



Morphological and behavioral differences in the gastropod *Trophon geversianus* associated to distinct environmental conditions, as revealed by a multidisciplinary approach



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ABSTRACT

The gastropod *Trophon geversianus* exhibits shell polymorphisms along its distribution in subtidal and intertidal habitats. Our hypothesis is that morphological and behavioral patterns of *T. geversianus* represent habitat-specific constraints; subsequently we expect an association between shell morphology, attachment behavior, and habitat. In order to test this hypothesis we compared individuals from intertidal and subtidal habitats, at three sites in Golfo Nuevo (Argentina). We analyzed shell morphology using classic morphometric variables, 3D geometric morphometrics and computing tomography scan. The results were complemented with field observations of attachment to substrate and turning time behavior, as well as of the number of shell scars produced by crab predation. Our results showed differences in shell size and shape between intertidal and subtidal-collected individuals. Centroid size, total weight and shell weight, as well as shell density and thickness were significantly lower in intertidal individuals than in subtidal ones. Gastropods from intertidal habitats presented a low-spined shell and an expanded aperture which might allow better attachment to the bottom substrate, while subtidal individuals presented a slender and narrower shell shape. The number of crab scars was significantly higher in shells from subtidal individuals. Observations of the behavior of gastropods placed at the intertidal splash zone showed 100% of attachment to the bottom in the intertidal individuals, while subtidal specimens only attached in average in 32% of the cases. These latter took 12 times longer to re-attach to the bottom when faced up. Phylogenetic analysis of COI gene fragments showed no consistent differences among individuals sampled in both habitats. All these results suggest that *T. geversianus* has developed two ecomorphs with distinct morphological and behavioral responses to physically stressful conditions registered in north Patagonian intertidals, as opposed to lower physical stress but higher predation pressure in the subtidal habitats. The findings of this work constitute a starting point in the study of ecological adaptation processes in gastropods from Patagonian coastal environments.

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1. Introduction

The dioecious gastropod *Trophon geversianus* (Pallas, 1774) has a wide distribution range in Patagonia (Argentina, inhabiting both rocky intertidal and shallow subtidal shores). *T. geversianus* reproduces by intra-capsular development without a free larval phase, with egg-capsules attached upon bivalves of the family Mytilidae, from which the species usually feeds (Cumplido et al., 2010; Pastorino, 2005; Penchazadeh, 1976).

T. geversianus uses the accessory boring organ (ABO) alternated with the radula during drilling of its prey shells (Pío, 2010). In rocky shores of Golfo Nuevo, individuals of this species are more abundant in the intertidal habitat than in the subtidal (Rechimont et al., 2013). Egg-capsules from intertidal individuals have different size compared with the subtidal ones (Cumplido et al., 2010, author's unpublished data). In addition, shell length of egg-laying females and maturation sizes from intertidal individuals are smaller than the subtidal ones (Cumplido comm. pers.). These facts could be denoting different subpopulations in those habitats.

Rocky intertidals are fluctuating environments characterized by a wide range of physical conditions, including some of the most severe in the world (Denny and Wethey, 2001). In general, the main factors influencing the intertidal are wave exposure and tidal excursion gradients; the combination of these two environmental gradients can result

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in a complex set of conditions in rocky intertidal habitats (Menge and Branch, 2001). In contrast, the subtidal is characterized by more homogeneous physical conditions. In particular, Patagonian intertidal rocky shore communities are exposed to unusually harsh physical conditions, not registered in any other intertidal over the world, and consequently they are strongly structured by physical stress (Bertness et al., 2006). Subtidal habitats are more homogeneous and stable, as they lack the direct action of wave splash (despite “groundswell” wave action) and show less temperature variation compared with intertidal shores. The predators (crabs species) also differ between habitats, being subtidal predators more aggressive and with bigger claws (Rechimont, 2011; www.proyectosub.com.ar).

Due to the different environmental pressure registered in the studied zone and different egg-capsule sizes in intertidal and subtidal habitats, we hypothesized that *T. gerversianus* exhibits morphological variations as a result of habitat-specific adaptation. In particular, differences in shell morphology would result in differential abilities to avoid detachment during wave splash (Carvajal-Rodríguez et al., 2005; Denny and Wethey, 2001), and in different efficiencies in defense to predators (Kitching and Lockwood, 1974). Particularly, we predict that: the specimens from the intertidal have a smaller and globular shell shape and bigger relative size of the shell aperture (wave splash response: better capacity of attachment to the bottom), while specimens from subtidal habitats have thicker shells and fusiform shape with smaller relative size of the shell aperture (predator response).

In order to test this hypothesis, we studied specimens of *T. gerversianus* from intertidal and subtidal habitats at three sites in Golfo Nuevo (Patagonia, Argentina), comparing shell morphology using classic morphometric variables and 3D GM methods. Shell structure was studied by gravimetric methods and complemented by computing tomography scan.

We also performed field observations such as number of shell scars produced by predation, and substrate attachment ability. As an additional evidence of the species identity of the individuals from both habitats, a preliminary phylogenetic analysis of cytochrome oxidase I gene fragments was performed.

2. Materials and methods

2.1. Study area

The study was conducted in rocky shores of Golfo Nuevo, northern Patagonian shelf (southern South America; Fig. 1). The annual mean air temperature is 13.4 °C, with a monthly mean of 20.4 °C in January

(summer) and 6.4 °C in July (winter). Annual air temperature amplitude can reach up to 40 °C, and daily variations up to 20 °C (Labraga and Davies Last visit, 2013). Surface water temperature shows mean annual values of around 14 °C, with a maximum of 18–20 °C at the end of the summer, and a minimum of 8–9 °C in spring (Dellatorre et al., 2012). Mean annual precipitation in the area is 235.9 mm (Labraga and Davies Last visit, 2013). Dominant tides are semidiurnal, and have amplitudes of 1.83 m during the neap cycle and 5.73 m during the spring cycle (Tide Tables, SHN-Argentine Hydrographic Service). Wind circulation over Golfo Nuevo is characterized by strong and persistent westerly winds, which are driven by the two anti-cyclones located in the Atlantic and the Pacific sectors and a low-pressure belt located around 60° S (Paruelo et al., 1998). Annual mean wind speed is 4.6 m/s (16.6 km/h), reaching up to 25 m/s (90 km/h). Southwestern and Western winds present a frequency of approximately 38% in summer and more than 50% in winter (Rechimont, 2011).

2.2. Sampling

A total of 267 *T. gerversianus* individuals were collected between June and September 2012 at three sites along the Golfo Nuevo (Patagonia, Argentina) (Fig. 1). At each site, two habitats were sampled: subtidal (SUB) and intertidal (IN). The distance between habitats at each site was not more than 4 km. Samples and observations from Garipe, which is located in the natural protected area of “El Doradillo”, were performed with the corresponding permissions of the local authorities (Organismo Provincial de Turismo and Fauna, Chubut province). Adult specimens of *T. gerversianus* were randomly collected in the SUB by scuba diving at 5–10 m depth and in the IN zone manually during low tides. After collection, specimens were transported to the laboratory and frozen (–20 °C) for preservation. Specimens were also collected and placed into tide pools for their evaluation during field observations.

2.3. Size, weight and predation

Total body weight was recorded to the nearest 0.1 g. After removing the soft parts, the weight without shell (body weight) and the weight of the shell separately (shell weight) were registered. To evaluate differences in shell size, body weight and shell weight between habitats, the last two variables were normalized by dividing the values by their corresponding total length. The number of shell damage marks (scars) due to potential predation was registered by visual examination in collected specimens. Potential predators of *T. gerversianus* that could mark shells leaving scars were visually registered in the field. Statistical

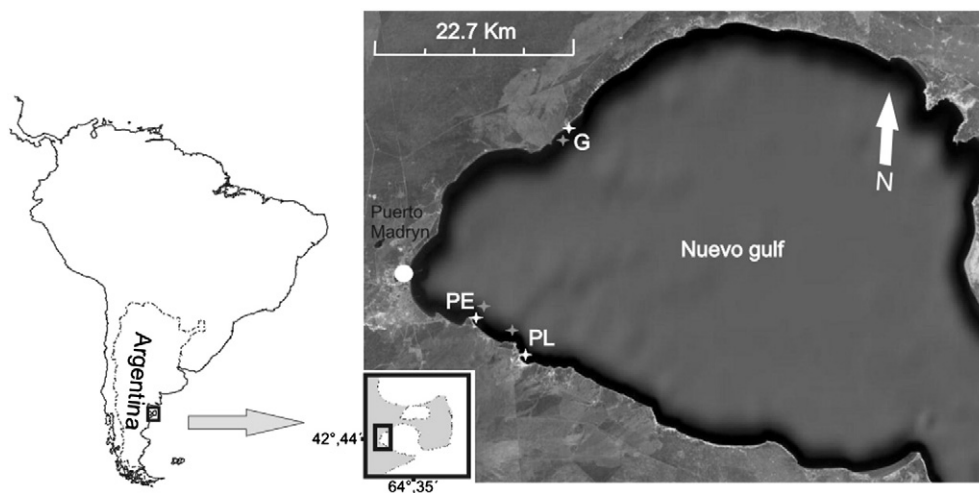


Fig. 1. Sample collection sites at Golfo Nuevo, on each intertidal (white star) and subtidal (grey star). Sampled sites were Garipe (G): IN 42°37'03.29"S, 64°51'15.32"W; SUB 42°37'05.27"S, 64°51'14.21"W; Punta Este (PE): IN 42°47'06.90"S, 64°57'10.51"W; SUB 42°46'01.99"S, 64°56'33.86"W, and Punta Loma (PL): IN 42°49'17.95"S, 64°53'12.29"W; SUB 42°48'30.79"S, 64°54'51.76"W).

significance of the differences was evaluated by Kruskal–Wallis test (Zar, 1999), since the assumptions for parametric tests were not met. For significant results ($P < 0.05$) pairwise comparisons test of subgroups according to Conover (1999) was applied. Statistical analyses were run using Infostat software (Di Rienzo et al., 2009). Computing tomography scans (CTs) were performed for one intertidal and one subtidal individual (randomly chosen). Density and lip thickness were measured in coronal sections of the shell and in 10 transverse sections of the lip, and visualized graphically using the software packages COMPUDENT NAVIGATOR 3D.

2.4. 3D Geometric morphometrics

Analyses of shell shape in gastropods collected from each habitat within each site were performed using the software MorphoJ version 1.05b (Klingenberg, 2008). The shapes of individuals were captured by the Cartesian coordinates of a three-dimensional configuration of 16 anatomical landmarks (Fig. 2). All specimens were measured by one observer (RANV) using a Microscribe G2X digitizer. Landmark configurations were superimposed by generalized Procrustes analysis (Rohlf and Slice, 1990; Slice et al., 1996). This procedure translates and rotates the landmark configurations to a common origin and scales them to unit centroid size, which is defined as the square root of the summed squared distances of all landmarks from the centroid (Rohlf and Slice, 1990; Slice et al., 1996). The centroid size was used as proxy for shell size.

To assess and control putative allometric effects (changes in shape associated with size differences), we computed pooled within-habitat multivariate regression of shape (Procrustes coordinates) on size (centroid size) (Bookstein, 1991; Klingenberg, 2011; Monteiro, 1999). To evaluate the independence between the shape and size variables, we carried out a permutation test with 10,000 rounds (Bookstein, 1991; Zelditch et al., 2004).

The principal components of shape were calculated from a principal component (PC) analysis of the variance–covariance matrix of the

Procrustes coordinates to reduce the quantity of shape variables and to display the major features of shape variation along the axes (Zelditch et al., 2004).

Finally, to visualize and test the separation of shell shapes between each SUB and IN groups, we performed a linear discriminant analysis (DA) 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 and warping surfaces (Wiley et al., 2005). The data analyses were performed with the MorphoJ v1.05c (Klingenberg, 2011). As suggested by Klingenberg (2011) for a better visualization of the shell shape discriminate factor in 3D data, we produced in the MorphoJ program, coordinate files for input into the Landmark software (Wiley et al., 2005) to deform surfaces from CT scans using the thin-plate spline function. The statistical significance of pairwise differences in mean shapes was assessed with permutation tests using Mahalanobis distance as the test statistic (10,000 permutations per test).

2.5. Field observations of adhesive ability and turning behavior

Observations of adhesive ability and time to turn down when faced up (defined as the time taken to place the foot on the substrate) were performed in the field. Fifty individuals were randomly collected from the subtidal (5 m deep) and 50 from the intertidal at Garipe site in August 3rd (2012). From those collected, 25 specimens from each habitat were randomly chosen and placed in 10 individual exclusion cages (five replicate cages, containing 5 snails from each habitat). This was done only at Garipe site, due to the fact that the other sites studied are frequently visited by people (mainly for recreational and fishing purposes) and previous experiences demonstrated that visitors detach the exclusion cages. The individuals were placed inside a stainless-steel mesh (5 mm) exclusion cage ($15 \times 15 \times 4$ cm, L \times W \times H). Each cage was bolted to the substrate (covered 100% by mitylids *Brachidontes rodriguezii* and *Brachidontes purpuratus* preyed by IN *T. geversianus*) with a stainless-steel centered lock. Individuals attached to the bottom or turned up (not attached) in each cage were registered at two different moments (one week, and one month after).

On the other hand, turning time (re-attachment) of faced-up gastropods from both habitats was registered in the field at Garipe site. Fifteen individuals from each habitat were placed in separate small tide pools (less than 50 cm of diameter). Gastropods were covered by 1 cm of sea water and placed with the shell aperture facing up immediately after collection. Turning time re-attachment to the bottom was measured with a digital chronometer.

2.6. Cytochrome Oxidase I gene amplification and phylogenetic analysis

Cytochrome Oxidase I (COI) gene fragments from 9 individuals, 4 of them collected from the SUB and 5 from the IN habitat were amplified and sequenced as part of the International Barcode of Life initiative (iBOL, www.boldsystmes.org). Standard iBOL protocols for DNA extraction and COI gene amplification were followed. Briefly, DNA was extracted from ethanol-fixed tissue samples from foot muscle using a protocol based on the use of glass fiber filtration plates (Ivanova et al., 2006). Approximately 700 bp of the COI gene were amplified by PCR. Sequences were deposited in the project “Invertebrados marinos de Antártida y plataforma Argentina” [INV] in the Barcode of Life Data Systems (BOLD) website (www.barcodinglife.org).

For phylogenetic analysis, a total of 114 unique COI sequences, including the 9 sequences generated in this study and 106 sequences from various gastropod species available in GenBank database, were aligned using ClustalW. All positions containing gaps or missing data were eliminated, resulting in a total of 586 positions in the final dataset.

Phylogenetic reconstruction was performed using sequences from individuals belonging to the Trophoninae subfamily, including the genera *Trophon*, *Leptotrophon*, *Scabrotrophon*, *Boreotrophon*, and *Trophonella*.

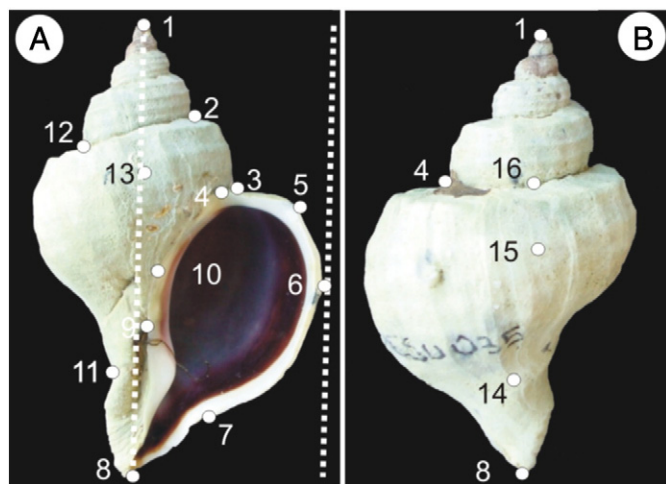


Fig. 2. Landmark configuration placements (white dots) on the shell were made using two different orientations, apertural (A) and abapertural (B). These landmarks include: (1) apex, (2) apertural extreme of the starting point of antepenultimate whorl, (3) apertural extreme of the starting point of last whorl, (4) posterior angle of the aperture, (5) posterior point of the lip, (6) tangent point in the lip, parallel to the line (dash line) formed by landmarks 1 and 8, (7) anterior most point of the lip, (8) anterior tip of columella, (9) start of umbilicus shell (10) depression at the external border of columella upper plicate, (11) external curvature of the siphonal lip, (12) left border of suture in the penultimate whorl on a middle plane to the axis from landmarks 1 to 8, (13) apertural maximum height, (14) depression at the last whorl and siphonal canal in the abapertural view, (15) apertural maximum height, and intersection between the border of suture in the penultimate whorl and middle plane (dash line) to the axis from landmarks 1 to 8 (16). All landmarks were painted with small points of indelible marker to aid in a more precise identification.

Although *T. shackletoni* and *T. longstaffi* are currently included in the genus *Trophonella* (Harasewych and Pastorino, 2008) within the new subfamily Pagodulinae (Barco et al., 2012), in this work their names were reproduced exactly as they appear in Genbank database (*Trophon shackletoni* and *Trophon longstaffi*). Distances were computed using the Jukes–Cantor method (Jukes and Cantor, 1969). The evolutionary history was inferred using the Neighbor–Joining method and branch stability was assessed by bootstrap test (1000 permutations). Phylogenetic analyses were performed using the software MEGA version 5 (Tamura et al., 2011).

3. Results

3.1. Size, weight, and predation marks

Centroid size, body and shell weight of the individuals differed significantly between habitats (Kruskal–Wallis test: $H_{cs} = 211.90$, $H_{bw} = 206.31$, $H_{sw} = 204.88$ respectively, $P < 0.0001$ in all cases; Fig. 3). Individuals from SUB were larger and heavier than IN ones. Number of scars in the shell of the individuals differed among sites: the number of scars were lower in shells from IN than from SUB (Kruskal–Wallis test: $H = 67.84$ $P < 0.0001$). A mean of 0.8 scars (SE = 0.06) in gastropods from IN and 2.1 (SE = 0.16) in SUB animals were recorded. Three species of crabs that could be potential predators of *T. gerversianus*, capable of damaging the snail's shell lip, were registered in the SUB: *Ovalipes trimaculatus*, *Peltarion spinosolum* and *Leucippa pentagona*. On the other hand, the crabs *Cyrtograpsus angulatus* and *C. altimanus* were registered at the IN habitat. Crabs from SUB are bigger and present more robust claws than IN ones.

Computing tomography scans (Fig. 4) revealed that, in general terms, the shell density (mean = $88.54\% \pm SE = 3.84$) and thickness (mean = $1.44 \text{ mm} \pm SE = 0.9$) registered from intertidal habitat were lower than those from subtidal (mean shell density = $96.33\% \pm SE = 1.7$; mean thickness = $2.81 \text{ mm} \pm SE = 0.74$).

3.2. Shell shape variation

The relation between shape and size pooled within-habitat was allometric (permutation test with 10,000 random permutations, $P < 0.0001$). For subsequent analyses, we used the regression residuals as a new size-unrelated shell shape variable. The shell shape variation is summarized in Fig. 5 with scatter plots for the first 2 PCs of shape. The specimens from the three groups presented the same patterns of ordination in the morphospace, with the major shell shape variations between IN–SUB represented along the first axis (PC1). The geometrical interpretation of the positive extremes of PC1 was associated with globular shells (shorter spire and bigger relative size of the shell aperture) while the negative was associated with the development of a fusiform shape (elongated-spined shell and smaller relative size of the shell aperture—Fig. 5).

The discriminant analysis (DA) of the three sites showed that the range of shell shapes was different between the two habitats in similar way. As there are only two groups, there is a single axis of shape differences and scores are shown with histogram bars proportional to their frequency (Fig. 6).

In addition, the shape deformations depicted by the discriminant vector are shown in Fig. 6 as deformation of a surface representation of shell of *T. gerversianus* using tomography computed scan. The mean shell shapes of gastropods from each group of SUB are slender and more lengthened than those from the IN habitat. The cross-validated classification table showed that the accuracy of shell shape in predicting habitat is better than a 93% random chance. Results of significance tests, the percentage of individuals that were correctly classified and Mahalanobis distances are shown in Table 1.

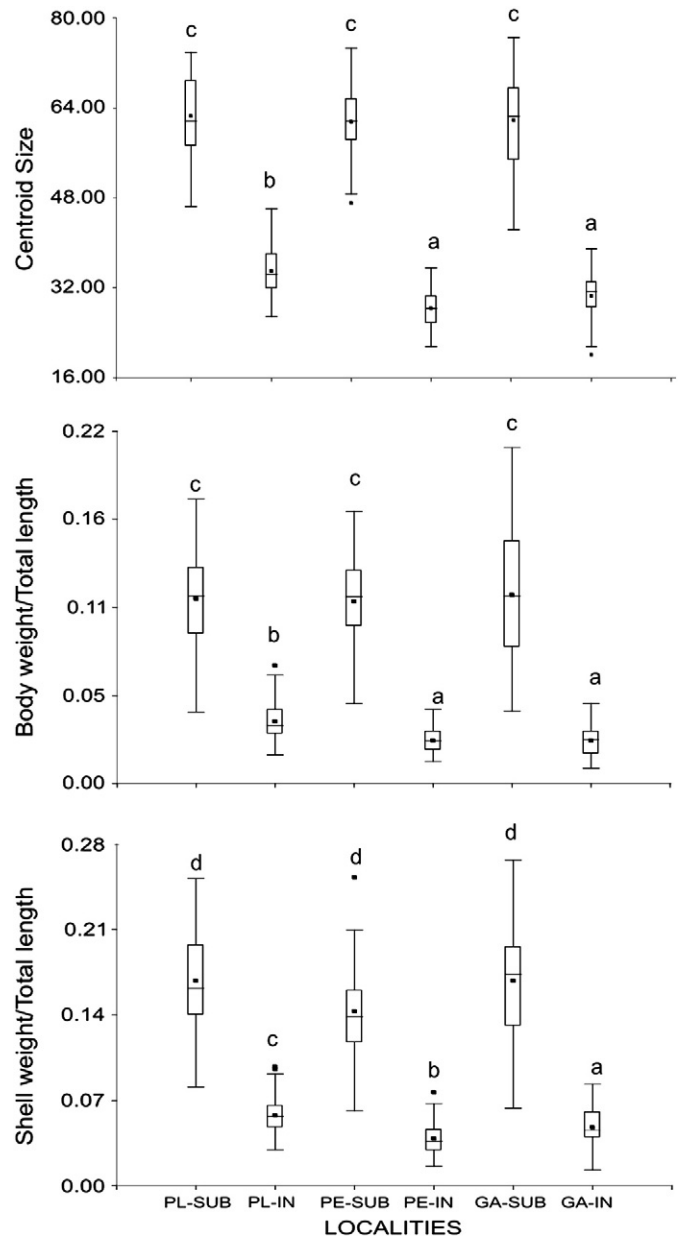


Fig. 3. Size and weight variations between intertidal and subtidal sites. The central dot represents the mean; the median is represented as a central line; the limits of the box, the first and third quartiles, and the whiskers the 95% confidence interval; the dots out the whiskers are outliers. Different letters indicate significant differences ($P < 0.05$) in pairwise comparisons test. Abbreviations: PL-SUB, PL-IN: Punta Loma subtidal and intertidal respectively; PE-SUB, PE-IN: Punta Este subtidal and intertidal respectively and GA-SUB, GA-IN: Garipe subtidal and intertidal respectively.

3.3. Adhesive ability and turning behavior

All animals placed in the exclusion cages were alive after one month of exclusion, and evidences of feeding on *Brachidontes* mussels in all cages were registered (data not shown).

Differences in the number of specimens attached to the substrate were registered in two opportunities during one month. Along this period 2 swell events occurred in addition to the daily wave splash. Gastropods from SUB were attached only in 36% and 28% of the cases (corresponding to one week and one month, respectively), while the IN individuals were 100% attached in both time periods. Also, after one month of exclusion, we registered shell breakage (mainly in the apex and siphonal canal) in 70% of the SUB individuals, due to rolling

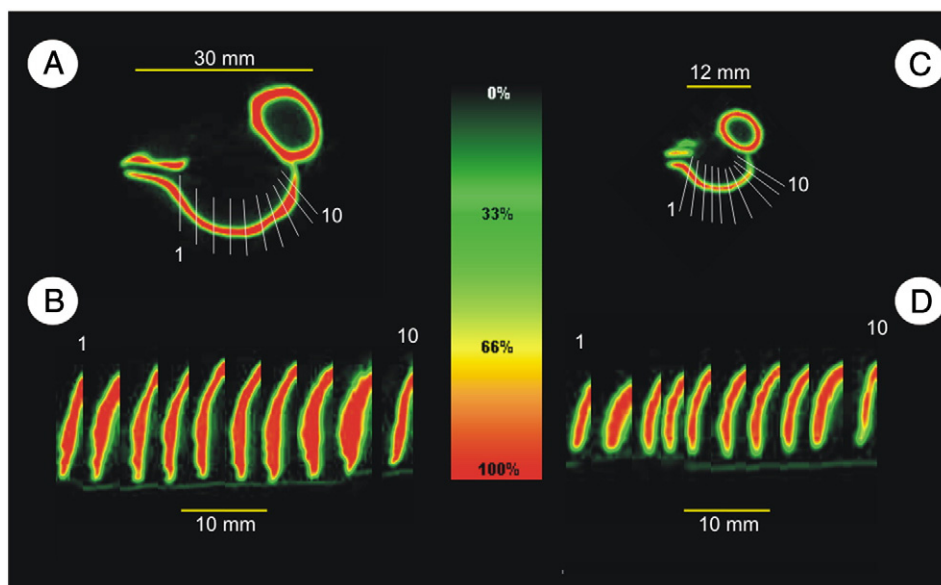


Fig. 4. Percentage densitometry analyses in shell cross-section by CT scan; A and B: specimen from subtidal habitat (SU); C and D: specimen from intertidal habitat (IN). White lines on the coronal images A and C shows the transverse section made in the shell's lip zone. B and D detail of ten transverse sections of A and C. Densities are represented by a color scale (center) as 100% red and 0% black.

produced by detachment and wave action. No shell damage due to rolling was registered for IN gastropods.

Field observations revealed that, in general terms, the IN specimens turned down faster than the subtidal individuals. The mean time of substrate re-attachment in intertidal individuals was 4.96 min (SE \pm 2.73; N = 15) while for subtidal individuals mean turning time was 61.24 min (SE \pm 30.78; N = 7). The remaining 8 individuals from subtidal had not turned down after 2 h, when the high tide covered the place precluding us of continuing the observations.

3.4. Phylogenetic analysis of the COI gene fragments

A phylogenetic analysis of cytochrome oxidase I sequences was performed using sequences from individuals belonging to the Trophoninae subfamily, including the genera *Trophon*, *Leptotrophon*, *Scabrotrophon*, *Boreotrophon*, and *Trophonella* (Fig. 7). The COI gene showed, in general, good level of resolution for this subfamily: highly supported clusters were formed, clearly distinguishing among the different genera as well as between the two true species of *Trophon* (*T. geversianus* and *T. plicatum*, Fig. 7).

The sequences generated in this work clearly clustered with sequences from another individual also assigned to *T. geversianus*, identified in Playa Larralde (situated in San José Gulf, Argentina, near Golfo Nuevo). Although there was certain variability in the sequences, this was minimal (genetic distances between sequences ranged from 0–0.6% at the nucleotide level and 0–1.5% at the amino acid level). Moreover, this variability was not associated to habitat of origin of the individuals, as sequences from individuals of different habitats clustered together, some of them being 100% identical. In other words, no distinction between individuals of *T. geversianus* collected from different habitats was observed based on this gene. Trees based on Maximum Parsimony and Maximum Likelihood methods, as well as trees based on amino acid sequence alignments, showed similar results (data not shown).

4. Discussion

The combination of classical morphometry, 3D geometric morphometrics methods and computer tomography scan allowed us to characterize, with high resolution, the shell size, shape, and shell density

variations in *T. geversianus*, allowing us to identify distinct ecomorphs from intertidal and subtidal habitats from Golfo Nuevo. Geometric morphometrics (GM) methods have the advantage of allowing the detection of intrapopulation subtle shape changes (Carvajal-Rodríguez et al., 2005; Idaszkin et al., 2013; Márquez et al., 2010) and allows to graphically visualize the results by means of multivariate statistical analysis (Adams et al., 2004). GM has been used to study marine gastropod shell shape variations in different research contexts (a brief review of applications of GM landmarks methods in marine snails is provided as Supplementary Table). Of these methods, 3D GM maintains the original dimensionality of the study object, allowing a better interpretation of results. However, up to now, only a few studies have used this approach to analyze shell shape variations in gastropods (Supplementary Table).

Body size and shape are influenced by three of the principal factors that affect intertidal organisms (heat, water loss and wave shock) (Levinton, 2001). Small shell sizes found on the intertidal ecomorph of *T. geversianus* could be related to an increased tolerance to high and low temperatures and desiccation stress. On the other hand, the bigger size and shell density of the subtidal individuals could be an adaptation to predation pressure, principally by crabs. The main differences in shell shape between these ecomorphs is the fusiform shape with elongated-spined shell and smaller aperture (related to the shell profile) in the subtidal individuals, whereas the intertidal specimens have a low-spined shell and an expanded aperture. Carvajal-Rodríguez et al. (2005) and Conde-Padín et al. (2008) registered shell shape variations in the gastropods *Littorina saxatilis* and Guerra-Varela et al. (2009) in *Nucella lapillus*, and related them to different shore levels and micro-habitats of rocky shores. These authors stated that gastropods from wave-exposed micro-habitat of the lower shore had larger muscular foot, favoring a larger shell aperture and better fixing of the gastropods to the substratum. In contrast, in the upper shore micro-habitat, desiccation and predation may favor sheltered specimens showing a relatively smaller shell aperture (Carvajal-Rodríguez et al., 2005; Guerra-Varela et al., 2009). On the other hand, changes in morphology patterns are a common defense against shell-breaking predators (Vermeij, 1993; Palmer, 1990; Trussell, 1996; Bourdeau, 2009). Accordingly, we found that the subtidal ecomorph presents a fusiform shape, a relatively slender shell aperture, thicker and denser shells than intertidal ones. The same pattern of shell shape was recorded for other species of gastropods under predation by crabs (Appleton and Palmer, 1988;

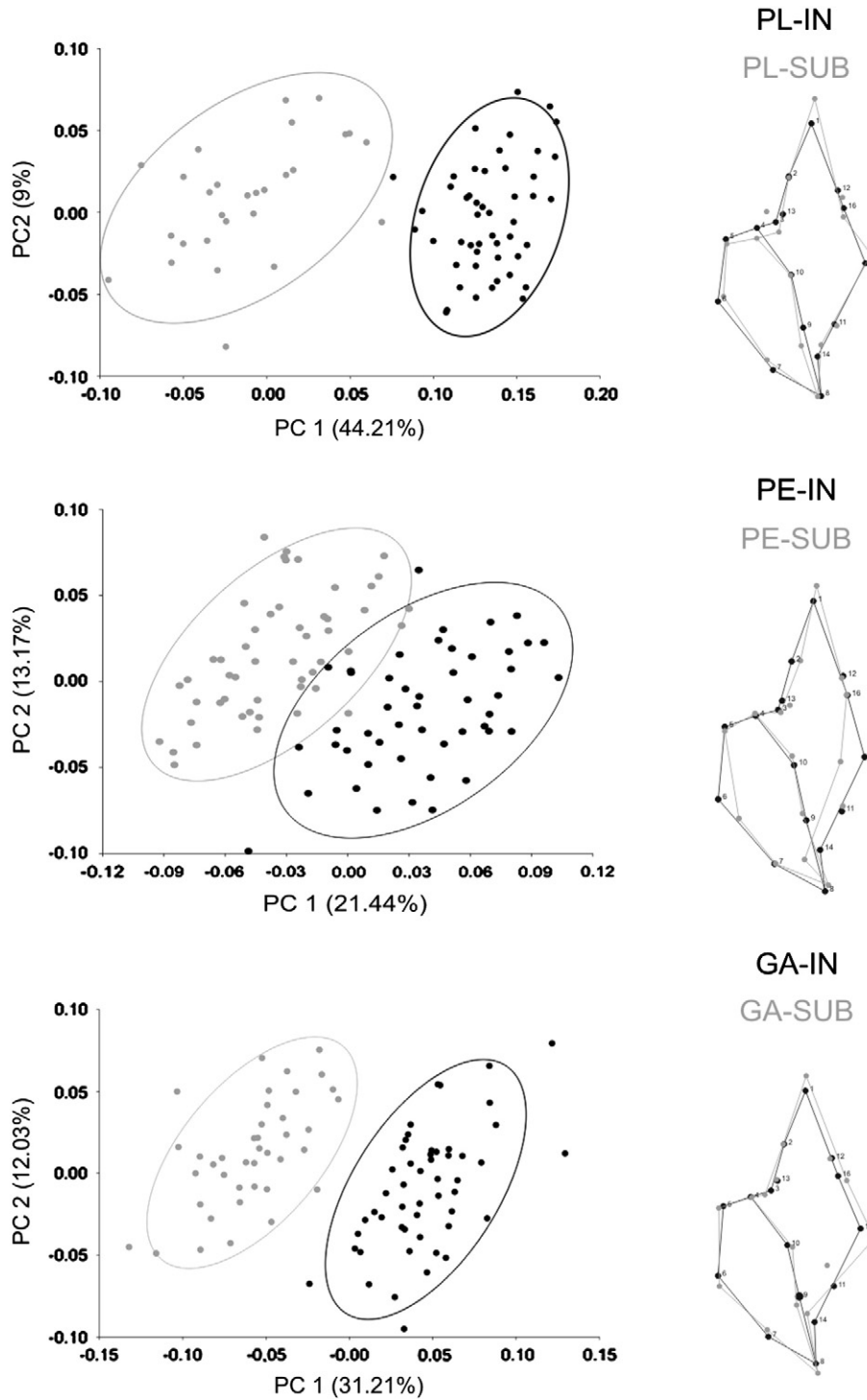


Fig. 5. Plot of the principal components (PCs) for different habitat based on Procrustes distances (left). The figures represent displacement vectors from the overall mean shape (black dot) to the positive extreme shape (vector) for PC1. Shape changes have been exaggerated (scale factor 0.15). Percentages of explained variance for each axis are in parentheses. Abbreviations: PL-SUB, PL-IN: Punta Loma subtidal and intertidal respectively; PE-SUB, PE-IN: Punta Este subtidal and intertidal respectively and GA-IN, GA-SUB: Garipe subtidal and intertidal respectively.

Carvajal-Rodríguez et al., 2005; Conde-Padín et al., 2007; Johannesson, 1986; Johannesson and Johannesson, 1996; Rolán-Alvarez et al., 1997; Trussell, 2000). As predicted, the intertidal ecomorph was more resistant to wave exposure (by attachment to the mussel matrix) and turned down faster than the subtidal ecomorph. It is reasonable to assume that the relatively large shell aperture of individuals from exposed shores increases the ability to resist strong wave action, probably by allowing a better attachment of the foot. Also, short shells in the intertidal

specimens could produce less dragging, thus decreasing the risk of being detached from the substratum (Carvajal-Rodríguez et al., 2005; Rolán-Alvarez et al., 1997). In field observations, the intertidal individuals took less than 12 folds less time than the subtidal ones to re-attach to the substratum when dragged by the wave splash. These results are in agreement with the extreme environmental conditions of intertidal habitats in the Patagonian region, that force the animals to live attached to the mussel matrix constituted by *B. purpuratus* and

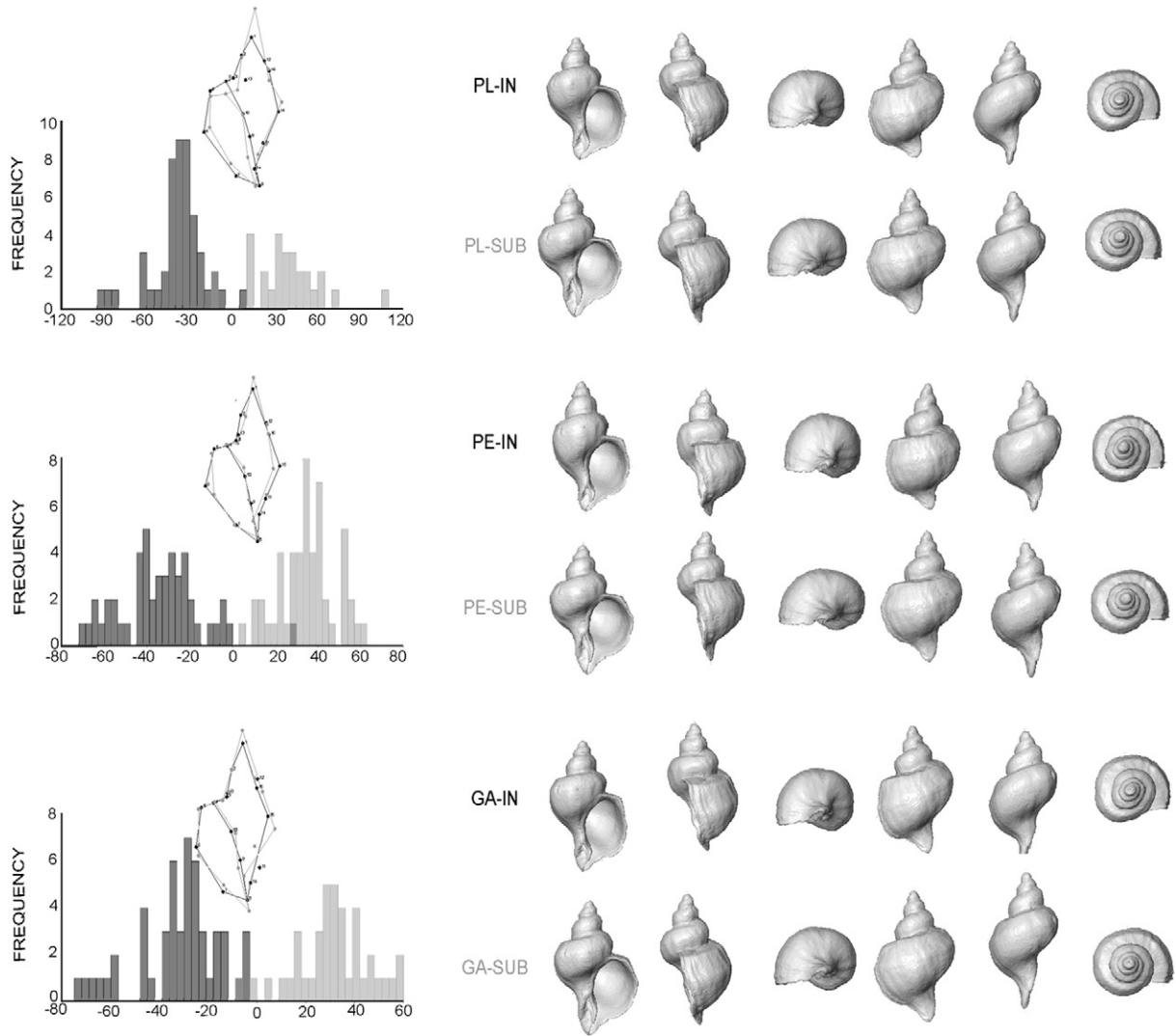


Fig. 6. Discriminant analysis of shell shape differences between each groups (intertidal vs subtidal). Frequencies of the discriminant scores predicted by a jackknife (leave-one-out) cross-validation are shown using histogram bars. Intertidal (black vector) and subtidal (grey vector) mean shapes are visualized using wireframe drawings magnified 2 times. At right, the column of image shows the visualization of the extreme of discriminant vector between habitats (IN-SUB) as shell shape deformations (to the right in each row) in different views of the shell.

B. rodriguezii which protects them from desiccation (Bertness et al., 2006; Rechimont et al., 2013; Silliman et al., 2011).

Sequence analysis of a phylogenetically informative gene (Cytochrome Oxidase I) preliminarily suggested the species identity of all studied specimens from both habitats, and indicated that the two ecomorphs associated with distinct habitats do not differ significantly at this level of phylogenetic resolution. However, as it has been found in other gastropods that two distinct species can show lack of divergence in their mitochondrial markers (Kemppainen et al., 2009), these

results must be taken with caution. The COI-based phylogenetic tree also suggests that the genus *Boreotrophon* might be related to members of the subfamily Pagadulinae (Barco et al., 2012). Further studies are needed to corroborate this hypothesis.

Organisms are known to present alternative strategies for survival in heterogeneous habitats. These are phenotypic plasticity, heritable trait variation (genetic polymorphism) or the development of a generalist response (Hollander et al., 2006; Scheiner, 1998). In this work we could exclude the possibility of a generalist response in members of *T. geversianus*, as two ecomorphs associated with specific habitats were identified. However it is not possible from the findings of this work to distinguish if the source of variation is due to phenotypic plasticity or to genetic polymorphism, or both. Moreover, the possibility that they constitute sibling species cannot be completely excluded. All these scenarios must be further evaluated by means of the use of other molecular markers (such as microsatellites or AFLPs) and by laboratory crosses (Rolán-Alvarez et al., 2004).

In conclusion, the morphological and behavioral differences registered in the gastropod *T. geversianus* from north patagonian rocky shores could be associated to differences in physical stress and predation pressure by crabs between intertidal and subtidal habitats. 3D geometric morphometrics proved to be a powerful tool to discriminate ecomorphs in this species. Given the fact that other gastropod species

Table 1

Classification matrix showing the jackknife cross-validated classification, differences between means and Mahalanobis distances of each group.

Habitat	Intertidal	Subtidal	Total	Percentage correct	T-square	D ²
		Punta Loma			<0.0001	81.25
Intertidal	48	1	49	98		
Subtidal	2	26	28	93		
		Punta Este			<0.0001	87.40
Intertidal	47	1	48	98		
Subtidal	0	51	51	100		
		Garipe			<0.0001	85.22
Intertidal	51	0	51	100		
Subtidal	2	38	40	95		

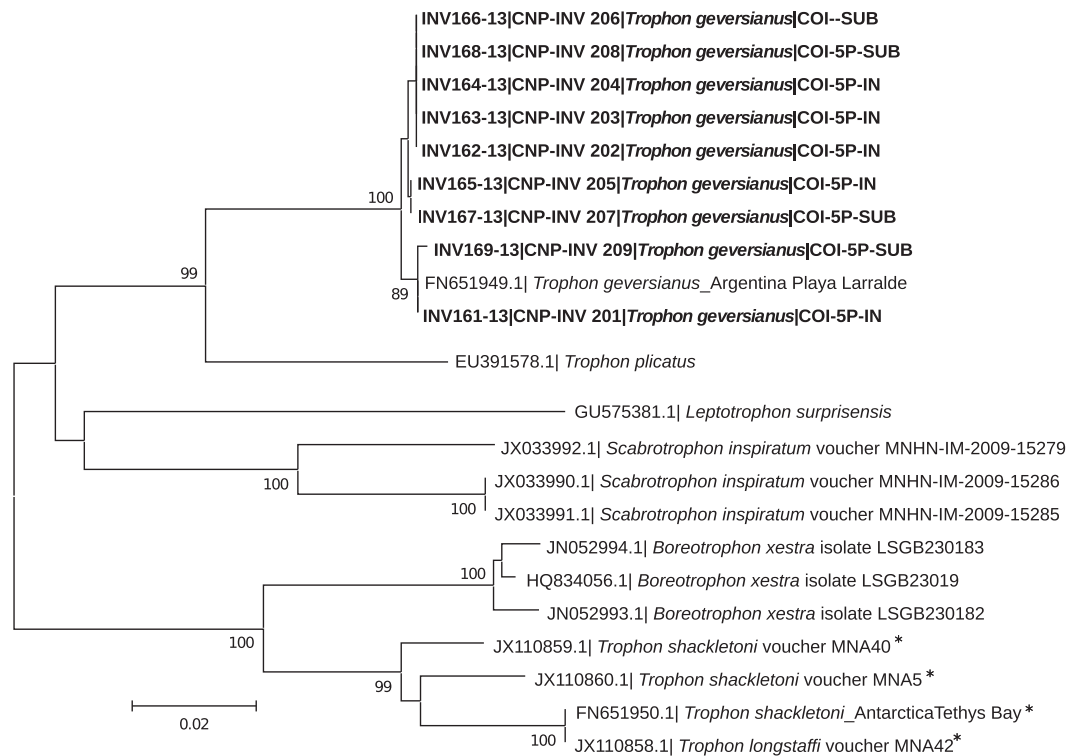


Fig. 7. Molecular phylogeny based on partial COI sequences of the gastropods collected in this study and related species from public databases. The analysis was based on evolutionary distances and the tree constructed by neighbor joining (for details see **Materials and methods** section). Values at the nodes are percentages over 1000 bootstrap permutations (only values higher than 50% are shown). The bar corresponds to 0.02 nucleotide substitutions per site. Sequences generated in this study are depicted in bold. For database sequences, accession numbers are shown before species name. SUB: subtidal individuals. IN: intertidal individuals. *Although registered as *Trophon* in Genbank database, these species have been recently re-classified and are currently included in the genus *Trophonella* (Harasewych and Pastorino, 2008) within the new subfamily Pagodulinae (Barco et al., 2012).

are known to bear coexisting subpopulations in intertidal and subtidal rocky shores in Patagonia, this work constitutes a starting point to study ecological adaptation processes in gastropods from Patagonia by means of the use of multiple complementary techniques.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.seares.2014.05.002>.

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