DECOMPOSING SHELL FORM INTO SIZE AND SHAPE BY GEOMETRIC MORPHOMETRIC METHODS IN TWO SYMPATRIC ECOTYPES OF *LITTORINA SAXATILIS*

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ABSTRACT

Two sympatric snail ecotypes (RB and SU) of *Littorina saxatilis* from exposed rocky shores of NW Spain differ in many life history traits, but classical morphometric analysis has failed to reveal significant shell shape differences between them. We used geometric morphometric methods on landmark data from digitized shell images to study size and shape components in both ecotypes at two localities. The results showed significant differences between ecotypes in both shell size and shape (both uniform and non-uniform components). Allometry was also detected for some component of the local variation in shape, although it did not explain the observed differences between ecotypes. The SU ecotype had a relatively rounded shell shape with a big aperture, whereas the RB ecotype had higher spire and smaller aperture. We suggest that shape differentiation is correlated with adaptive differences between ecotypes.

INTRODUCTION

The rough periwinkle, *Littorina saxatilis* (Olivi 1792), is an intertidal rocky shore gastropod, which is gonochoristic and undergoes direct development (Reid, 1996). The species has a very low dispersal capability because of its ovoviviparity (females carry a brood pouch with shelled embryos) and the low mobility of adults (Janson, 1983; Erlandsson, Rolán-Alvarez & Johannesson, 1998). As a consequence, the species typically shows high genetic differentiation at local as well as at broader geographical scales (Ward, 1990). *Littorina saxatilis* lives under different physical and ecological conditions depending on tidal reach (Raffaelli & Hawkins, 1996). Furthermore, local conditions due to slope of the rocky shore, wave energy, substrates and exposure are able to create different habitats at a scale of even a few metres (Janson, 1983; Johannesson, Johannesson & Rolán-Alvarez, 1993; Reid, 1996). Therefore, this species is highly polymorphic and a great number of adaptive ecotypes/morphs have been described (Janson, 1983; Johannesson *et al*., 1993; Reid, 1996; Wilding, Grahame & Mill, 2001).

Shell morphology is an extremely polymorphic trait in littorinids (Reid, 1996), and has been extensively studied in rough periwinkles by using distances and ratio variables in classical multivariate analysis (Janson & Sundberg, 1983; Johannesson, 1986; Grahame, Mill & Brown, 1990; Mill & Grahame, 1995; Johannesson & Johannesson, 1996; Cruz, Rolán-Alvarez & García, 2001). However, this strategy for measuring shell variation can confound size and shape, and the results are very sensitive to the particular distances and ratios chosen in the study (Bookstein, 1991). This happens because variation is only quantified between the endpoints of linear distance, and even then does not specify which endpoint moves relative to the other. In addition, it is well known that ratios in biometrical analysis have serious statistical drawbacks (Sokal & Rohlf, 1995: 17). The new landmark-based technique of geometric morphometrics involves no restriction in the direction of variation among the chosen landmarks and is the most effective way to capture information about the shape of an organism, especially when combined with multivariate statistical procedures (Rohlf & Marcus, 1993; Rohlf, Loy & Corti, 1996). A few pioneering

works have used the landmark-based geometric morphometric approach to decompose shell form in shell size and shape in gastropods (Stone, 1998; Guralnick & Kurpius, 2001). Here, we used a similar strategy (using a different shell positioning to define the landmarks) to study a polymorphism in *L. saxatilis*, for which classical distance and multivariate methods have failed to detect shape differentiation between ecotypes (see Johannesson *et al*., 1993).

In Galicia (NW Spain), two ecotypes of *L. saxatilis* are found on exposed rocky shores, adapted to different shore levels and habitats. The ridged and banded (RB) ecotype lives preferentially on the upper shore among barnacles, while the smooth and unbanded (SU) form can be found in the mussel belt on the lower shore (Johannesson *et al*., 1993). Both 'pure' forms and a variable percentage of intermediates (putative hybrids) can be found on the mid shore, living in true sympatry (Rolán-Alvarez *et al*., 1999, 2004). Although the pure forms mate partly assortatively on the mid shore (Rolán-Alvarez *et al*., 1999), there remains some gene flow between them and they are therefore considered conspecifics (Johannesson *et al*., 1993; Rolán-Alvarez, Rolán & Johannesson, 1996, Rolán-Alvarez *et al*., 2004). However, the ecotypes differ in various morphological and life history characteristics which correlate with habitat differences (Johannesson *et al*., 1993; Rolán-Alvarez *et al*., 1996; Rolán-Alvarez, Johannesson & Erlandsson, 1997; Cruz *et al*., 2004). For instance, RB snails are exposed to changes in salinity, sun exposure (heat and desiccation stresses) and predation on the higher intertidal shore, while the SU form survives in a more wave-exposed habitat on the lower shore.

There are conspicuous shell size and sculpture differences between these ecotypes (Johannesson *et al*., 1993; Fig. 1). Adaptive advantages have been shown for each ecotype: the larger and more-sculptured morph (RB) resists crab attacks better than does the smaller and thinner-shelled morph (SU), while the SU morph is less affected by waves (Rolán-Alvarez *et al*., 1997; Cruz *et al*., 2001). Johannesson *et al*. (1993) used 13 shell measurements and principal component analysis to study shell form in several populations of these two ecotypes. These authors observed significant differences in the first principal component (argued to be mainly due to size variation because all loadings were positive and of similar magnitude), but they could not detect any significant difference in other principal

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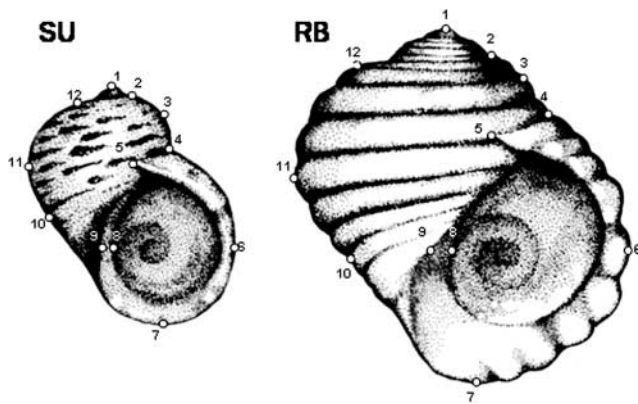
Figure 1. Specimens representative of both ecotypes of *Littorina saxatilis*: the RB is ridged and banded (right) and the SU is smooth and unbanded (left). The landmarks (1–12) used in the morphological study to describe shell size and shape are indicated on both drawings.

components. Apparently, however, 'the SU ecotype had less pronounced aperture lips, thinner shells, and higher whorls than the other morph' (Johannesson *et al.*, 1993: 1777). The inefficacy in detecting shape differences in their study could be caused by the analytical shortcomings of the geometric properties of distance variables and of standard multivariate analyses.

To check this possibility, we studied shell size and shape in these two ecotypes at two localities using landmarks and geometric morphometric methods. Our results show that these ecotypes differ in shell size as well as in the first uniform and non-uniform (first relative warp *sensu* Rohlf *et al.*, 1996) components of shell shape. These results confirm the advantages of using landmark techniques and geometric morphometric methods to study shell size and shape in littorinids. The potential causes of the observed differences in shell shape are discussed in the light of the existing knowledge related to this polymorphism.

MATERIAL AND METHODS

Specimens of both ecotypes from upper (RB) and lower shore (SU) areas (20 m apart) were obtained during June 2003 at Silleiro and La Cetarea (25 km apart). At each sampling site all snails within an area of 1 m^2 were captured and taken to the laboratory, where they were placed on a surface in rows and columns and numbered. In each sample 15 specimens were randomly chosen for morphological analysis (60 snails in total) using the random number generator from GWBASIC. The specimens used were larger than 3 mm (range 3–11 mm) in shell height (otherwise excluded and a new specimen chosen) because we were interested in comparing adults of both ecotypes. Specimens smaller than 3 mm are typically immature in these populations (Johannesson, Rolán-Alvarez & Ekendahl, 1995), and former fitness estimation of these ecotypes in the wild have been based on snails larger than 3 mm (Rolán-Alvarez *et al.*, 1997). Shells were examined using a Leica MZ12 stereoscopic microscope, and colour images were captured and digitized using a Leica digital ICA video camera and QWin Lite version 2.2 software, always with the specimens in the same position (with the axis of the shell on the *y*-axis and the aperture in the same plane as the objective; Fig. 1). Shell variables were obtained using 12 landmarks (LM) representative of the shell, as shown in Figure 1. LM1 represents the apex of the shell; LM2 is on the right border of the profile of the shell at the end of the upper suture of the penultimate whorl and LM4 is in the lower suture; LM3 marks the intermediate position between LM2 and LM4 along the curvature of the whorl;

LM5 is at the end of the suture; LM6 is the most external position in the external part of the outer lip; LM7 is the lowest point at the base; LM8 and LM9 show the internal and external border, respectively, of the columella on a perpendicular line to the axis from LM6; LM11 is the most external point in the last whorl at the left profile of the shell; LM10 is the profile point between LM7 and LM11 closest to LM5; and finally LM12 is on the left border of the profile of the shell where the last whorl starts to curve (Fig. 1). These points do not necessarily represent homologous landmarks from a developmental point of view in different specimens, although they allow us to capture and decompose, objectively and repeatably, shell size and shape in this species. In fact, only LM1, 2, 4 and 5 are type I landmarks (the most preferred type), while LM3, 6, 7, 8, 9, 10, 11 and 12 are type III landmarks, following Bookstein's (1991) classification. Furthermore, we made a pilot experiment repeating capture of the shell image in the same specimen 20 times and obtaining the 12 landmarks for each replicate. The mean error of landmark coordinates obtained in this pilot experiment was 0.15 mm (about 0.9% of the measurements relative to the size). We could also calculate the errors of the shape variables analysing these 20 repeated measurements together with the whole experimental data. The errors of shape variables should be expected to be smaller *a priori* (see below), because under the geometric morphometric approach some of the errors caused by shell rotation are corrected (Bookstein, 1991; see below).

For each specimen, centroid size (estimating the specimen size) and uniform (affine) and non-uniform (non-affine) components of shell shape were obtained. Centroid size is the square root of the sum of squared distances of landmarks to their centroid (the average *x* and *y* coordinate points) of the landmark configuration (Bookstein, 1991). The uniform transformation is wholly linear, i.e. a kind of transformation that leaves the set of parallel lines parallel. Any landmark rearrangement has some component of this sort, and some local component (Rohlf & Bookstein, 2003). Thus, the uniform components account for shell variation at a global scale (all landmarks simultaneously), while the local components express variation in the vicinity of different landmarks. The first uniform component (U1) holds the vertical coordinate fixed and allows the horizontal coordinate to shift (expressing changes at the horizontal scale), while the second component (U2) holds the horizontal coordinate fixed and allows the vertical coordinate to shift (expressing changes at the vertical scale) (Rohlf & Bookstein, 2003). On the other hand, non-uniform components describe local shape deformations from a reference configuration at different spatial scales. Non-uniform shape measurements were computed via relative warp analysis (RWA) (Bookstein, 1991; Rohlf, 1993) and the uniform part of shape variation was computed using the space complement of the non-uniform component (Rohlf & Bookstein, 2003).

The estimation of shell shape components was accomplished by aligning the raw coordinates of the specimens using the Procrustes generalized orthogonal method (GLS; Rohlf & Slice, 1990), which determines a reference configuration by minimizing the sum of squared distances between homologous landmarks from different specimens. The coordinates of aligned specimens were used for a relative warp analysis (RWA). The RWA finds a function fitting all landmarks to the reference configuration. This produces the principal warps that describe shape deformations of the reference configuration at different spatial scales. The specimen deviations from the reference configuration are called the partial warp scores. The relative warps (RWs) are the principal components of the variation among specimens in the space of the principal warps (see Bookstein, 1991; Rohlf, 1993). The RWs were computed, excluding the uniform component, using the algorithm given

by Rohlf (1993). We used the scaling option $\alpha = 0$, which weights all landmarks equally following Rohlf *et al.* (1996), except for detecting allometry when we used $\alpha = 1$. Each RW explains a percentage of the overall local variation in decreasing order, as with principal components (Rohlf, 1993). All calculations were performed by the program MODICOS, developed by one of us (Carvajal-Rodríguez & Rodríguez, 2005; http://life.-bio.sunysb.edu/morph/soft-comprehensive.html). The TPSRELW program developed by Rohlf (1998; http://life.bio.sunysb.edu/morph/soft-tps.html) was used to obtain the graphics.

Multiple regression analysis was used to detect allometry, testing for significant relationships between any of the independent variables (all uniform and non-uniform variables of shell shape) and the centroid size (Bookstein, 1991). The stepwise procedure was used in order to avoid erroneous fitting of variables to the regression model due to the large number of independent variables (20), with the 0.05 and 0.1 criteria for forward and backward selection, respectively (Sokal & Rohlf, 1995). Differences between ecotypes or localities were studied by one-way ANOVA and ANCOVA (Sokal & Rohlf, 1995). These analyses were computed with the SPSS/PC statistical package (version 12.01).

RESULTS

The step-wise multiple regression analysis of allometry showed that several of the relative warps contributed significantly to the regression model on the centroid size (Table 1). The presence of allometry in the main non-uniform components of shell shape made it necessary to use ANCOVA to show ecotype differences in shell shape independent of centroid size (see below).

We obtained the relative warps extracted from the matrix of the partial-warp scores. The two uniform components (non-correlated; $r = 0.001$) explained 76% (U1) and 20% (U2) of the shell variation on a global scale. The six main relative warps (non-correlated; $-0.001 \le r \le 0.001$) explained 90% of the overall variation in the non-uniform component (Table 2). The errors of the former shape variables were calculated using the same pilot experiment described above (repeating the capture of the same specimen 20 times). The mean error of centroid size was 0.0105 mm (about 0.08% of the measurement relative to the centroid size), while the mean error of the uniform component (U1 and U2; average mean error = 0.0008; SD = 0.0002) and the relative warps (RW1–RW6; average mean error = 0.0008; SD = 0.0003) were very similar, although due to the low values of their means in the data set, they were not negligible in certain cases (representing in average a 2.3% of the overall variation studied in those shape variables, range 0.04% for RW1 to 7.4% for RW6).

Table 1. Allometric analysis for shell shape measurements in every combination of ecotype and locality. A step-wise multiple regression analysis for centroid size (as dependent variable) and two uniform and 18 non-uniform measurements obtained for $\alpha = 1$, as independent variables, is presented. The F test of the regression analysis (F), the percentage of variation explained for the independent variables (r^2), and the variables included in the model as well as their regression coefficients (Beta) are also shown.

| Multiple regression		Variables in the model	
r^2	F	Name	Beta
0.83	41.4***	RW1	0.769***
		RW2	0.238**
		RW5	−0.203**

**P < 0.05, ***P < 0.001.

Table 2. Mean squares of the factor morph and their significance in the one-way ANOVA for different shell measurements: centroid size (CS), two uniform (U1 and U2) and the six main (explaining 90% of the overall variation) non-uniform estimates (RW1 to RW6) of shell shape for $\alpha = 0$. The mean squares for shape measurements are multiplied by 1000.

Measure	Variance explained	ANOVA			
		Silleiro		La Cetarea	
		Morph (MS$_{between}$)	Error (MS$_{within}$)	Morph (MS$_{between}$)	Error (MS$_{within}$)
CS	–	213.9***	2.3	435.8***	3.6
U1	76%	47.82***	0.45	10.30***	0.37
U2	20%	0.01	0.48	0.55	0.26
RW1	54%	166.45***	1.39	76.61***	0.79
RW2	13%	0.36	1.27	3.83	1.11
RW3	8%	1.37	1.24	0.01	0.47
RW4	7%	0.68	0.47	4.28*	0.91
RW5	5%	0.01	0.55	1.12	0.42
RW6	3%	0.58	0.38	0.84*	0.19

**P < 0.05, ***P < 0.001.

Ecotype differences in size, and uniform and non-uniform components of shell shape were studied by one-way ANOVA (Table 2). Centroid size, U1 (the first uniform component) and RW1 (the first non-uniform component) showed significant differences between ecotypes at both Silleiro and La Cetarea. These differences between ecotypes for U1 and RW1 remained significant in all cases using ANCOVA (when corrected for the covariable centroid size; $P < 0.01$). In La Cetarea, however, significant differences between ecotypes were detected for two further relative warps (RW4 and RW6). A similar ANOVA was also performed on the rest of the relative warps (RW7–RW18) but significant ecotype differences were only detected for RW11 in La Cetarea (not shown). The distribution of specimens from different ecotypes and localities are plotted in
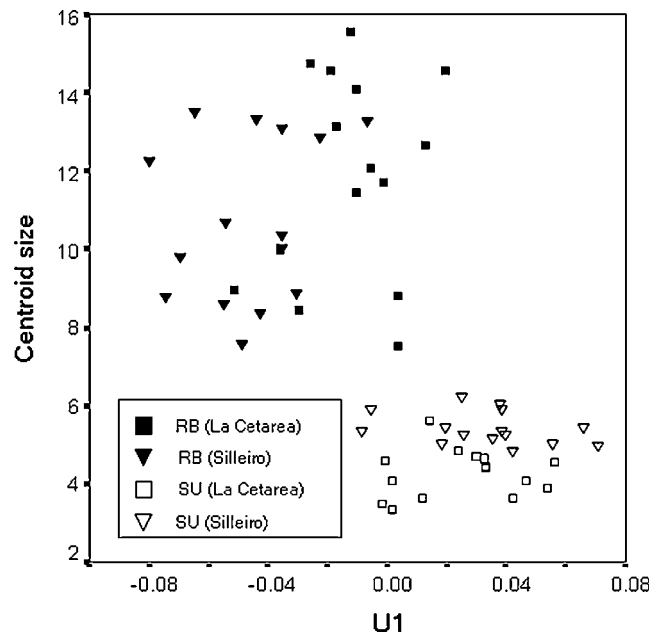
Figure 2. Individuals from different ecotypes and localities plotted for centroid size and the first uniform estimate of shell shape obtained for $\alpha = 0$.
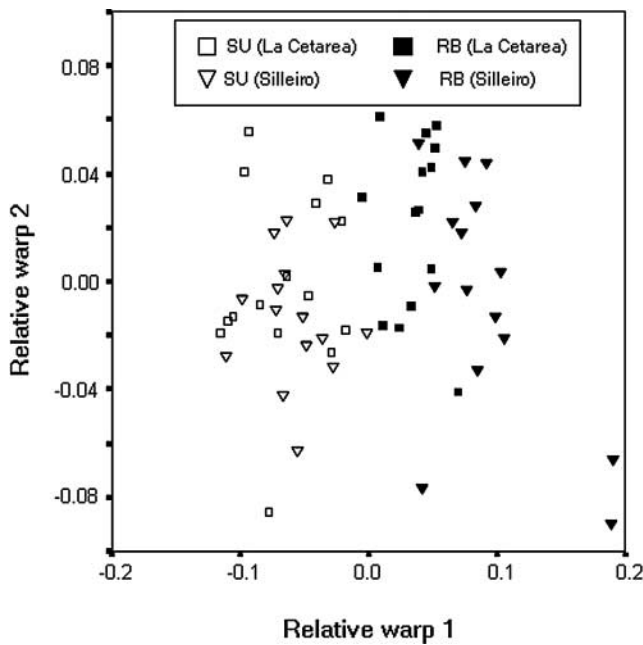
Figure 3. Individuals from different ecotypes and localities plotted for the first and second non-uniform estimates (relative warps) of shell shape obtained for $\alpha = 0$.

Figure 2 by centroid size and the main uniform shell shape (U1). Differences between localities within ecotypes are apparent, but they do not compromise the clear differences observed between ecotypes (Fig. 2) in agreement with statistical tests (Table 2). The same trends are also observed for RW1, when plotting the overall specimens for the first (RW1) and second (RW2) non-uniform (local) components of shell shape (Table 2; Fig. 3).

We repeated the ANOVA but using only the four type I landmarks (LM1, LM2, LM4 and LM5) instead of 12. These four landmarks alone were able to capture the general differences

of the shell shape between ecotypes. Among Silleiro samples we observed significant differences in centroid size, U1, U2 and RW1, whereas among La Cetarea samples significant differences were detected for centroid size, RW1 and RW2 ($P < 0.05$; results not plotted). These differences remained significant for RW1 in both localities, when corrected by the covariable centroid size (ANCOVA; $P < 0.05$), indicating the robust performance of geometric morphometric techniques in this system.

The interpretation of the RW1 variation, representing local variation, can be carried out using the interpolating function (thin-plate splines) describing shape change in RWA, as shown in Figure 4. The SU ecotype presented the most negative deformations, while the RB showed the most positive ones (Figs 3, 4). The external landmarks (LM1, LM2, LM3, LM4, LM6, LM7, LM10, LM11 and LM12) and those that best represent the aperture (LM5, LM6, LM7 and LM8) were connected by lines for an easier visualization of the meaning of RW1 deformations in each ecotype in Figure 4. RW1 can be described mainly as variation in the relative size and shape of the aperture, showing the relatively bigger aperture of the SU ecotype than the RB.

DISCUSSION

The applicability of geometric morphometric methods to the study of gastropod shells is in its infancy. Johnston, Tobachnick & Bookstein (1991) claimed that landmarks should be preferentially used along axial sculpturing (varices), only available in some gastropods, while others (Stone, 1988; Guralnick & Kurpius, 2001) suggested that every point between two whorls is an appropriate landmark. These authors, however, defined their landmarks on the apical view in order to study the ontogenetic trajectory of the shell shape (see Guralnick & Kurpius, 2001). Here, in contrast, the landmarks are objectively located on the shell profile of the frontal view, capturing the variation of shell shape, but without limiting our effort to exclusively homologous (from a developmental point of view) landmarks. This parallels traditional distances and ratios used in taxonomy or microevolutionary studies (see Janson & Sundberg, 1983; Johannesson, 1986; Grahame *et al.*, 1990; Mill & Grahame,
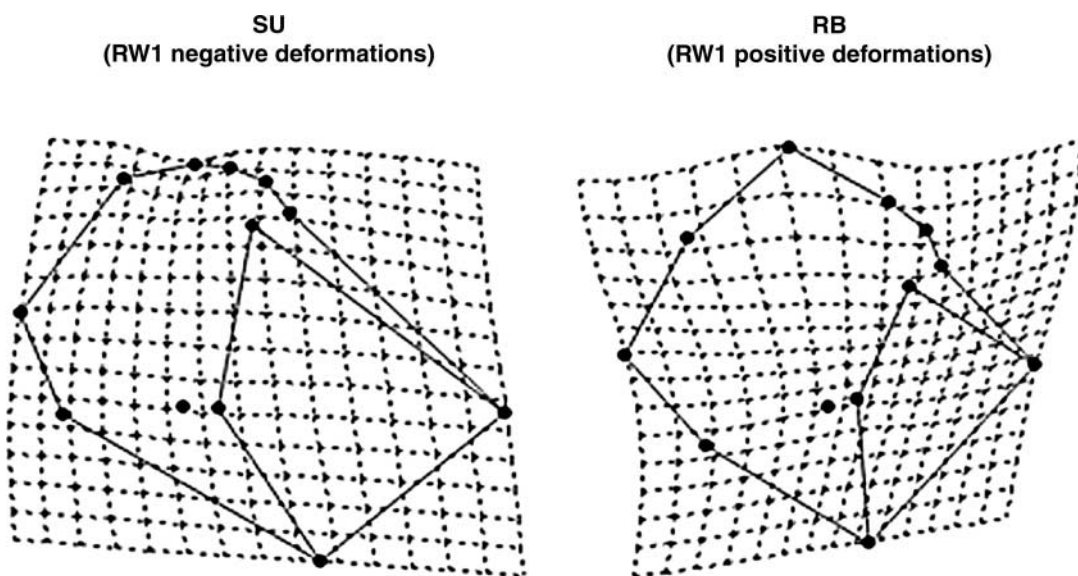
Figure 4. Thin-plate spline representation, from the TPSRELW software (Rohlf, 1998) for showing the most extreme positive (as in RB; see Fig. 3) and negative (as in SU) deformation of the landmarks for x and y axis, for $\alpha = 0$, in the populations studied. Some landmarks are connected by lines to facilitate the interpretation of the differences between ecotypes (see text).

1995; Johannesson & Johannesson, 1996; Cruz *et al.*, 2001), but using morphometric analysis of relative warps. In addition, the methods used here were extremely robust to changes in the number of landmarks chosen, as we obtained nearly the same result using 12 and four points.

Differences in size (centroid size in this study), morphology (presence of ridges and bands), and behaviour and life history characteristics between the two sympatric ecotypes of *Littorina saxatilis* have been noted throughout the literature (Johannesson *et al.*, 1993; Rolán-Alvarez *et al.*, 1996, 1997; this study). However, classical distance variables analysed with multivariate methods on hundreds of specimens only detected significant differences between ecotypes in the first principal component of shell variation (Johannesson *et al.*, 1993). A clear quantitative demonstration of ecotype differentiation in shell shape was lacking for this polymorphism. Our use of geometric morphometric methods, however, has revealed not only significant variation in size, but also in uniform and non-uniform components of shell shape. The more structured variation obtained by our method can be explained by the more sensitive geometric and statistical properties of the landmark-based geometric morphometric methods compared to distance variables and indices (Rohlf, 2000; Monteiro, Bordin & Furtado dos Reis, 2000; Douglas *et al.*, 2001). This new approach also allows an intuitive and objective interpretation of shell shape variation (see Fig. 4).

The differentiation in shell shape between these two ecotypes affected both uniform and non-uniform (local) components of shell shape. The first uniform component shows that the SU ecotype presented a significantly larger separation of its landmarks with respect to the RB ecotype along the horizontal scale. This may be due to the need for a more globular and robust shell in the RB in order to resist crab attacks, which are common on the upper shore (Rolán-Alvarez *et al.*, 1997). The main shell shape differentiation between ecotypes, at a local scale, is observed in the first relative warp (RW1). This variation reveals that these ecotypes differ in the relative area of the aperture (Figs 3, 4). It is known that these two ecotypes are maintained by strong divergent selection in upper and lower-shore habitats, with SU adapted to a wave-exposed habitat and RB to a more desiccated habitat and more frequent crab predation (Rolán-Alvarez *et al.*, 1997). The differences in RW1 between these two ecotypes match the previous interpretation, as the larger aperture found in the SU specimens is needed to accommodate a large foot necessary to avoid being dislodged by waves. In fact, the SU ecotype attached significantly better to a glass surface than the RB ecotype in a wave-effect simulation (Rolán-Alvarez *et al.*, 1997). On the other hand, the smaller aperture observed (related to the shell profile) in the RB specimens protects the animal from desiccation or crab predation. In fact, the RB ecotype resists sun exposure and crab predation significantly better than the SU ecotype (Rolán-Alvarez *et al.*, 1997). A similar relationship between the opercular shape and tidal zonation level has been also described in the related genus *Nodilittorina* (Reid, 1996).

The geometric morphometric methods used here can be easily applied to other gastropods. These methods are cheap, although a method to digitize the shell is still needed, the software available is user-friendly and it is less time consuming than classical alternatives. In addition, they represent a fundamental improvement both to statistically detect subtle differences between groups and to understand the patterns of shell shape variation, representing a clear advance for taxonomic and microevolutionary studies of gastropods.

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