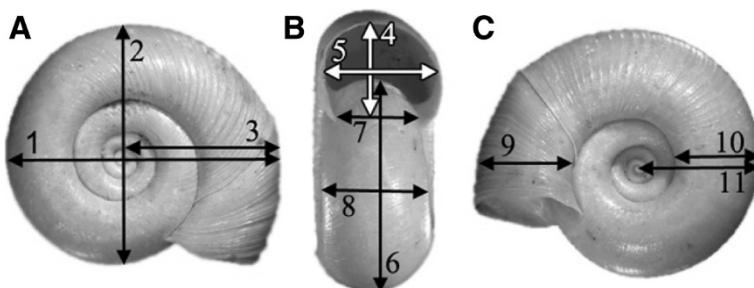
Figure 2 External shell morphology of *Biomphalaria* showing the 11 linear variables measured in this study. (A) Right side, **(B)** ventral side, and **(C)** left side. 1 Shell length, 2 shell height, 3 length of anterior extreme to the umbo, 4 peristome length, 5 peristome width, 6 ventral shell length, 7 length of peristomal callus, 8 ventral shell width, 9 anterior length of last whorl, 10 posterior length of last whorl, 11 length of posterior extreme to the umbo.

Results

Haplotype network

The alignment of the COI sequences was of 663 nucleotides. The overall nucleotide composition was 43.2% T, 13.2% C, 26.1% A, and 17.5% G. The COI haplotype network recovered two independent groups separated by 10 mutational steps, one integrated by snail sequences from the Lauca basin, including Parinacota, and the other integrated by snail sequences from the Caquena basin, including Caquena and Colpa (Figure 3). The genetic distance between the groups was 1.9%. Only one haplotype was found in the Caquena basin, thus, the diversity indexes were 0. For the Lauca basin, the diversity indices were $k = 8$, $S = 13$, $H = 0.838$ y $\pi = 0.00332$. Tajima's D and Fu and Li's F did not reject the hypothesis of selective neutrality for this basin ($D = -1.78$, $0.10 > p > 0.05$, $F = -1.92$, $P > 0.10$). We did not find haplotypes shared among haplogroups. Considering these results, in the analysis of the shell morphology and life history traits, we assessed whether the variance in these traits was better explained by the effect of lineages or localities using a nested two-way ANOVA using localities as a factor nested within the lineages.

Univariate analysis of the shell length of snails

The shell length of snails from Parinacota lineage was larger than those of Caquena-Colpa in snails captured from the field [nested ANOVA (lineages), $F_{(1,142)} = 190.058$, $p < 0.01$] (Figure 4A). No significant differences at locality level, nested under lineages, were found, implying that there was no difference between Caquena and Colpa. In the F_1 generation, lineages and populations showed significant differences in shell length [nested ANOVA (lineages), $F_{(1,272)} = 133.968$, $p < 0.01$; nested ANOVA (localities) $F_{(1,272)} = 8.140$, $p < 0.01$] (Figure 4B); snails from the Parinacota lineage were larger than those of Caquena-Colpa, and the snails from Caquena were

slightly higher than those of Colpa. The *a posteriori* analysis showed significant differences among all three localities.

Multivariate analysis of shell length of snails captured in the field

For snails captured in the field, PC1 explained 94.1% of the total variance in the samples. The visual inspection of the factor loadings suggests that this component was strongly influenced by the size of the individuals, while PC2 explains 2.3% of the variance and would be related to the shape of individuals. The first two orthogonal axes explained 96.4% of the variation in shell morphology among sites (Figure 5A). The characters with greatest loading for PC1 were shell length (–0.993), shell height (–0.990), and ventral shell length (–0.981). For PC2 the greatest loading was the length of the peristomal callus (–0.422). The PCA showed that in the morphometric space the snails of Parinacota separated partially from those of Colpa and Caquena, while these latter two populations had a high superposition (Figure 5A). This result is due to the individuals found in Parinacota were larger than those found in the two other populations. The LDA showed a significant discrimination capacity (Wilk's $\lambda = 0.3979$, $F_{(22, 264)} = 7.024$, $p < 0.001$) and found a mean of 73.8% correct classification for the three localities (Table 1). The difference in morphology was estimated using the square of the Mahalanobis distance, and this value was significantly lower among localities that belonged to lineage Caquena-Colpa than between these and Parinacota (squared Mahalanobis distance: Caquena-Colpa = 1.67; Caquena-Parinacota = 5.14; Colpa-Parinacota = 4.69). In this context, it is important to note that 14 of the 17 snails from Caquena incorrectly classified were assigned to Colpa and only 3 to Parinacota. We then also performed a PCA of the variables corrected by the length of the individuals. The PC1 explained 27.3% of the
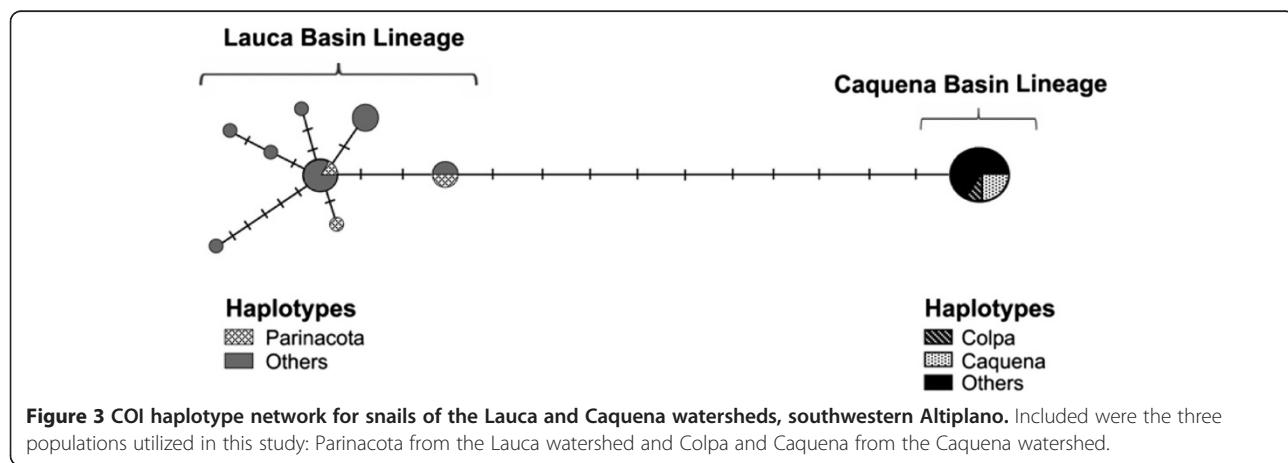
Figure 3 COI haplotype network for snails of the Lauca and Caquena watersheds, southwestern Altiplano. Included were the three populations utilized in this study: Parinacota from the Lauca watershed and Colpa and Caquena from the Caquena watershed.
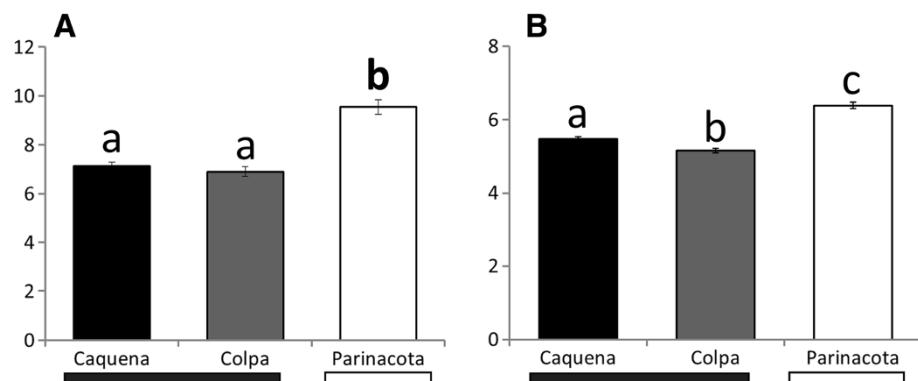
Figure 4 Shell length (mm). (A) Snails captured in the field. **(B)** Snails raised in the laboratory. Error bars indicate standard error. Characters on the error bars indicate significance groups (Bonferroni *post hoc* test *p* < 0,05). Bar under chart labels indicates lineages: black = Colpa-Caquena; white = Parinacota.

variance of the shell shape, while PC2 explained 15.7% and PC3 12.9% of the variance. Together, these three variables explained 56% of the variance. These three components were analyzed by an LDA that presented significant values (Wilk's $\lambda = 0.6794$, $F_{(6, 280)} = 9950$, $p < 0.001$). Classification values were low for all three localities (less than 67%), and the average classification rate was 56%. These findings indicate that adult individuals from different populations cannot be differentiated due to the shape of the shell.

Multivariate analysis of shell length in snails captured in the field vs. their descendents reared in the laboratory

To test for differences in morphometric variables between generations (G_0 and F_1), we performed a PCA with the individuals grouped by locality. The percentages of the variance explained are given in Figure 5B,C,D. The characters that showed the greatest loadings in the first principal component were the shell length and shell height in the three localities. In all three localities, the greatest variance
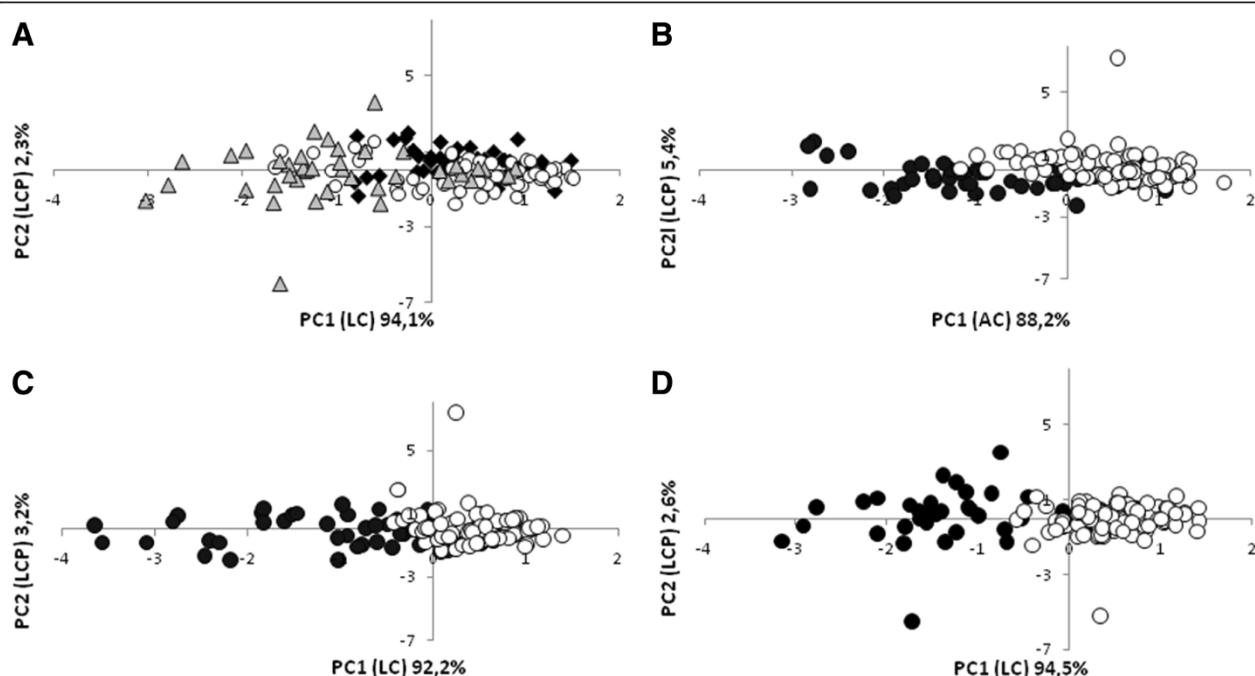
Figure 5 Morphometric analysis of snails as function of the first two principal components of 11 variables. (A) Individuals collected in the field by locality (filled diamond, Caquena; unfilled circle, Colpa; filled triangle, Parinacota). **(B**, **C**, and **D)** Individuals of Caquena, Colpa, and Parinacota collected in the field *vs.* descendents raised in the laboratory (filled circle, G_0, unfilled circle, F_1).

Table 1 Classification table obtained from LDA for the snails collected in the field

Locality	Caquena	Colpa	Parinacota	P
Caquena	31	14	3	64.6
Colpa	6	44	6	78.6
Parinacota	4	5	32	73
Total	41	63	41	73.8

P, percentage of correct classification.

was associated with the size of individuals (PC1). In the morphometric space, the G_0 snails were mainly distributed to the left of the F_1 individuals, and their amplitude range was greater for both principal components. These results would relate to the sampling method of G_0 individuals for whom no age classes were considered and in general were bigger and more variable in size, whereas the laboratory-grown individuals were measured in one moment at the same age. The LDA had a significant discrimination capacity (Wilk's $\lambda = 0.4324$, $F_{(22, 498)} = 11.78$, $p < 0.001$) and indicated that the percentage of correct classification was greater for those snails reared in the laboratory compared to their parents collected in the field (data not shown). The LDA of the F_1 snails produced 66.3% correct classification for the three localities (Table 2). We also performed a principal component analysis with the corrected variables by the length of the individuals. The PC1 explained 23.6% of the variance of the shell shape, while the PC2 explained 16.3% and PC3, 11.8% of the variance. The first three principal components explained 51.2% of the variance. The three first principal components were analyzed together using a LDA (Wilk's $\lambda = 0.6147$, $F_{(15, 1101)} = 14.16$, $p < 0.001$). The classifications values were very low for all three locations (under 55%), and the average classification rate was 37%. These results indicate that absence of morphological groups that could be associated with the localities, lineages, or generations.

Oviposition rate

The daily rate of oviposition was 0.56 for Caquena, 0.47 for Colpa, and 0.50 for Parinacota (Figure 6A). The differences were not statistically significant between lineages or localities.

Table 2 Classification table obtained from LDA for the snails raised in the laboratory

Locality	Caquena	Colpa	Parinacota	P
Caquena	51	23	14	58
Colpa	26	55	4	65
Parinacota	14	7	68	76
Total	91	85	86	66.3

P, percentage of correct classification.

Number of eggs per ovicapsule

The snails from Parinacota lineage had the greatest clutch size, with 16 eggs per ovicapsule, followed by Caquena and Colpa (9 and 7 eggs, respectively, Figure 6B), these differences were significant only at lineage level (nested ANOVA, $F_{(1,31)} = 479.419$, $p < 0.01$). This analysis was followed by an ANCOVA using the progenitors size as covariate, and any variance between lineages was explained by the size of the adult progenitor [ANCOVA (adult size), $F_{(1,30)} = 31.256$, $p < 0.01$].
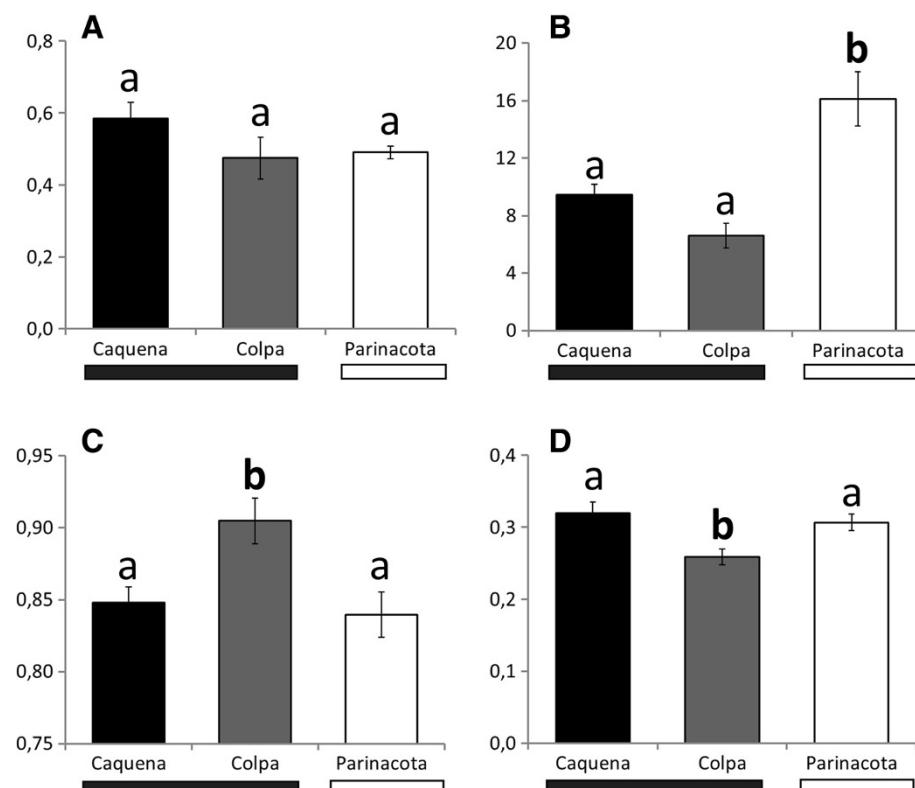
Hatching size

The snails from Colpa had the largest size (0.90 mm), followed by Caquena (0.84 mm) and Parinacota (0.83 mm) (Figure 6C). The differences were statistically significant only at the level of localities [nested ANOVA (locality), $F_{(1,118)} = 7.617$, $p < 0.01$]. The one-way ANOVA using as factor only the localities showed significant differences (ANOVA, $F_{(2,118)} = 5.08$, $p < 0.01$). The Bonferroni *a posteriori* analysis showed significant differences between the populations of Colpa and Caquena ($p = 0.02$) and between Colpa and Parinacota ($p = 0.01$) but not between Caquena and Parinacota.

Growth rate

The snails from Caquena had the greatest growth rate (0.32 mm/week), followed by Parinacota (0.31 mm/week) and Colpa (0.26 mm/week) (Figure 6D). The differences were statistically significant only at the level of localities [nested ANOVA (locality), $F_{(1,33)} = 10.331$, $p < 0.01$] but not at the level of lineages. The one way ANOVA using as factor only the localities showed significant differences (ANOVA, $F_{(2,33)} = 6.073$, $p < 0.01$). The Bonferroni *a posteriori* analysis indicated that Colpa was significantly different from the other two localities, while the difference between Caquena and Parinacota was not significant.

Discussion

The magnitude of selection in natural populations can be approached by complementing spatial studies of neutral variability with distribution patterns of traits (Tian-Bi et al. 2013). In this paper we evaluated genetic, morphological, and life history traits variation in snails of the genus *Biomphalaria* from different populations of the Chilean Altiplano. Considering the COI mitochondrial sequences, the three Altiplano populations of *Biomphalaria* studied form part of two distinct lineages. One lineage was composed of populations from the Lauca basin, among which is the Parinacota wetland, while a second lineage was composed of populations from the Caquena basin, which included snails from the Colpa and Caquena wetlands. The two lineages belong to different taxa, as yet undescribed, which were recently inferred by phylogenetic analysis (Collado et al. 2011; Collado and Méndez 2012).

Figure 6 Life history characters in *Biomphalaria* snails of the southwestern Altiplano. (A) Rate of oviposition (eggs per day). **(B)** Number of eggs per ovicapsule (eggs per ovicapsule). **(C)** Hatching size (mm). **(D)** Weekly growth rate (mm). Error bars indicate standard error. Characters on the error bars indicate significance groups (Bonferroni *post hoc* test *p* < 0.05). Bar under chart labels indicates lineages: black = Colpa-Caquena; white = Parinacota.

The percentage of sequence divergence between lineages (1.9%) is within the range of nucleotide variation estimated between closely related species of *Biomphalaria* (0.5% to 3.3%, Jørgensen et al. 2007; Collado et al. 2011). The allopatric distribution of these lineages may be attributed to vicariance events which have occurred in this Altiplano region, as has been suggested to explain the presence of different freshwater fishes of the genus *Orestias* Valenciennes, 1839, which are co-distributed with *Biomphalaria* in this area (Lüssen et al. 2003; Vila 2006; Vila et al. 2011). The snails from Caquena and Colpa share a single haplotype. This could be due the fact that both wetlands became isolated recently, so they have not reached genetic differences or still have connectivity through the Caquena River, which is fed with water from Caquena and Colpa wetlands and could be used by the snails to move between them, especially during the Altiplano winter events when the level of water rises. Also, this connectivity could be achieved by passive dispersal mediated by other organism, as described in other snails (Malone 1965; Rees 1965; Wesselingh et al. 1999; Wada et al. 2012; Boag 1986; Van Leeuwen et al. 2013).

It has been reported that the shell morphology of mollusks depends upon a variety of factors, for instance, predation (Palmer 1990; Trussell 1996), temperature (Trussell 2000; Anderson et al. 2007), population density (Goodfriend 1986; Anderson et al. 2007), precipitation, and availability of calcium (Goodfriend 1986; McMahon and Whitehead 1987), among others. We found differences in shell length between lineages, suggesting that part of the variation may have a genetic basis. In fact, although the visual inspection of PC1 and PC2 indicated a considerable overlap in the shell morphology of the three populations, the snails of Colpa and Caquena had an even greater overlap, indicating that these two populations are more similar with respect to Parinacota; this pattern was maintained in the F_1 generation. The LDA also indicated morphological similarities between Caquena and Colpa and greater differences with Parinacota. The differences found were associated mainly to the size of individuals since there was no evidence of differences in the form of the shell. The snails cultured in laboratory tended to converge in shell morphology, although snails from Parinacota remain showing greater difference respect to the others localities. The convergence of shells is probably due to phenotypic plasticity,

considering that genetic response to selection in the laboratory is unlikely in one generation. This implies that the variation in shell size of the snails could be explained by the interaction between genetic and environmental components. Morphological plasticity has been found in *Lymnaea ovata* (Draparnaud, 1805) and *Lymnaea peregra* (Müller, 1774) in response to laboratory conditions; snails of this two species showed convergence to a similar form in just two laboratory generations (Wullschleger and Jokela 2002).

Many life history characters during ontogeny are related to body size (e.g., clutch size, egg size, and age at first reproduction (Farnesi et al. 1984; Baker and Hawke 1991; Bengtsson and Baur 1993; Anderson et al. 2007). In the present study, we found variation in life history traits within lineages of *Biomphalaria* and minor differences between lineages that can be attributed to body size variation. For example, the snails from Parinacota put a greater number of eggs per ovicapsule than Caquena and Colpa, but when considering the size of individuals as a covariate, only this factor explains all variation. Variation in other life history traits like hatching size and growth rate was found only associated with the locality (not lineage), which would be due to proximal causes. Additionally, we found no differences in the daily rate of oviposition. Our findings contrast with those of Wullschleger and Jokela (2002) that found life history traits variation associated to the genotype in *L. ovata* and *L. peregra* suggesting that these two snails follow a different genetically determined life cycle. The variation found in life history traits of *Biomphalaria* may be due to phenotypic plasticity associated to the extreme environment of Altiplano.

The southern Altiplano includes a number of freshwater taxa which must bear temperature ranges that oscillate from −20°C in winter nights to 20°C during the day in the summer (Fornari et al. 2001) and inhabit hydrological systems with different physicochemical characteristics (Vila 1975; Mühlhauser et al. 1995; Dorador et al. 2003; Risacher et al. 2003; Márquez-García et al. 2009). This study suggests that a small part of the variation in shell morphology in the populations of *Biomphalaria* in the southwestern Andean Altiplano has a genetic base, while the variation in life history characters is due to proximal causes. We consider that this phenotypic plasticity may contribute to the persistence of the populations of these gastropods in these extremely high environments.

Conclusion

In this paper, we performed genetic and morphometric analyses using snail samples from wetlands found to more than 4,380 m altitude in the Chilean Altiplano. The results suggest that the shell size of the snails has a genetic basis associated to the phenotype, while the expression and evolution of life history traits in these extreme high environments are highly influenced by proximal causes.

Authors' contributions
GAC carried out the sequence alignment, performed the molecular genetic analysis, and drafted the manuscript. HFS carried out the morphological and life history traits studies, performed the statistical analysis and drafted the manuscript. MAM conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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