Vol. 475: 145–153, 2013
doi: 10.3354/meps10138

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published February 14

Proteomic and morphological divergence in micro-allopatric morphotypes of *Melarhaphe neritoides* in the absence of genetic differentiation

Sara D. García[1], Angel P. Diz[1,*], Alexandra Sá-Pinto[2], Emilio Rolán-Alvarez[1]

[1]Departamento de Bioquímica, Genética e Inmunología, Facultad de Biología, Universidad de Vigo, Vigo 36310, Spain
[2]Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO/UP), Campus Agrário de Vairão, Vairão 4485-661, Portugal

ABSTRACT: The intertidal zone represents an interesting model system to study adaptation because several ecological factors change abruptly across this area, causing strong divergent selective pressures on a scale of meters. On the Galician shore (NW Spain), 3 species (*Littorina saxatilis, Nucella lapillus* and *Melarhaphe neritoides*) show similar adaptation to survive these abrupt changes. The 3 species have independently evolved morphotypes with similar morphological features that occur either in sheltered or exposed environments. However, while in *L. saxatilis* and *N. lapillus* the morphotypes are genetically based, no genetic divergence has been found between the morphotypes of *M. neritoides*. To better understand the extent of divergence between the 2 morphotypes of *M. neritoides* and the causes of this polymorphism, protein expression patterns of morphotypes collected from 2 Galician localities were studied. Additionally, mtDNA genetic diversity and shell morphometrics were studied for a reference population at Silleiro. A significant and geographically consistent divergence in protein expression was found between morphotypes kept under controlled laboratory conditions for 1 mo. Morphometric analyses showed that the morphotypes maintained the differences in shell shape even after being kept for 15 mo under similar laboratory conditions. No significant genetic differentiation was found between the 2 morphotypes at the mtDNA level despite the high genetic diversity observed. Together, our data suggest phenotypic plasticity as the most likely cause for the morphotypes and provide putative candidate loci that can be further studied for a better understanding of the origin of this adaptive polymorphism.

KEY WORDS: Evolutionary ecology · Phenotypic plasticity · Adaptation · Speciation · Proteomics · mtDNA · Morphometrics

INTRODUCTION

When exposed to different environments, the same genotype may result in distinct phenotypes, a phenomenon known as phenotypic plasticity or standard reaction norm in an ecological and quantitative genetic context, respectively (Falconer & Mackay 1996, Pigliucci 2005). The genes involved in such a plastic response may potentially contribute to adaptation and therefore to evolution (Pigliucci 2001, 2005, Langerhans & DeWitt 2002, DeWitt & Scheiner 2004, Hollander et al. 2006). In fact, natural selection may maintain adaptive polymorphisms in a population by acting either directly on loci coding for the trait ('direct adaptation') or on loci controlling a phenotypic plastic response for that trait ('indirect adaptation') (Garland & Kelly 2006, Pigliucci et al. 2006, Price 2006). Phenotypic plasticity is expected to be preferentially associated with species with high dispersal abilities, despite some well-known exceptions (Hollander 2008).

*Corresponding author. Email: angel.p.diz@uvigo.es

Intertidal rocky shore areas are extremely heterogeneous habitats with strong environmental gradients from lower to upper shores (Raffaelli & Hawkins 1996). In exposed areas of Galician shores, these environmental gradients are steep and impose very different ecological pressures on organisms living at distinct levels, from the strong impact of wave action on the lower shore to desiccation, heat and osmotic stress on the upper shore (Rolán-Alvarez 2007). Accordingly, marine intertidal gastropods from such microhabitats are very interesting models to study mechanisms of ecological adaptation (Etter 1996). Two gastropods with direct development (*Littorina saxatilis* and *Nucella lapillus*; Rolán-Alvarez 2007, Guerra-Varela et al. 2009) and one with planktotrophic larvae (*Melarhaphe neritoides*; Cuña et al. 2011) show similar and convergent adaptations to the steep ecological gradient of exposed Galician shores: the lower shore form (exposed: E) has a relatively larger shell aperture compared to the upper shore form (sheltered: S) to lodge a stronger muscular foot that increases the ability to resist rocky-shore dislodgment during wave impact. The smaller shell aperture of the upper shore form may reduce desiccation and heat stress (Cuña et al. 2011).

Melarhaphe neritoides (L.) is a small dioecious littorinid gastropod that typically lives on intertidal rocky shores from Mediterranean and Iberian Atlantic coasts (Fretter & Graham 1980). This species shows seasonal reproduction, possessing pelagic eggs and larvae that spend up to 4 to 8 wk in the water column until juveniles settle on a particular rocky shore (Johannesson 1992, Cronin et al. 2000). On exposed rocky shores from Galicia (NW Spain), 2 morphotypes have been found related to different shore levels and microhabitats. These 2 morphotypes of *M. neritoides* can be found in distant geographic locations, but no genetic differentiation at a micro-geographical scale was observed in one study of hundreds of AFLP markers (Cuña et al. 2011). Shell shape features characteristic of each of these morphotypes were still maintained after 9 mo under similar environmental conditions (Cuña et al. 2011), suggesting that differences between morphotypes are genetically determined or caused by phenotypic plasticity mechanisms acting during early developmental stages.

To fully answer classic adaptive and evolutionary questions, it is important to follow an integrative approach at distinct levels. Proteomics — the study of the protein complement expressed by a genome — is usually an underrepresented level despite the evidence supporting the importance of studying protein expression patterns (reviewed by Diz et al. 2012a). The importance of proteomics in the study of phenotypic plasticity has been recently discussed in the context of global warming and environmental pollution effects on aquatic organisms (Silvestre et al. 2012). Accordingly, to enhance our understanding of this evolutionary phenomenon, we tested for significant differences between morphotypes at several levels, including morphology, mtDNA and protein expression. The proteomic analyses showed high proteome differentiation between morphotypes within locality, a trend maintained at a wide geographical scale. Morphometric analyses revealed significant differences in shell shape between morphotypes after 15 mo under similar ecological conditions in the laboratory. Despite this, no significant genetic differentiation was found in the mtDNA between the 2 morphotypes collected in the same locality. Together, these data suggest phenotypic plasticity to be the most likely cause for this adaptive polymorphism, although alternative explanations cannot be excluded.

MATERIALS AND METHODS

Sampling and experimental design

Individuals of *Melarhaphe neritoides* were sampled according to their position on the shore: sheltered (S; at the upper level) and exposed (E; at the lower level), in August 2010. Samples were collected from 2 rocky shore areas: Silleiro Cape, Baiona (42° 06′ 15″ N, 8° 53′ 56″ W) and Roncudo Cape, Ponteceso (43° 16′ 25″ N, 8° 59′ 12″ W). These localities are linearly and geographically separated by 200 km of rocky shore and Cape Finisterre. A barrier to gene flow has been described across this area and has been suggested as the most likely cause for the small but consistent genetic differentiation found in populations of both direct-developing species and species with planktonic dispersal (see Fig. 1 from Piñeira et al. 2008, Diz & Presa 2009). Distances between upper- and lower-shore microhabitats differed between these 2 sampling localities (30 and 6 m at Silleiro and Roncudo, respectively) due to different shore slopes. After collection, specimens were immediately transported to our marine station facilities (ECIMAT) and kept in aquaria with seawater from an open circuit. Technical details of the breeding system for this and other related littorinid species are available in previous works (Conde-Padín et al. 2007, 2009). The specimens were placed in aquaria randomly positioned in the system to avoid uncontrolled environmental biases. Ten to 15 individuals, sorted

by morphotype (S and E) and locality (Silleiro and Roncudo), were maintained under identical environmental conditions for 1 mo to minimise environmental effects on protein expression. After this period, a batch of 240 specimens were collected, immediately snap frozen in liquid nitrogen, labelled and stored at −80°C until further proteomic analyses. A second batch of 40 juvenile specimens from Silleiro was maintained under similar laboratory conditions for 15 mo. Shell morphometric analyses were performed in 2 sub-samples of 12 specimens per morphotype. For genetic analyses, individuals from lower- and upper-shore microhabitats were collected in Silleiro and preserved in ethanol until DNA extraction.

Morphometric analysis

Sampled specimens were examined using a Leica MZ12 stereoscopic microscope. Colour images were captured with a digital ICA video camera. Shell images (N = 20) were analysed using 10 landmarks positioned on the digitalised shell images (see Cuña et al. 2011), following the approach used in other related snail species (Carvajal-Rodríguez et al. 2005, Conde-Padín et al. 2007, Guerra-Varela et al. 2009). For each specimen, size was measured by the centroid size (CS), whereas shape was measured using both the uniform component (U1 and U2) and the relative warps (RW) (Rohlf & Slice 1990, Bookstein 1991, Rohlf & Marcus 1993, Rohlf & Bookstein 2003, Zelditch et al. 2004, Carvajal-Rodríguez et al. 2005). The relative warps were computed excluding the uniform component (to study globosity separately), using the algorithm described by Rohlf & Marcus (1993), following the procedure applied by Carvajal-Rodríguez et al. (2005). We used the scaling option $\alpha = 0$, which weights all landmarks equally (Rohlf et al. 1996). All calculations were performed using the software TpsDig and TpsRelw (both available at http://life.bio.sunysb.edu/ee/rohlf/software.html), and MODICOS (Carvajal-Rodríguez & Rodríguez 2005).

mtDNA analyses

The high mutation rate of mtDNA and its usually lower effective population size make this genetic marker a useful tool to reveal weak genetic divergence between populations, providing an additional source of data that can be compared with results from AFLP markers. To test for genetic divergence between the 2 morphotypes, we sequenced a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (*COI*) for 31 and 18 individuals of the exposed and sheltered morphotypes, respectively. Total genomic DNA was extracted from a portion of foot muscle following standard procedures (Sambrook et al. 1989). *COI* was amplified according to Folmer et al. (1994) and Sanger sequenced with the primers used in the PCR amplification. Sequences were trimmed to a 606 bp fragment and aligned manually with Bioedit 7.0.9.0 (Hall 1999). All haplotypes were submitted to EMBL Nucleotide Sequence Database (accession numbers: HF548151–HF548199). Haplotype networks were estimated with TCS (version 1.21; Clement et al. 2000) using 94% statistical confidence as the parsimony connection limit (Templeton et al. 1992). To test for genetic differentiation between morphotypes, exact-tests of population differentiation (Raymond & Rousset 1995) were performed, and F_{ST} (Wright's fixation index) values between morphotypes and their statistical significance (1000 permutations) were estimated using ARLEQUIN version 3.11 (Excoffier et al. 2005). DnaSP (Rozas et al. 2003) was used to calculate population diversity indexes.

Proteomic analyses

A pooling sample strategy was followed for protein extraction to reduce the biological variation and increase the statistical power in the experimental design (Diz et al. 2009). This strategy was successfully applied in 2 previous studies in another marine snail (Martínez-Fernández et al. 2008, 2010). Each sample included 20 similarly sized specimens to diminish any within-pooled sample bias (see Diz et al. 2012a). Three samples (biological replicates) were prepared for each morphotype (E and S) and locality (Roncudo and Silleiro), i.e. a total of 12 biological replicates. Two of these 12 samples were analysed twice (i.e. technically replicated) to estimate the technical noise in the experimental procedure. Proteins were extracted from each pooled sample (without shells) and solubilised in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 1% IPG), at a constant ratio of ~30 mg of tissue per 1 ml of lysis buffer. This procedure was accelerated by tissue disruption by sonication (Branson Digital Sonicator 250) using 10 blasts of 25% amplitude and 5 s each, with 10 s breaks. This was done on ice to avoid protein overheating. After centrifugation for 30 min at 21 000 × *g* and 15°C, the pellet was discarded, and the protein supernatant was stored at −80°C until

electrophoresis. Protein concentration was measured with a modification of the Bradford method (Ramagli & Rodriguez 1985).

Proteins were analysed by 2-dimensional electrophoresis (2-DE) in batches of 6 gels/samples per run following a randomising block design for minimising technical variation (see Diz & Skibinski 2007). A total of 80 µg of total protein was used per 2-DE gel. The first dimension of separation was performed through isoelectric focusing (IEF), carried out on immobilised pH gradient strips (pH 5–8/17 cm, BioRad) with a Protean IF System (BioRad) and including an active strip-rehydration step, followed by 2 consecutive steps of strip equilibration (2 × 15 min) with dithiothreitol (DTT) and iodoacetamide (IAA), respectively. The second dimension of gel electrophoresis was carried out with labcast 12.5% polyacrylamide gels ($22 \times 27 \times 0.1$ cm^3 from GE Healthcare) using an electrophoresis system (Ettan Dalt*six,* GE Healthcare). Electrophoresis was carried out at 20°C, using an F12-MC refrigerated/heating circulator system (Julabo labortechnik), and at a constant current of 15 W gel^{-1} for ~5 h until the bromophenol blue front reached the bottom of gels. Protein spots were visualised by a modified version of the silver staining method described by Heukeshoven & Dernick (1985). Silver stained gels were scanned at high resolution by an Image Scanner (BioRad), and 2-DE gel images were saved as TIFF files. Semi-automatic alignment of 2-DE gel images, protein spot detection and volume measurements were accomplished by Progenesis SameSpots software version 3.3 (Nonlinear Dynamics). A careful final spot-filtering step was carried out to exclude technical artefacts and saturated or not well defined spots (e.g. due to streaking). SameSpots ensures that zero or negative spot intensity values are not returned by the software, a convenience for subsequent statistical analysis.

Absolute spot volumes provided by 2-DE software were normalised for each gel and then transformed to a logarithmic scale in order to better fit normality and homoscedasticity (see Diz & Skibinski 2007). The transformed volume of each protein spot (dependant variable) was analysed by a 2-way ANOVA with factors Morphotype (fixed; with S and E treatments), Locality (random; Silleiro and Roncudo) and their interaction. The SGoF multitest adjustment (Carvajal-Rodríguez et al. 2009) was applied on the whole set of p-values obtained from previous analyses at 5% and 1% significance level to account for the multiple hypothesis testing problem (reviewed by Diz et al. 2011). Statistical analyses were done with SPSS/PC version 17.0. The frequency of significant spots (after multitest adjustment) between treatments or factors in the ANOVA was compared with likelihood *G*-tests performed with POPTOOLS (Hood 2010), allowing a statistical robust comparison across factors (Allison et al. 2006).

RESULTS

Proteomic comparison

Normalised and log-transformed protein spot volumes were individually analysed by a 2-way ANOVA (n = 532 spots). Significant differences in the proteome analysed were observed between morphotypes but not between localities (or interaction), regardless of the significance level used (5 and 1%; Table 1). Interestingly, 1-way ANOVA returned slightly different results, i.e. the quantitative differentiation in protein expression between morphotypes significantly differed between localities (16% in Silleiro and 6.4% in Roncudo; *G*-test = 24.7, df = 1, $p < 0.001$). According to our results, 14 (41.2%) of the protein spots showing significant differences in expression between morphotypes in Roncudo also showed significant expression differences in Silleiro. Such percentage increased to 88% when using those spots that showed significant differences in expression ($p < 0.05$) between the 2 morphotypes in Silleiro without applying any multitest adjustment. The fold-change parameter (the relative expression ratio between the 2 morphotypes) obtained for each spot showed a high correlation ($r = 0.8$; n = 14; $p < 0.01$) between the 2 localities. Table 2 shows the results from this comparative analysis for the 14 spots that showed significant differences in expression in both localities. Of these 14 protein spots, 6 showed an up-regulated expression in the sheltered morphotype, while 8 spots were up-regulated in the exposed morphotype. The location of these protein spots in the 2-DE protein map is shown in Fig. 1.

Table 1. Percentage of significant protein spots (n = 532) from a 2-way ANOVA for each factor (Morphotype, Locality and Interaction) at 5% and 1% significance level, after applying the corresponding multitest adjustment. A likelihood *G*-test was used to check the frequency of significant loci between factors. ***$p < 0.001$

Factors	Significance level	
	5%	1%
Morphotype	22.6	14.3
Locality	0	0
Interaction	0	0
G-test	245***	158***

Table 2. Fold changes of protein expression (sheltered/exposed morphotypes) in each sampling locality observed for protein spots that significantly differed in expression between morphotypes in both localities after applying a multitest correction ($p < 0.05$). Notice that differences in protein expression between morphotypes (fold change) are significantly similar across localities in all cases (p of binomial test is < 0.0005). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

SPOT	Fold change (ratio sheltered/exposed)	
	Silleiro	Roncudo
29	4.63***	1.91*
32	3.50***	2.40*
248	2.52***	3.11**
303	0.55***	0.47*
311	1.59***	1.75***
313	0.30***	0.29***
332	0.49**	0.55*
354	0.56**	0.53*
380	1.18*	1.39*
461	0.49*	0.36**
505	0.44***	0.46**
512	0.29***	0.30**
519	0.59*	0.48***
520	1.27**	1.70***

Morphometry

Significant differences in shell size (CS) and shape (for RW1) (Table 3) were still observed between individuals collected in Silleiro at different shore levels after spending 15 mo under similar laboratory conditions. These results mirror those previously reported for individuals from different shore levels kept under similar conditions for 9 mo (Cuña et al. 2011). In agreement with this work, the individuals from the lower shore presented a slightly larger shell aperture than those collected from the upper shore (data not shown).

Table 3. *Melarhaphe neritoides*. Morphometric measurements taken from specimens sampled in Silleiro and maintained for 15 mo under identical laboratory conditions. Mean values per morph (n = 12) and SD in parentheses. CS: centroid size (in mm); U: uniform component; RW: relative warp; ns: not significant, $^{**}p < 0.01$

Morph	CS	U1	U2	RW1	RW2	RW3
Exposed (Lower)	0.33 (0.011)	0.007 (0.006)	−0.005 (0.003)	−0.02 (0.009)	−0.004 (0.006)	0.009 (0.008)
Sheltered (Upper)	0.37 (0.009)	−0.007 (0.008)	0.005 (0.003)	0.02 (0.009)	0.003 (0.010)	−0.009 (0.005)
F ANOVA	10.3**	1.8ns	3.9ns	10.5**	0.4ns	3.4ns

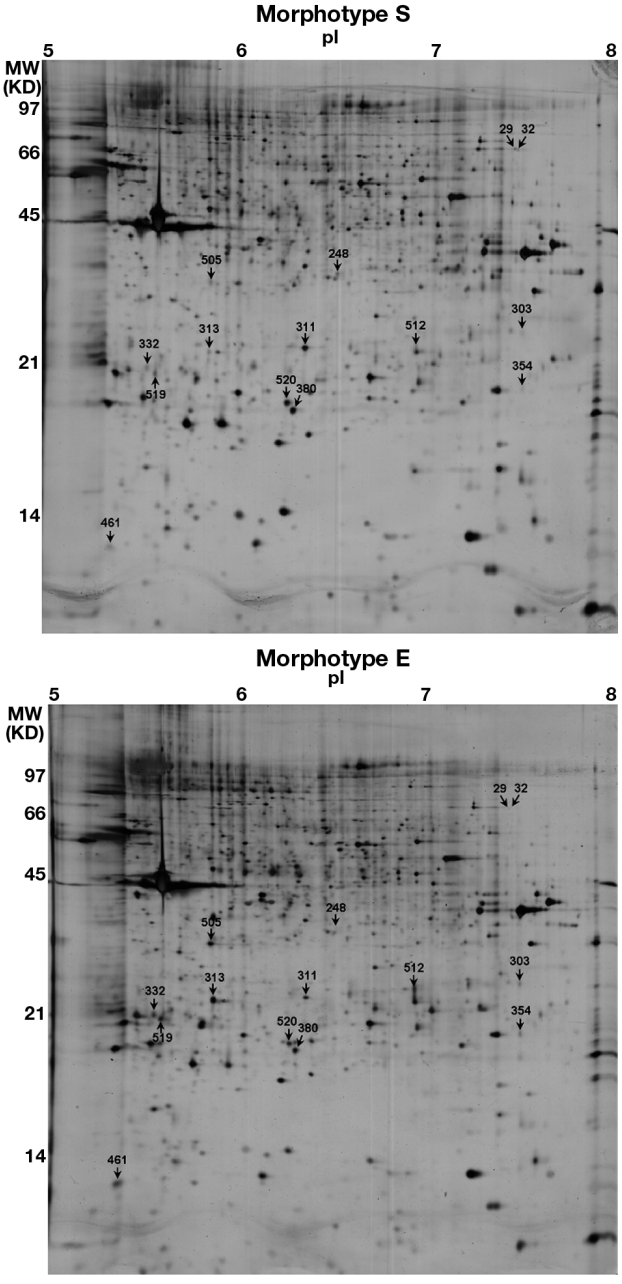

Fig. 1. Two-dimensional electrophoresis gels showing the proteome of *Melarhaphe neritoides* from exposed (E) and sheltered (S) morphotypes. Arrows show numbered protein spots with significant differences in expression between morphotypes in both sampling localities. MW: molecular weight; pI: isoelectric point. Statistical information for these protein spots is given in Table 2

mtDNA analyses

The results from mtDNA sequencing (summarised in Table 4 and depicted in the haplotype network represented in Fig. 2) reveal very high levels of

Table 4. Summary statistics of mtDNA diversity observed within each morphotype and in the entire sample at Silleiro Cape. n: sample size; S: number of segregating sites; Hd: haplotype diversity; π: nucleotide diversity

Morphotype	n	S	Hd	π
Exposed	31	72	0.998	0.01951
Sheltered	18	49	0.994	0.01883
Total	49	95	0.995	0.01922

genetic diversity within Silleiro, with 46 haplotypes detected in the 49 individuals sampled: 30 haplotypes in the E morphotype (n = 31) and 17 in the S morphotype (n = 18). From the 606 nucleotide positions analysed, 95 (16%) were polymorphic, resulting in 21 (10%) amino acid substitutions. No evidence for nuclear copies, such as stop codons or heterozygous positions, was detected. Despite this high genetic diversity, no significant genetic divergence was observed between the 2 morphotypes according to the results of the exact-tests ($p \gg 0.05$) and F_{ST} analysis ($F_{ST} = -0.005$; $p \gg 0.05$).

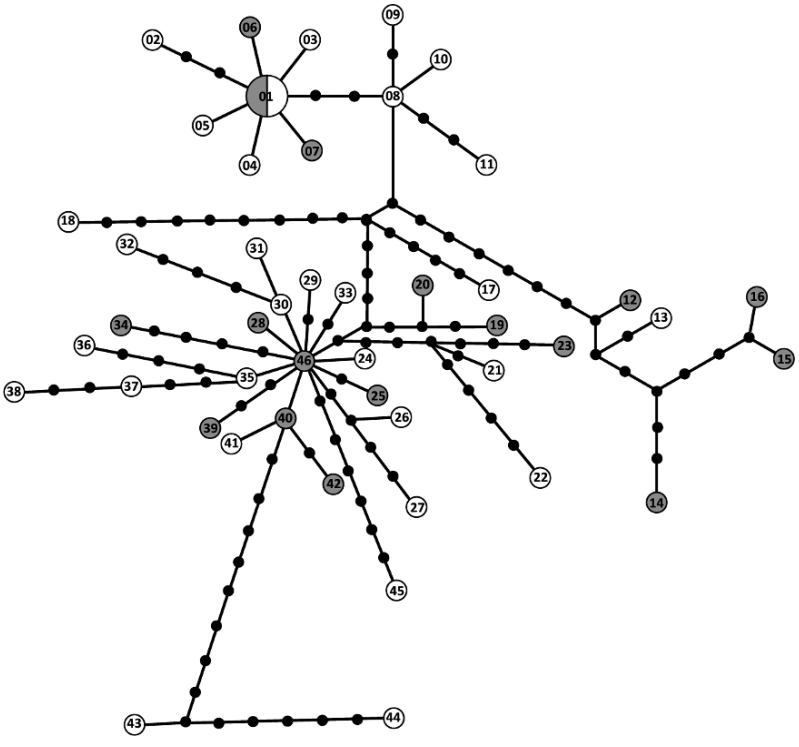

Fig. 2. Statistical haplotype network of a 606 bp fragment of cytochrome *c* oxidase I of *Melarhaphe neritoides*. The area of each circle is proportional to haplotype frequency; numbers inside the circles represent haplotype codes. For each haplotype, the area filled (white/grey) is proportional to the number of individuals from each morphotype: (white) lower shore (exposed) morphotype and (grey) upper shore (sheltered) morphotype. Black dots represent inferred missing haplotypes, and black lines connecting haplotypes or missing haplotypes represent a single mutation

DISCUSSION

Results from the present study show that the morphological divergence previously reported for the Galician morphotypes of *Melarhaphe neritoides* (Cuña et al. 2011) further extends to the proteomic level, with significant expression differences detected in up to 20% of the protein spots analysed. Similar to the pattern of morphological divergence (Cuña et al. 2011 and the present work), the reported proteomic differences between morphotypes are maintained after individuals are kept under controlled laboratorial conditions, suggesting that these differences are due to genetic differences and/or to permanent or long-term persistent phenotypic plasticity induced during early ontogenetic stages. Under the genetic hypothesis and given the large proportion of protein spots showing significant differences between morphotypes, we would expect substantial genetic divergence between these forms. Contrary to these expectations, the results of F_{ST} analyses and exact-tests show no significant genetic differences between the 2 morphotypes in the mtDNA, despite the high genetic diversity observed among the individuals sampled (see Table 4, Fig. 2). Although patterns of mtDNA diversity and divergence may be strongly influenced by historical demography and/or selective events (see Gillespie 2000, 2001, Ballard & Whitlock 2004, Bazin et al. 2006, Galtier et al. 2009), the lack of genetic divergence reported here is in total agreement with previous results obtained with 657 AFLP markers (Cuña et al. 2011) and further supports phenotypic plasticity as the main cause for the protein and morphological polymorphism. However, the (genetic) hypothesis that a few regulatory genes could be influencing expression levels in a large number of genes (i.e. those involved in the protein expression patterns observed) cannot be ruled out (see different mechanisms in Jaenisch 1997). Note that these 2 hypotheses are not mutually exclusive, and both factors may be contributing to the observed differences. In this context, the 14 protein spots that show significant and consistent differences in expression between morphotypes

in 2 distinct sampling locations (Table 2) represent good candidates to further study the evolutionary mechanism promoting the divergence between these 2 morphotypes. Information regarding the expression of these proteins under different environmental conditions and developmental stages can provide important information to support one or even both hypotheses. Also, the identification of these proteins, their coding genes and promoter regions will allow testing for the presence of genetic differences that could explain the expression differences.

Significant differences in the percentage of significant protein spots between morphotypes were observed between Silleiro and Roncudo (16 and 6%, respectively) when the analysis was done within locality. Under the phenotypic plasticity hypothesis, these differences could be explained by ecological differences between the 2 sampling localities. In fact, the lower- and upper-shore microhabitats in the 2 localities are slightly different, as these habitats are separated by >30 m in Silleiro but by <10 m in Roncudo (E. Rolán-Alvarez pers. obs.). Alternatively, these differences could be due to a sex or developmental stage bias in the samples compared. However, 3 biological replicates of 20 individuals each were performed for each morphotype in each sampling locality, thus making sampling bias less likely. Additionally, low differences were found in gene expression between sexes in *Littorina saxatilis,* another littorinid species: ~1.8% for transcripts (Martínez-Fernández et al. 2010) and <5% for proteins analysed (A. Diz unpubl. data), while ecotype differences in protein expression remained almost constant across ontogeny (Diz et al. 2012b). Nevertheless, sexual or anatomical differentiation between *Melarhaphe neritoides* morphotypes was not apparent in the studied samples.

Taken together, our results better support phenotypic plasticity as the most likely hypothesis to explain the proteomic and morphologic divergence observed between the 2 morphotypes of *Melarhaphe neritoides.* Phenotypic plasticity would also better conform to the expectations for a species with pelagic larvae, for which dispersal is mostly controlled by external factors, such as sea currents, with the scale of dispersal being much larger than the scale of ecological shifts. If so, *M. neritoides* may have evolved specific mechanisms that cause stable environmentally mediated changes in patterns of gene expression during early ontogeny, perhaps as a general strategy to deal with the environmental variation faced by the planktonic larvae. Such early environmental imprinting has already been described for several species (see Whitman & Agrawal 2009) and suggested under different epigenetic phenomena, e.g. via differential DNA methylation levels (Jaenisch 1997). In fact, several cases of adaptive phenotypic plasticity in plants have been shown to be due to differences in the environment experienced by parents that influence the developmental trajectories of progeny (either due to methylation or other epigenetic mechanisms; Uller 2008). To confirm the role of phenotypic plasticity on divergence observed between the morphotypes of *M. neritoides*, further experimental efforts are required, including the laboratory breeding of individuals from both morphotypes under a variety of environmental conditions.

If phenotypic plasticity proves to be the mechanism causing shell and proteomic variation in *Melarhaphe neritoides*, this species would represent an interesting contrasting model to *Littorina saxatilis* and *Nucella lapillus*. These latter species present ecotypes with similar morphological adaptations to those found in *M. neritoides*. However, *L. saxatilis* is a clear example of direct adaptation, as strong genetic differentiation can be found in some markers, suggesting a genetic basis for the morphological and anatomical divergence (reviewed by Rolán-Alvarez 2007). In *N. lapillus,* the ecotypes can be linked to a chromosome polymorphism, thus supporting genetic determination as the most plausible explanation for this adaptive process (Guerra-Varela et al. 2009). When compared to *L. saxatilis, M. neritoides* shows a similar degree of proteome differentiation between morphotypes (Martínez-Fernández et al. 2008, Diz et al. 2012b). These results suggest that phenotypic plasticity mechanisms can be as effective as genetically determined mechanisms in causing protein expression divergence and may play an important role in adaptation and evolution. In this context, and bearing in mind that knowledge on the genetic basis and molecular mechanisms of phenotypic plasticity is still rather limited (Pigliucci 2001, 2005), *M. neritoides* may represent a key model system to study this adaptive process. Note that such an important plastic response in gene expression (affecting up to 20% of the proteome studied) should probably be adaptive. In fact, it has been suggested that the main evolutionary relevance of plastic responses in the wild comes from the opportunity to maintain adaptive polymorphism without fitness costs (Pigliucci 2005). In any case, the study of morphotype formation and its maintenance in *M. neritoides* can provide valuable information for understanding adaptation through phenotypic plasticity.

Acknowledgements. We thank the ECIMAT institution for providing marine laboratory facilities, especially R. Sampedro and N. González-Lavín who provided technical help during the sampling and maintenance of specimens. We are also grateful to N. Santamaría for administrative contributions. We thank P. Cardia for his suggestions and review of this manuscript. This work has been funded by the Ministerio de Ciencia e Innovación (MCI) (CGL2008-00135/BOS), Fondos Feder: 'Unha maneira de facer Europa' and Xunta de Galicia (INCITE09 310 006 PR, 10PXIB 310044PR and 'Grupos de Referencia Competitiva' 2010/80), FEDER funds through the Programa Operacional Factores de Competitividade (COMPETE) and national funds through FCT (Fundação para a Ciência e a Tecnologia) within the scope of the project FCOMP-01-0124-FEDER- PTDC/BIA-BEC/105221/2008. A.P.D. is currently supported by an 'I. Parga Pondal' fellowship from Xunta de Galicia, and A.S.P. is supported by the FCT grant SFRH/BPD/48750/2008. The authors declare no conflict of interest.

LITERATURE CITED

Allison DB, Cui X, Page GP, Sabripour M (2006) Microarray data analysis: from disarray to consolidation and consensus. Nat Rev Genet 7:55–65

Ballard JW, Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol 13:729–744

Bazin E, Glémin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. Science 312:570–572

Bookstein FL (1991) Morphometric tools for landmark data: geometry and biology. Cambridge University Press, New York, NY

Carvajal-Rodríguez A, Rodríguez MG (2005) Morphometric and distance computation software oriented for evolutionary studies. Online J Bioinformatics 6:34–41

Carvajal-Rodríguez A, Conde Padín P, Rolán Álvarez E (2005) Decomposing shell form into size and shape by geometric-morphometric methods in two sympatric ecotypes of *Littorina saxatilis.* J Molluscan Stud 71:313–318

Carvajal-Rodríguez A, de Uña-Alvarez J, Rolán-Alvarez E (2009) A new multitest correction (SGoF) that increases its statistical power when increasing the number of tests. BMC Bioinformatics 10:209

Clement M, Posada D, Crandall KA (2000) Tcs: a computer program to estimate gene genealogies. Mol Ecol 9: 1657–1659

Conde-Padín P, Grahame JW, Rolán-Alvarez E (2007) Detecting shape differences in species of the *Littorina saxatilis* complex by morphometric analysis. J Molluscan Stud 73:147–154

Conde-Padín P, Caballero A, Rolán-Alvarez E (2009) Relative role of genetic determination and plastic response during ontogeny for shell-shape traits subjected to diversifying selection. Evolution 63:1356–1363

Cronin MA, Myers AA, O'Riordan RM (2000) The reproductive cycle of the intertidal gastropod *Melarhaphe neritoides* on the west and south coasts of Ireland. Biol Environ Proc R Ir Acad B 100B:97–106

Cuña V, Saura M, Quesada H, Rolán-Alvarez E (2011) Extensive micro-geographical shell polymorphism in a planktotrophic marine intertidal snail. Mar Ecol Prog Ser 427:133–143

DeWitt TJ, Scheiner SM (2004) Phenotypic variation from single genotypes: a primer. In: DeWitt TJ, Scheiner SM (eds) Phenotypic plasticity. Functional and conceptual approaches. Oxford University Press, New York, NY, p 1–9

Diz AP, Presa P (2009) The genetic diversity pattern of *Mytilus galloprovincialis* in Galician Rías (NW Iberian estuaries). Aquaculture 287:278–285

Diz AP, Skibinski DOF (2007) Evolution of 2-DE protein patterns in a mussel hybrid zone. Proteomics 7:2111–2120

Diz AP, Truebano M, Skibinski DOF (2009) The consequences of sample pooling in proteomics: an empirical study. Electrophoresis 30:2967–2975

Diz AP, Carvajal-Rodríguez A, Skibinski DOF (2011) Multiple hypothesis testing in proteomics: a strategy for experimental work. Mol Cell Proteomics 10:M110.004374

Diz AP, Martínez-Fernández M, Rolán-Alvarez E (2012a) Proteomics in evolutionary ecology: linking the genotype with the phenotype. Mol Ecol 21:1060–1080

Diz AP, Páez de la Cadena M, Rolán-Alvarez E (2012b) Proteomic evidence of a paedomorphic evolutionary process within a marine snail species: a strategy for adapting to extreme ecological conditions? J Evol Biol 25:2569–2581

Etter R (1996) The effect of wave action, prey type, and foraging time on growth of the predatory snail *Nucella lapillus* (L.). J Exp Mar Biol Ecol 196:341–356

Excoffier L, Laval LG, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longman, Harlow

Folmer O, Black M, Hoeh W, Lutz RA, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299

Fretter V, Graham A (1980) The prosobranch molluscs of Britain and Denmark. Part 5—*Littorinacea.* J Molluscan Stud 7:243–284

Galtier N, Nabholz B, Glémin S, Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Mol Ecol 18:4541–4550

Garland T Jr, Kelly SA (2006) Phenotypic plasticity and experimental evolution. J Exp Biol 209:2344–2361

Gillespie JH (2000) Genetic drift in an infinite population: the pseudohitchiking model. Genetics 155:909–919

Gillespie JH (2001) Is the population size of a species relevant to its evolution? Evolution 55:2161–2169

Guerra-Varela J, Colson I, Backeljau T, Breugelmans K, Hughes RN, Rolán-Alvarez E (2009) The evolutionary mechanism maintaining shell shape and molecular differentiation between two ecotypes of the dogwhelk *Nucella lapillus.* Evol Ecol 23:261–280

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6:103–112

Hollander J (2008) Testing the grain-size model for the evolution of phenotypic plasticity. Evolution 62:1381–1389

Hollander J, Collyer ML, Adams DC, Johannesson K (2006) Phenotypic plasticity in two marine snails: constraints superseding life history. J Evol Biol 19:1861–1872

Hood GM (2010) PopTools version 3.2.5. Available at www.poptools.org

Jaenisch R (1997) DNA methylation and imprinting: Why

bother? Trends Genet 13:323–329
Johannesson K (1992) Genetic-variability and large scale differentiation in two species of littorinid gastropods with planktotrophic development, *Littorina littorea* (L.) and *Melarhaphe* (*Littorina*) *neritoides* (L.) (*Prosobranchia, Littorinacea*), with notes on a mass occurrence of *M. neritoides* in Sweden. Biol J Linn Soc 47:285–299
Langerhans RB, DeWitt TJ (2002) Plasticity constrained: overgeneralized induction cues cause maladaptive phenotypes. Evol Ecol Res 4:857–870
Martínez-Fernández M, Rodríguez-Piñeiro AM, Oliveira E, de la Cadena MP, Rolán-Alvarez E (2008) Proteomic comparison between two marine snail ecotypes reveals details about the biochemistry of adaptation. J Proteome Res 7:4926–4934
Martínez-Fernández M, Páez de la Cadena M, Rolán-Alvarez E (2010) The role of phenotypic plasticity on the proteome differences between two sympatric marine snail ecotypes adapted to distinct micro-habitats. BMC Evol Biol 10:65
Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture. Johns Hopkins University Press, Baltimore, MD
Pigliucci M (2005) Evolution of phenotypic plasticity: Where are we going now? Trends Ecol Evol 20:481–486
Pigliucci M, Murren CJ, Schlichting CD (2006) Phenotypic plasticity and evolution by genetic assimilation. J Exp Biol 209:2362–2367
Piñeira J, Quesada H, Rolán-Alvarez E, Caballero A (2008) Genetic discontinuity associated with an environmentally induced barrier to gene exchange in the marine snail *Littorina saxatilis.* Mar Ecol Prog Ser 357:175–184
Price TD (2006) Phenotypic plasticity, sexual selection and the evolution of colour patterns. J Exp Biol 209: 2368–2376
Raffaelli DG, Hawkins SJ (1996) Intertidal ecology. Chapman & Hall, London
Ramagli LS, Rodriguez LV (1985) Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis 6: 559–563
Raymond M, Rousset F (1995) An exact test for population differentiation. Evolution 49:1280–1283
Rohlf FJ, Bookstein FL (2003) Computing the uniform component of shape variation. Syst Biol 52:66–69
Rohlf FJ, Marcus LF (1993) A revolution in morphometrics. Trends Ecol Evol 8:129–132
Rohlf FJ, Slice DE (1990) Extensions of the Procrustes method for the optimal superimposition of landmarks. Syst Zool 39:40–59
Rohlf FJ, Loy A, Corti M (1996) Morphometric analysis of old world Talpidae (Mammalia, Insectivora) using partial-warp scores. Syst Biol 45:344–362
Rolán-Alvarez E (2007) Sympatric speciation as a by-product of ecological adaptation in the Galician *Littorina saxatilis* hybrid zone. J Molluscan Stud 73:1–10
Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–2497
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Press, New York, NY
Silvestre F, Gillardin V, Dorts J (2012) Proteomics to assess the role of phenotypic plasticity in aquatic organisms exposed to pollution and global warming. Integr Comp Biol 52:681–694
Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619–633
Uller T (2008) Developmental plasticity and the evolution of parental effects. Trends Ecol Evol 23:432–438
Whitman DW, Agrawal AA (2009) What is phenotypic plasticity and why is it important? In: Whitman DW, Ananthakrishnan TN (eds) Phenotypic plasticity of insects: mechanisms and consequences. Science Publishers, Enfield, NH, p 1–63
Zelditch ML, Swiderski DL, Sheets HD, Fink WL (2004) Geometric morphometric for biologists: a primer. Academic Press, London

Editorial responsibility: Roger Hughes, Bangor, UK

Submitted: July 30, 2012; Accepted: October 24, 2012
Proofs received from author(s): February 2, 2013