MORPHOLOGICAL AND GENETIC ANALYSIS OF TWO SYMPATRIC MORPHS OF THE DOGWHELK *NUCELLA LAPILLUS* (GASTROPODA: MURICIDAE) FROM GALICIA (NORTHWESTERN SPAIN)

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ABSTRACT

There are two morphs, exposed and sheltered, of the dogwhelk *Nucella lapillus* associated with different degrees of exposure on the rocky shores of Galicia (northwestern Spain). These two morphs are typically found at different localities (allopatrically), but in a few sites they can be found in sympatry. We have analysed the shell and radular characteristics of these two morphs from a locality where they appear in sympatry. Genetic analysis using microsatellites was also performed. Morphological analysis was applied to shelled embryos, juveniles and adults. The results indicate consistent morphological differentiation across life-history stages, suggesting genetic determination. Differentiation in radular morphology (width of the rachidian teeth) was also detected in adults. Other taxonomically relevant traits such as protoconch morphology did not show differences between morphs. These results agree with the intraspecific polymorphism found in this species in other parts of Europe, hence the two morphs may represent ecotypes adapted to contrasted habitats. Although the relative contributions of classical genetic variation and phenotypic plasticity to variation in shell morphology remains unknown, our observations of genetic differentiation between the two morphs indicate that the genetic component is significant.

INTRODUCTION

Nucella lapillus (Linnaeus, 1758) is a common species of the family Muricidae and is distributed along the North Atlantic coast from the Strait of Gibraltar to Connecticut, including England, Iceland and Greenland (Moore, 1936; Fretter & Graham, 1985; Wares & Cunningham, 2001). *Nucella lapillus* is dioecious; laying benthic capsules that each contains 15–30 shelled embryos. Crawling young emerge from the capsules and, as in other gastropods with direct development, the limited dispersal capability is correlated with pronounced spatial variability in shell morphology (Crothers, 1975; Fretter & Graham, 1985; Graham, 1988; Kitching, Muntz & Ebling, 1966; Marko, 1998).

There is a conspicuous phenotypic and genetic polymorphism in *N. lapillus*, associated with the degree of wave exposure on intertidal rocky shores (Ebling *et al.*, 1964; Kitching *et al.*, 1966; Crothers, 1973, 1975; Day, 1990; Dixon *et al.*, 1994; Kirby, 2000). Other ecotypes exist that are not directly related to the degree of exposure (Fretter & Graham, 1985). The exposed ecotypes are typically squatter, with relatively larger apertures and thinner shells than the sheltered ecotypes. Interestingly, in some localities the sheltered/exposed polymorphism reaches its maximum distinctness in sub-adults and then decreases with ageing (Berry & Crothers, 1968), probably because adults undergo shell erosion (Fretter & Graham, 1985).

Variation in gastropod shell size and morphology can be both genetically and environmentally determined (Boulding & Hay, 1993; Johannesson & Johannesson, 1996; Carballo, García & Rolán-Alvarez, 2001), but it is difficult to quantify the relative importance of the two components. In *Nucella*, however, there are some reported data that suggest a particularly strong contribution of environmental determination for certain traits (Trussell & Etter, 2001). For example, shell colour is very variable, and a part of this variation has been explained as a side-effect of feeding on different organisms (Moore, 1936). Similarly, shell thickness and shell shape in general have been claimed to change during growth due to induction by environmental factors (Gibbs, 1993; Trussell & Etter, 2001). Nevertheless, genetic differences are also involved in at least some populations showing the sheltered/exposed polymorphism (Fretter & Graham, 1985; Day, 1990; Dixon *et al.*, 1994; Kirby, 2000).

On Galician shores (northwestern Spain) there are distinct morphs of *N. lapillus* that inhabit different locations across an environmental gradient (from exposed to sheltered sites; Rolán, 1983). The exposed morph lives preferentially outside the estuaries in the most exposed areas, predating mostly on mussels (*Mytilus galloprovincialis*). This morph typically shows dark colour, large aperture and a surface without spiral cords or scales (Fig. 1E–H). The sheltered ecotype is typically found inside the Rias (estuaries) shows white or yellow shells with scales and spiral cords (Fig. 1A–D) and predates mostly on barnacles (*Chthamalus stellatus*). In the inner most parts of the estuaries an extremely sheltered ecotype can be found, which is larger and thicker than the other morphs. Furthermore, in certain areas of the exposed rocky shores (outside the estuaries), both morphs can appear nearly sympatrically (separated by a few meters or even meeting together). In those localities the exposed morph is found on the lower shore in the mussel zone, while the sheltered form is found on the mid-upper shore in the barnacle zone (see Rolán, 1983), as expected from the relative wave exposure of these different shore levels (see Johannesson, Johannesson & Rolán-Alvarez, 1993).

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We have investigated specimens of these two sympatric forms of *N. lapillus* from one exposed locality. We compared shell morphology of distinct age classes and also radular morphology between morphs. The results confirm that the two morphs differ morphologically at all stages (from shelled embryos to adulthood), suggesting that the polymorphism has a genetic basis, so that these two morphs may represent true adaptive ecotypes (genetic forms adapted to different habitats). The genetic analysis presented here also supports this hypothesis.

MATERIAL AND METHODS

Adults and juveniles of *Nucella lapillus* were collected during December 2002 at Cape Silleiro, an exposed locality in the south of the Ria of Vigo (northwestern Spain). Specimens (and egg capsules) of the exposed (dark) forms were picked from the lower shore in crevices or among mussels, which supposedly are their prey. Egg capsules were found in clutches close to groups of spawning adults, and each capsule was collected from a different clutch in order to to increase the likelihood that it was laid by a different female. Specimens of the sheltered (light) morph were collected from the mid-upper shore, in crevices or under rocks protected from the waves. These two habitats (exposed and sheltered) were nearly contiguous (separated by 1–2 m) at this locality. Images of individuals (20 juveniles and 20 adults) were digitized using an image analyser and Leica binocular, 11 points (coordinates) being recorded for each image (Fig. 1L). The morphology of pre-emerging snails was studied in 30 shelled embryos from 10 capsules (three shelled embryos per capsule) from the upper shore and 27 shelled embryos from nine capsules (three per capsule) from the lower shore, belonging probably to sheltered and exposed forms, respectively. In the shelled embryos, we recorded 10 coordinate points from the digitized images (see Fig. 2D). All the coordinates were transformed to distances, and so we obtained 55 new variables for juveniles and adults and 45 for shelled embryos. These variables were reduced within each age class (shelled embryos, juveniles and adults) to three main (non-correlated) factors by principal components analysis (Manly, 1986). In addition, we used the sum of the square deviations of every coordinate to the centroid (obtained by averaging the coordinates from all the studied points) as the best estimate of shell size (centroid size), being uninfluenced by shell shape (Bookstein, 1991). Juveniles of both forms were also used to study the diameter of the nucleus and protoconch following Verduin (1977; see also Fig. 2A,B). The radula was extracted from the soft parts of specimens preserved in alcohol. The excised radula was cleaned for a few minutes in sodium hydroxide solution, then washed in distilled water, and finally placed on a microscope slide for examination by phase-contrast microscopy. The rachidian tooth was compared between sheltered and exposed morphs, using samples of 10 similarly sized adults in each case (see Fig. 2E,F).

We investigated the relative importance of genetic effects in determining shell size differences between wild families by applying a one-way ANOVA with the random factor capsule (assuming that each capsule belonged to a different female and thus to a different family). Such design could be used to estimate heritabilities (percentage of additive genetic variance in the population), providing that shelled embryos from the same capsule could be classified as full- or half-sibs (Falconer & Mackay, 1991). However, because we did not know if embryos were full- or half-sibs, we used the ANOVA only to check if the additive genetic variance existed or not, as has been done for other related gastropods (Carballo *et al.*, 2001).

We estimated genetic differentiation between exposed and sheltered morphs by genotyping 15 individuals of each morph at seven microsatellite loci. DNA was extracted by grinding 3–10 mg of foot tissue and incubating for 30 min at 60°C in cetyl trimethylammonium bromide (CTAB) buffer (100 mM Tris–HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB; 2% polyvynilpyrrolidone 40,000 MW; 0.2% 2-mercaptoethanol), followed by chloroform/isoamyl alcohol extraction and ethanol precipitation. Seven microsatellite loci (Nlw2, Nlw3, Nlw8, Nlw11, Nlw21, Nlw25 and Nlw27, described in Kawai, Hughes & Takenaka, 2001) were amplified and scored using an ABI377 automated sequencer. Genetic differentiation was tested with an exact G-test (Goudet, Raymond, Demeeus *et al.*, 1996), and F_{st} estimated using the method of Weir & Cockerham (1984). Both tests were performed with FSTAT software (Goudet, 1995). A matrix of allele-sharing genetic distances between individuals was constructed with the MSA software (Dieringer & Schlötterer, 2003) and used to construct a neighbour-joining tree with PHYLIP (Felsenstein, 1993).

RESULTS

The two morphs showed similar sizes (centroid sizes) in sexually mature specimens (adults), but significantly differed in younger age classes (shelled embryos and juveniles; Table 1). Shell size and morphology were also investigated by reducing all distances by principal components analysis. The three main components and the percentage explained for each age class are presented in Table 1. The first principal component (PC1) was highly correlated with centroid size for shelled embryos ($r = 0.999$, $N = 57$, $P < 0.001$), juveniles ($r = 0.997$, $N = 20$, $P < 0.001$) and adults ($r = 0.998$, $N = 20$, $P < 0.001$). This suggests that PC1 represents size differences, whereas PC2 and PC3 represent different components of shape independent of size (principal components are not correlated). Thus we can focus on PC2 and PC3 to study morphological differentiation. There were significant differences between morphs for PC2 in the three age classes and for PC3 only in shelled embryos (Table 1). The relationships between morphs for these two PCs are shown in Figure 3. Sheltered and exposed morphs typically segregated in component-space, although a few individuals overlapped (Fig. 3). Differences are invariably maintained throughout the life cycle, at least for PC2 (Table 1).

We could investigate the relative importance of genetic effects for determining shell size and morphology by a one-way ANOVA with the random factor capsule (assuming that each capsule belonged to a different female and thus to a different family), although the results should be considered with caution due to the small number of families analysed. This analysis revealed that the centroid size was significantly different among families in both the sheltered ($F = 13.8$, $n_1 = 9$, $n_2 = 20$, $P < 0.001$) and the exposed morphs ($F = 13.8$, $n_1 = 8$, $n_2 = 18$, $P < 0.001$). The third principal component was also significant for the exposed ecotype ($F = 11.5$, $n_1 = 8$, $n_2 = 18$, $P < 0.001$), but the remaining PCs were not significant for either ecotype.

Additionally, we compared exposed and sheltered morphs for other traits, the diameters of nucleus and protoconch (measured in juveniles) and the radula (measured in adult individuals). The protoconch (Fig. 2A, B) is always white, smooth and one whorl (or a little less) 840–1410 μm in diameter. Nucleus and protoconch diameters were similar for these two morphs (Table 1). Radulae are typically rachiglossan with a rachidian tooth showing a prominent central cusp and three more on each side; the innermost of these is more prominent and has lateral microcusps; the two outer ones are smaller and closely spaced. The width of the rachidian teeth differed significantly between morphs (Table 1).

Microsatellite analysis revealed significant genetic differentiation between exposed and sheltered morphs ($P = 0.002$). In particular, two loci (11 and 25) showed significant differentiation ($P = 0.003$ and 0.001, respectively, with a Bonferoni cut-off at 0.007). The estimated value of F_{st} between exposed and

sheltered morphs, although relatively low (0.025), is significantly different from 0 (95% CI 0.006–0.051). Genetic differentiation is, however, not complete, as can be seen in Figure 4.

DISCUSSION

Intraspecific polymorphisms, related to the degree of wave exposure in natural populations of the intertidal dogwhelk *Nucella lapillus*, have been described in several parts of Europe (Crothers, 1973, 1975; Day, 1990; Dixon *et al.*, 1994; Kirby, 2000). Some of these exposed and sheltered forms of *Nucella* are known to differ in their chromosome number and allele frequencies (Day, 1990; Kirby, 2000), although Galician population of *Nucella* did not show any chromosome polymorphism ($2N = 26$) associated with wave-exposure gradients (Dixon *et al.*, 1994; J. J. Pasantes, personal communication). The Galician morphs differ from those from other locations in certain morphological characteristics, but these differences are within

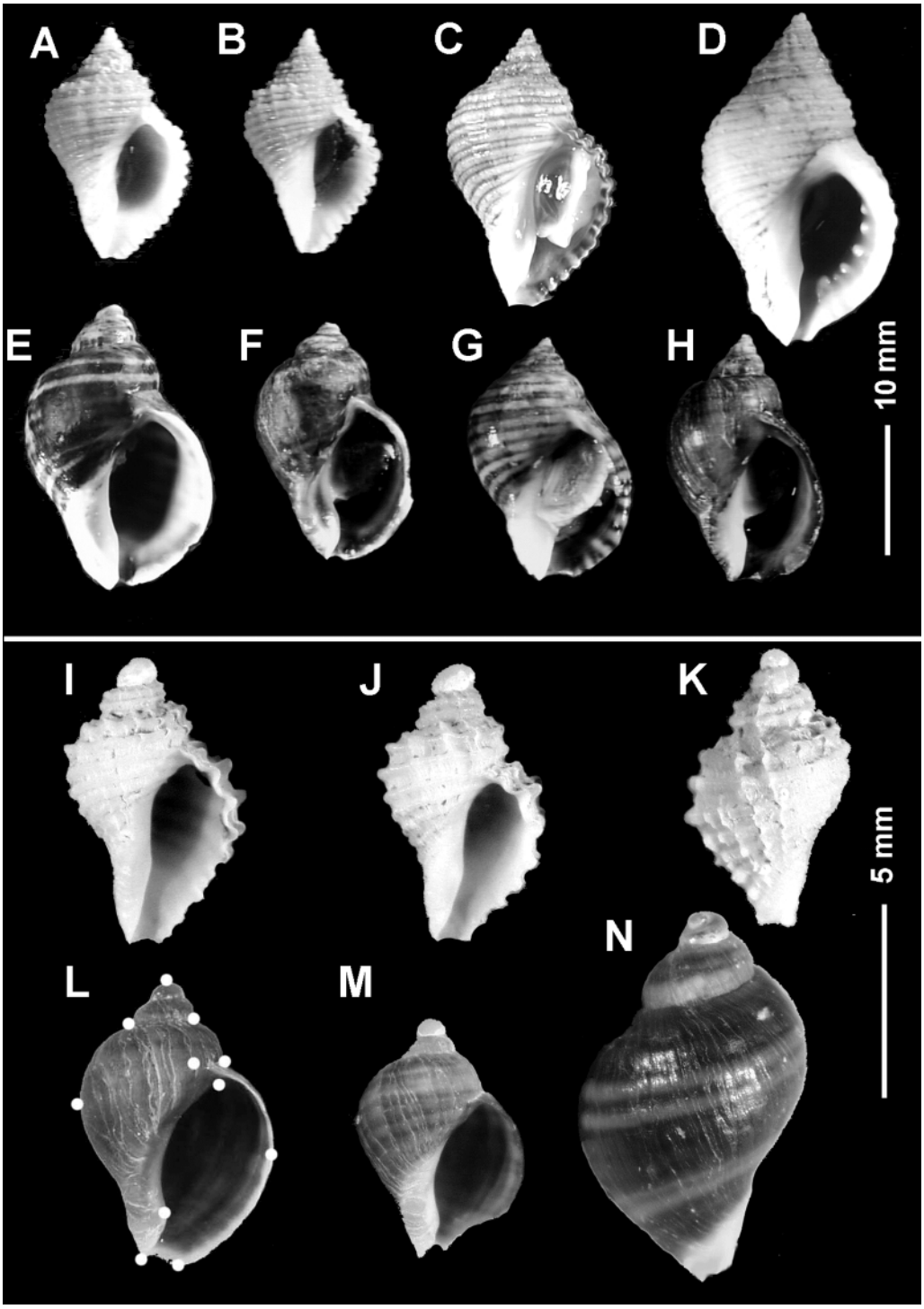

Figure 1. *Nucella lapillus* from Silleiro, Baiona, Spain. **A–D.** Adult specimens from the sheltered population. **A.** 19.0 mm. **B.** 17.5 mm. **C.** 22.6 mm. **D.** 27.1 mm. **E–H.** Adult specimens of the exposed population. **E.** 23.3 mm. **F.** 19.6 mm. **G.** 19.8 mm. **H.** 20.4 mm. **I–K.** Juvenile specimens of the sheltered population. **I.** 7.2 mm. **J.** 7.0 mm. **K.** 6.9 mm. **L–N.** Juvenile specimens from the exposed population. **L.** 6.9 mm, showing the coordinates points. **M.** 5.7 mm. **N.** 8.9 mm.

the range of variability typical of a species with low dispersal capability (Fretter & Graham, 1985). An exception is the size of the protoconch (800–1400 μm), which was rather larger than for British *N. lapillus* populations (500–700 μm; Fretter & Graham, 1985). Accordingly, the number of shelled embryos within the capsules was considerably smaller in the Galician populations (mean 11.9 embryos). However, there was no significant difference in protoconch size between the Galician morphs (Table 1), and so contrasts with other European populations may be a consequence of their relative isolation these populations. Additionally, there are intermediate forms (in the contact zone or close to it) in the studied populations and although the two morphs show small but significant genetic differentiation, it is unlikely that gene flow is totally prevented between them. Thus there is no reason to consider the exposed and sheltered morphotypes as valid species (see also Rolán, 1983).

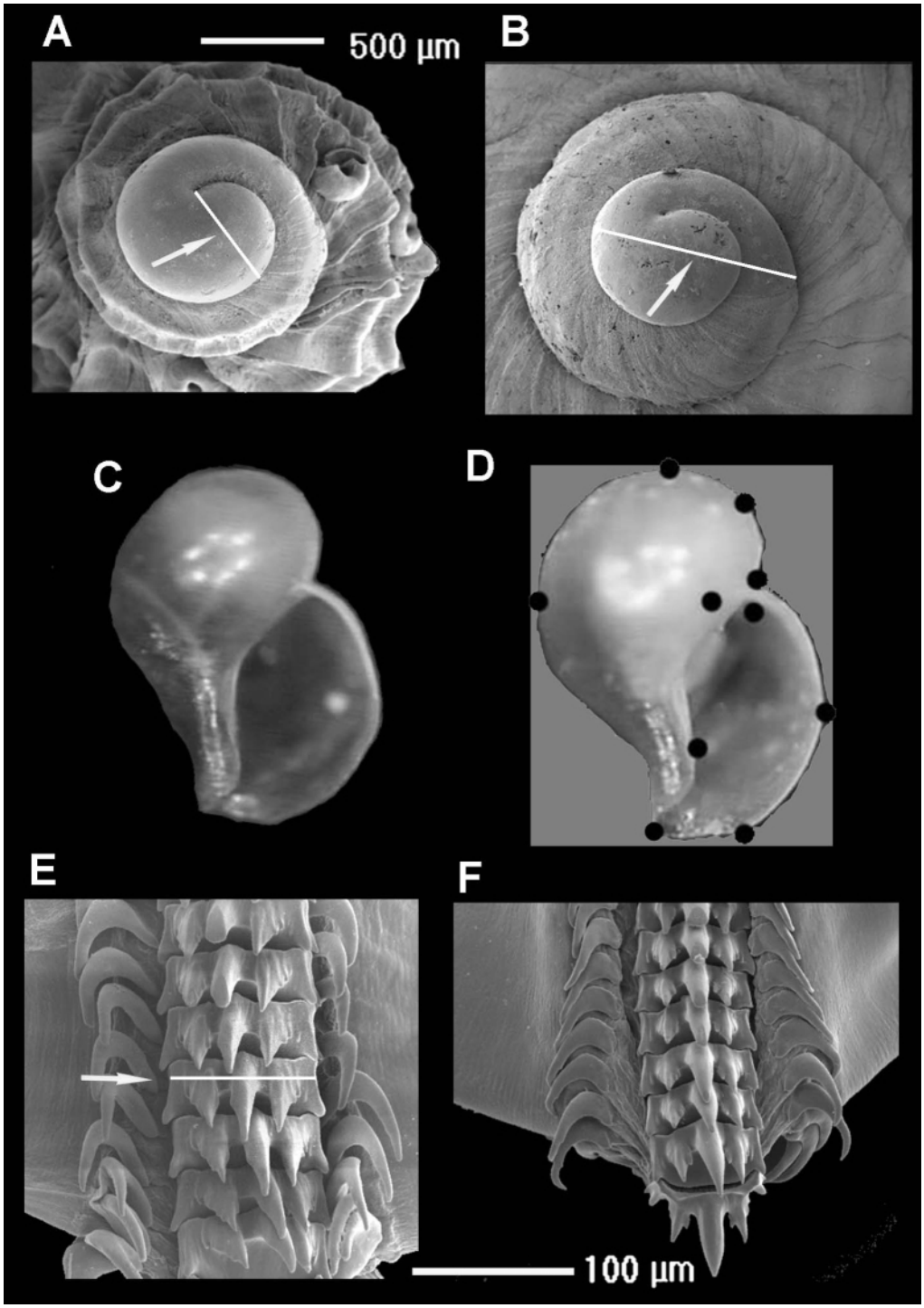

Figure 2. *Nucella lapillus* from Silleiro, Baiona, Spain. **A.** Protoconch of a specimen from the sheltered population, showing the line measuring the diameter of the nucleus. **B.** Protoconch of a specimen from the exposed population, showing the line measuring the diameter of the protoconch. **C.** Larval shell from the sheltered population. **D.** Larval shell from the exposed population, with the coordinate points. **E.** Radula from shell of 26.2 mm from the sheltered population, with the line measuring the width of the rachidian tooth. **F.** Radula from shell of 25.9 mm from the exposed population.

Table 1. Mean values of centroid size in the three age classes, nucleous and protoconch diameter in juveniles and radulae width in adults from both exposed and sheltered ecotypes of *Nucella lapillus* (in mm).

	Shelled embryos			Juveniles			Adults		
Traits	Sheltered	Exposed	*F*	Sheltered	Exposed	*F*	Sheltered	Exposed	*F*
Mean centroid size	1.74	1.45	22.1*	5.61	6.61	5.0*	25.01	25.15	0.1^{ns}
SD	0.223	0.239		1.237	0.691		1.404	3.478	
N	30	27		10	10		10	10	
PC1	77.35%		22.5*	83.99%		4.8*	61.91%		0.1^{ns}
PC2	5.87%		12.3*	7.46%		34.9*	14.7%		9.0^{*}
PC3	4.43%		8.2*	3.04%		0.1^{ns}	8.25%		1.6^{ns}
Nucleus diameter				0.56	0.57	0.1^{ns}			
SD				0.059	0.056				
N				10	10				
Protoconch diameter				1.13	1.19	0.9^{ns}			
SD				0.164	0.079				
N				10	10				
Width of rachidian tooth							0.09	0.13	46.5*
SD							0.013	0.013	
N							10	10	

The percentage of variation explained by each of the three main principal components (PC1, PC2, PC3) is also presented for the three age classes. Differences in mean values between ecotypes were evaluated by a classical one-way ANOVA. *, $P < 0.05$; ns, not significant.

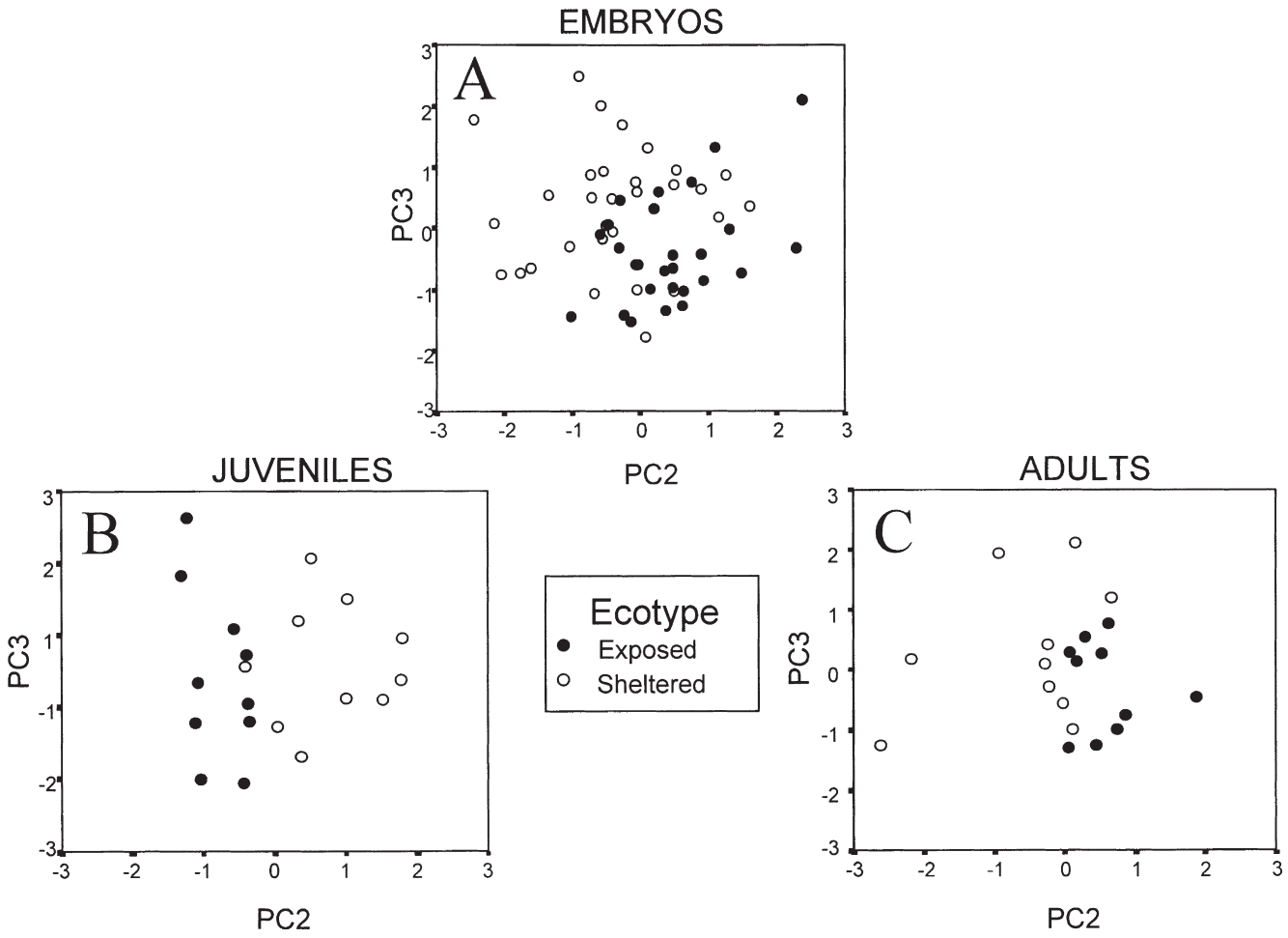

Figure 3. Distribution of the specimens studied in two-dimensional space generated by the second and third principal components. **A.** Shelled embryos. **B.** Juveniles. **C.** Adults.

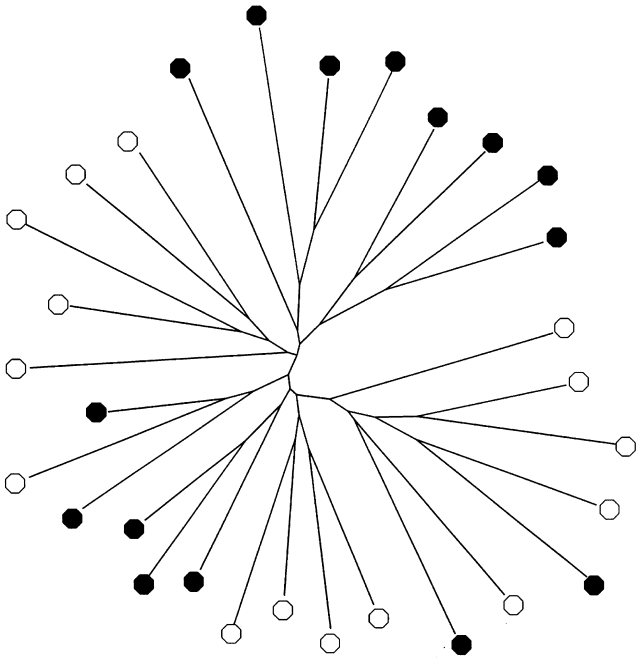

Figure 4. Neighbour-joining tree of individuals based on allele-sharing distance. Filled circles, exposed population; open circles, sheltered population.

The exposed and sheltered morphs of *N. lapillus* from Galicia were different from each other in shell and radular morphology, but not in protoconch size or morphology. Differences in the radulae may perhaps reflect the predominant diet of barnacles in the sheltered habitat and of mussels in the exposed, while shell differences suggest differences in adaptation to their corresponding habitats. In addition, this differentiation occurs even when the morphs occur in contiguous populations (separated by a few metres). The maintenance of shell-shape polymorphism from embryo to adulthood suggests that these traits are probably genetically determined. Moreover, the significant differences observed among families, not only for centroid size but also for the third principal component on the exposed morph, can be considered as further evidence of a genetic component for shell determination in these populations. The detected genetic variability for centroid size may be taken with some caution, because the size of shelled embryos at hatching can be influenced by maternal investment in some congeners (Moran & Emlet, 2001). However, this should not affect measures of shell morphology that are independent of size (like PC3).

Although environmental effects on shell morphology have been well documented in *Nucella lapillus* and other gastropod species (Boulding & Hay, 1993; Trussell & Etter, 2001), such environmental influence does not rule out a genetic contribution to the studied trait (Boulding & Hay, 1993), as typically both contributions have a role in natural populations. Indeed, the present genetic analysis strongly supports the hypothesis that the differentiation between exposed and sheltered morphs is at least partially under genetic control.

A related question is whether or not the ecotype differences are caused by phenotypic plasticity or different alleles at each ecotype affecting shell morphology. The intertidal rocky shore shows strong geographical and vertical environmental gradients (Raffaelli & Hawkins, 1996) and this may favour any of the above evolutionary strategies. Similarly, sheltered/exposed ecotypes are known to occur in other related gastropods, such as *Littorina saxatilis* (Olivi, 1792) (see Janson, 1983; Johannesson *et al.*, 1993; Reid, 1993; Rolán-Alvarez, Rolán & Johannesson, 1996) and *Ocenebra erinacea* (Linné, 1758) (see Rolán, 1983), and also in other groups of organisms (Trussell & Etter, 2001). In principle, it is more likely for species with limited dispersal ability to produce populations adapted to particular habitats, because (due to the lesser probability of receiving immigrants) the adaptive genomes are rarely disturbed by alleles (from other populations) adapted to different conditions. In summary, these morphs may represent ecotypes adapted to different environmental conditions (as at least some of the morphological variation may be inherited), although to determine the relative contribution of phenotypic plasticity would require experimentation on these populations.

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