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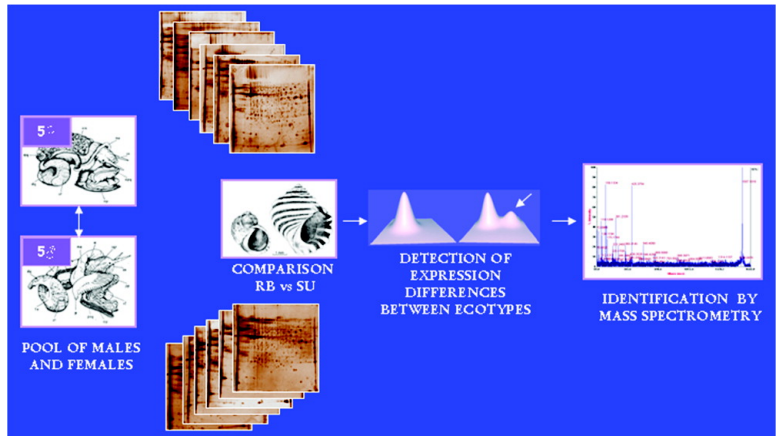


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# Proteomic Comparison between Two Marine Snail Ecotypes Reveals Details about the Biochemistry of Adaptation

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The proteomic changes occurring during speciation are fundamental to understand this process, though they have been rarely addressed until present. Therefore, we compared the proteome of two ecotypes (RB and SU) of the marine snail *Littorina saxatilis*, a case of sympatric incomplete speciation, originated as a byproduct of adaptation to distinct habitats. Thus, the RB ecotype is able to resist stresses of desiccation and temperature on the upper shore, whereas the SU ecotype defies strong physical disturbances due to wave action. Qualitative analyses of 2-DE gels demonstrated 21 proteins differentially expressed (1.4% of the proteome, 1.2% after considering type-I errors), while quantitative changes accounted for differences in 22 spots (16% of the proteome, 11% after considering type-I errors). These results suggest that adaptative phenotypic plasticity, natural selection, or both maintain these ecotypes in sympatry. Among the proteins identified by MS, we found that fructose-bisphosphate aldolase and arginine kinase were up-regulated in the SU ecotype, suggesting an enhancement of the level of energy available as ATP, in order to withstand its wave-exposed habitat.

**Keywords:** Differential expression • ecotypes • *Littorina saxatilis* • protein levels • sympatric speciation • two-dimensional gel electrophoresis • mass spectrometry

## 1. Introduction

In ecology, there has always been a general interest in understanding the detailed biochemical and physiological processes that occur during adaptation. Great progress has been made in the biochemistry of some well-known cases of adaptative variation, usually through the study of the levels of expression of one or a few particular genes.[1,2] Broadly, the study of adaptation has revealed at least three main evolutionary strategies at a biochemical scale: changes in the amino acid sequence of the protein, changes at the protein expression level, and changes in the protein environment.[2]

An array of methods has been used in biological, ecological and evolutionary studies to characterize and understand the phenotypic variability at the biochemical level, such as genomics, QTL mapping, transcriptomics and proteomics.[3−6] The genomic approach has fundamental practical and theoretical restrictions. First, it is only useful in well-known model species, where a considerable part of the genome has already been sequenced. Second, gene sequence data contain insufficient information to understand the function of the gene products. The QTL analysis would be most efficient when both detailed multigenerational pedigree information and a dense genetic linkage map are available,[7] something unusual for nonmodel species. Moreover, while the knowledge of the number and effect size of the genomic region(s) controlling the trait of interest is very valuable, the procedure to identify the quantitative trait nucleotide(s) of the QTLs is still very time-consuming.[8] In relation to transcriptomics, it is not possible to predict protein expression levels only from mRNA data and attempts to correlate protein abundance with transcript expression levels have had variable success.[9] This lack of correspondence has been used as a justification for the application of proteomics in studying expression differences between species.[10]

Among the multiple techniques that are used in proteomic studies nowadays, two-dimensional electrophoresis (2-DE) is currently one of the principal methods routinely applied for quantitative expression profiling of complex protein mixtures, together with mass spectrometry (MS). Hence, proteomic works published recently from marine systems were mainly focused on the identification of species[11,12] or on the search of biomarkers for pollutants in environmental studies.[13−16] Moreover, proteomics has provided highly valuable information both for phylogenetic inference and descriptive studies on genetic population diversity.[17−20] However, there are still few works dealing with detailed proteomic comparisons of ecotypes or incipient species in animals or plants.[21−25] Therefore, the proteomic approach is extremely promising, although there may be a slow progress for those species where there is no previous information on their proteome. In this study, we

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performed a preliminary proteomic analysis of two different ecotypes adapted to distinct habitats separated only by a few meters.

*Littorina saxatilis* (Olivi, 1792) is a marine gastropod presenting separate sexes, ovoviviparity (the female carry a brood pouch with nonplantonic shelled embryos) and typically high polymorphism. In the intertidal zone of the Galician coast, two ecotypes of *L. saxatilis* are adapted to different shore levels and habitats.[26,27] The large-sized ridged and banded ecotype (RB) is found on the upper shore where it lives among the barnacles. It has a larger and more robust shell, and a smaller aperture for reducing the loss of water due to the high sunshine exposition.[28,29] In contrast, the small-sized smooth and unbanded ecotype (SU) occupies the lower shore, living on mussels, and has a smaller shell with a relatively large aperture[29] to avoid dislodgement by the heavy wave action.[28] Although the habitats where these ecotypes live are typically separated by only 5−10 m of rocky shore, the adults have the capability to move from one habitat to the other, and therefore, they can be considered as effectively sympatric.[26] At the midshore, however, both ecotypes meet and occasionally mate in strict sympatry, although showing a partial prezygotic isolation barrier, with partial assortative mating.[30−32] Because of the ecotype distribution and its partial reproductive barrier, the gene flow among ecotypes is only slightly restricted.[33] Thus, the existence of phenotypic and genetic differences between ecotypes in the presence of gene flow can only be possible due to strong divergent natural selection acting across the environmental gradient. Experimental corroboration of such mechanism has already been provided in a repeatable way,[28,34,35] and hence, this model system is considered as a key example of incomplete and sympatric ecological speciation.[26,36−38]

To understand the importance of the physiological, ecological and behavioral differences between these ecotypes, we compared the protein expression of RB and SU specimens by 2-DE electrophoresis in two distinct situations: when they live at distinct shore level (upper and lower shore) and when they live in strict sympatry at the midshore. By definition, population proteomics will be based on the analysis of genetic polymorphisms detected by a qualitative approach (presence/absence of protein spots) and, second, by a quantitative analysis on abundance (relative volume) of the protein spots.[5] We found qualitative and quantitative changes in the expression of a number of proteins (about 12%), indicating that adaptation probably involves extensive changes of the proteome and the genome prior to completion of the speciation process. In particular, we identified two proteins with quantitative differences in their expression that could be interpreted as adaptative. However, we have not succeeded in the identification of most of the altered proteins, mainly due to the scarce representation of mollusc species in databases, which points to the future need of protein sequencing studies.

## 2. Materials and Methods

**2.1. Sample Collection and Preparation.** Specimens of *L. saxatilis* were collected in Silleiro (NW Spain; 42°6′15″ N, 8°53′56″ W), one of the most extensively studied localities for this model system.[26] In December 2003, samples of the RB ecotype were obtained from the upper shore together with samples of the SU ecotype from the midshore since it was not possible to sample the lower shore due to strong waves. In May 2004, RB individuals from the midshore and SU individuals from the lower shore were sampled. In this way, half of the specimens of each ecotype were obtained in the same date. These ecotypes do not show any systematic change in zonation, density or size across seasons.[39] For each of the shore levels and ecotypes, we collected 100 individuals which were labeled and transported to the laboratory. We prepared 6 samples within each ecotype (3 RB upper + 3 RB mid and 3 SU mid + 3 SU lower), each one including 5 males and 5 females. This design allows us to compare the proteomic expression between ecotypes in strict sympatry or in microallopatry (see below). The pooling strategy has shown an efficiency similar to the individual strategy for gene expression comparisons,[40] and it has been successfully used in proteomics when the amount of tissue did not allow an individual analysis[41,42] as it occurs in our case. Moreover, when proteomic differences are analyzed, it is important to consider that they could be due to interindividual or to interspecies (interecotype in our case) variations.[22] In fact, the main problem of the qualitative studies is the high variability among individuals.[14] Our experimental design is rather conservative in showing overall proteomic differences between ecotypes (RB versus SU), as variation among individuals, sexes and sampling dates are included in the within-group variation component. Samples were stored at −85 °C until analyzed.

Shells were removed and tissues were homogenized in lysis buffer [7 M urea, 2 M thiourea and 4% (w/v) CHAPS] with protease inhibitors (Complete Mini, Roche) so that the final ratio was 50 mg of tissue per 1 mL of lysis buffer. The homogenates were stored at −85 °C until used. Then, proteins were solubilized at 100 rpm and 25 °C for 1 h in an orbital shaker and centrifuged at 16 000*g* for 15 min. Supernatants were immediately used for electrophoresis. Protein concentration was measured according to Bradford[43] with modifications.[44]

**2.2. Two-Dimensional Gel Electrophoresis.** During the first dimension of the separation (isoelectric focusing, IEF), proteins were separated on the basis of their isoelectric point (p*I*) in a Protean IEF Cell system (Bio-Rad) using 17-cm ReadyStrip IPG strips (4%T, 3%C; 170 × 3.3 × 0.5 mm) with a pH range from 5 to 8. The weight of protein analyzed was 150 $\mu$g for analytical and preparative silver-stained gels, and 3 mg for preparative gels stained with Coomassie Brilliant Blue. Prior to IEF, samples were mixed with rehydration buffer [0.3% (w/v) dithiothreitol (DTT) and 0.5% (v/v) Bio-Lytes 3/10 ampholytes in lysis buffer up to 350 $\mu$L]. This mixture was used to rehydrate the gel strips with a constant voltage (50 V) for 12 h at 20 °C. Rehydrated gels were focused up to 60 000 Vh. Current was restricted to 50 $\mu$A per gel and the temperature was maintained at 20 °C.[45]

Before denaturing polyacrylamide gel electrophoresis (SDS-PAGE), the second dimension of the separation, IPG strips were incubated in equilibration buffer [50 mM Tris, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 1% (w/v) DTT] for 20 min with gentle shaking. The incubation was continued with a buffer containing 2.5% (w/v) iodoacetamide (IAA) instead of DTT, for another 20 min. Equilibrated strips were placed onto 12% polyacrylamide (12%T, 0.8%C; 195 × 184 × 1.5 mm) gels to perform the SDS-PAGE in a Protean II xi Cell at a constant temperature of 15 °C.[46] The separation was carried out at 20 mA per gel for 15 min and then 40 mA per gel until the bromophenol blue marker reached the bottom of the gel.

Analytical gels were stained with silver nitrate.[47] Preparative gels were silver-stained with a protocol compatible with protein digestion and mass spectrometry,[48] or alternatively, gels with higher protein loading were stained with 0.2% (w/v) Coomassie

Brilliant Blue R-350.[49] To load higher protein amount, samples were concentrated using dialysis and lyophilisation. The samples were dialyzed for 8 h with several changes of mQ water and subsequently centrifuged at 16 000*g* for 15 min. Supernatants were then lyophilized for 24 h and then dissolved in lysis buffer to carry out the IEF.

When 2-DE is used to analyze different protein maps, it is essential to obtain high resolution of the protein forms. Therefore, prior to the analytical study, we optimized the resolution in both dimensions of the 2-DE in order to achieve the best separation of the proteins. Regarding the IEF, we first tried strips with a pH ranging from 3 to 10 and from 4 to 7. However, a more uniform spot distribution was obtained with a pH range from 5 to 8. We also tested different protein loads, the most suitable being 150 $\mu$g, as shown in previous studies with molluscs.[50] The best SDS-PAGE separation was achieved with 12%-polyacrylamide gels. To verify the reproducibility of the 2-DE procedure, samples from the same set of individuals were run on separate gels on two different days. Well-resolved spots were detected in both gels and their relative volume was compared. The parameters used in the verification of the reproducibility were stored and employed in the following analytical comparisons.

**2.3. Image Acquisition and Analysis.** Analytical gels were scanned using a GS-800 calibrated densitometer (Bio-Rad), and protein patterns were analyzed with the PD-Quest 7.1.1 software package (Bio-Rad). Spots were automatically detected on the basis of the spot parameters chosen by the user such as the faintest, the smallest and the largest spot on the gel scan. Other parameters (as sensitivity, minimal peak value, size scale) were adjusted so that only true spots were identified. The background was then subtracted and the filtered images edited to correct possible errors by means of adding or removing inaccurate spots. The intensity levels detected during the acquisition of the images were expressed as the relative volume of the spots in each gel (in ppm),[12,51−53] representing the spot quantity and being a semiquantitative measure. Moreover, silver staining has a dynamic range of 1 to 2 orders of magnitude,[54] and in this study, the weakest and strongest spots fell within this range. For each ecotype, 6 gels were obtained and matched. Only well resolved spots were taken into account, discarding overlapped and streaked areas or spots near the edges.

**2.4. Statistical Assessment.** Both qualitative and quantitative differences in the relative volumes of matched spots were analyzed. For qualitative analyses, all spots were considered and their intensities were transformed into a matrix of 0 (absence) and 1 (presence). The statistical significance was assessed using contingency tables through *Fisher's exact test of independence*.[55] For quantitative analyses, normalized intensity data were exported only for those spots presented in all the replicates. The statistical significance was assessed by a two-way ANOVA since we could not detect any significant deviation from normality using the Kolmogorov−Smirnov nonparametric test.[55] The ANOVA included the fixed factors *ecotype* (RB versus SU ecotypes) and *distribution* (upper/lower versus midshore), and their interaction. The factor distribution allows verifying if the ecotype differences vary when the ecotypes are compared in strict sympatry or microallopatry.

The type-I error increases proportionally with the number of tests applied, and therefore, different multitest corrections have been suggested.[56] However, such corrections assume independence during expression of the different proteins, which clearly does not always occur, and thus, they are not recommended for proteomic studies.[57] Nevertheless, to exclude the chance that all of the observed univariate significant tests were just caused by a type-I error, we checked if the observed frequency of significant spots was significantly different from the expected frequency (based in the *a priori* level of significance) by a goodness of fit G-test using the conservative continuity correction.[55] Statistical analyses were done by the SPSS/PC release 12 or calculated in Microsoft Excel 2003.

**2.5. In-Gel Digestion of Proteins.** For identification, spots belonging exclusively to one ecotype or with differential expression were excised from preparative gels (either silver- or Coomassie-stained) and analyzed at the Proteomics and Mass Spectrometry Facility at Parc Cientific de Barcelona (Barcelona, Spain).

Proteins were in-gel digested with trypsin (Promega) in the automatic Investigator ProGest robot (Genomic Solutions). Briefly, protein pieces were washed sequentially with ammonium bicarbonate and acetonitrile (ACN). Proteins were reduced by treatment with 10 mM DTT for 30 min at 56 °C, and alkylated with 55 mM IAA. The digestion was accomplished with 0.27 nmol of trypsin at 37 °C overnight. Tryptic peptides were extracted from the gel matrix with 10% (v/v) formic acid and ACN, pooled and evaporated to dryness. Samples were redissolved in 5 $\mu$L of ACN and 0.1% trifluoroacetic acid (TFA) 1:1 (v/v), or in 15 $\mu$L of 1% (v/v) formic acid, to be analyzed by MALDI-MS or ESI-MS, respectively.

**2.6. Protein Identification by Tandem Mass Spectrometry (MS/MS) and Database Search.** Tryptic peptides were analyzed by matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) with a 4700 Proteomics Analyzer (Applied Biosystems) or by electrospray ionization-mass spectrometry/mass spectrometry (ESI-MS/MS) with a Q-TOF Global (Micromass-Waters). For MALDI-TOF/TOF, the digests were redissolved in 5 $\mu$L of 0.1% (v/v) TFA, 50% (v/v) ACN. Typically, 0.5 $\mu$L of sample was mixed with 0.5 $\mu$L of matrix solution (2 mg/mL α-cyano-4-hydroxycinnamic acid, Waters) in 0.1% (v/v) TFA and 50% (v/v) ACN. The major peaks obtained by MALDI-TOF were selected for further MS/MS analyses. Spectra were submitted for database searching with the MASCOT search engine (version 2.0; Matrix Science). MS/MS searches were conducted against the nrNCBI database (version 20071214; 5 742 110 sequences; 1 979 781 838 residues). Search parameters were set as follows: enzyme trypsin; allowance of one missed cleavage site; carbamidomethyl (Cys) as fixed modification; oxidation of Met as variable modification; monoisotopic mass values; protein mass unrestricted; peptide mass tolerance ±200 ppm; fragment mass tolerance ±0.25 Da. The threshold for positive identification was a MOWSE score variable depending on each search ($p < 0.05$).

Some tryptic digests were analyzed by capillary liquid chromatography coupled with tandem mass spectrometry (Cap-LC-nano-ESI-Q-ToF) (CapLC, Micromass-Waters). Samples were resuspended in 15 $\mu$L of 1% (v/v) formic acid solution and 4 $\mu$L of sample was injected for chromatographic separation in a reverse-phase capillary $C_{18}$ column (75 $\mu$m of internal diameter and 15 cm length, PepMap column, LC Packings). The eluted peptides were ionized via coated nanoelectrospray needles (PicoTip, New Objective). A capillary voltage of 1800−2200 V was applied together with a cone voltage of 80 V. The collision energy in the CID (collision-induced dissociation) was 20−35 eV, employing argon as collision gas. Data were submitted for database searching in a MASCOT and Blast server

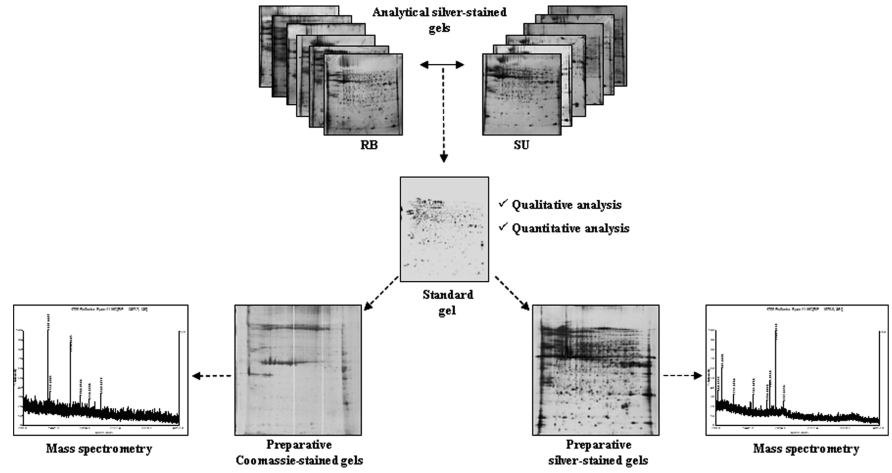


**Figure 1.** Workflow of the proteomic analysis of the *Littorina saxatilis* ecotypes RB and SU.

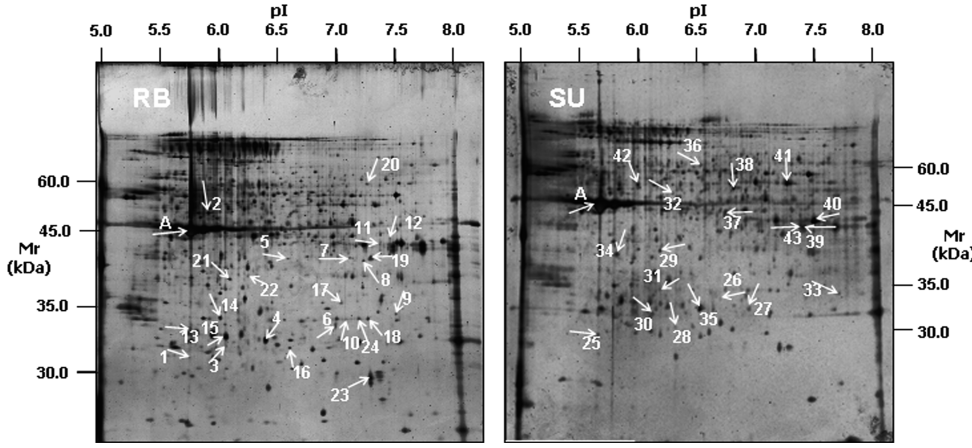


**Figure 2.** Two-dimensional standard protein maps for the RB and SU ecotypes. Spots with altered expression or exclusive to one of the ecotypes are indicated by arrows, and numbered as in Tables 1 and 2. The spot marked as "A" was used as a control for the MS methods and corresponds to actin.

against the NCBI database. Peptide sequences were obtained by *de novo* sequence analysis with the Protein Global Server (v.1.5) software. With the aim of increasing the number of positive identifications, searches were also carried out using Peaks and Phenyx search engines.

## 3. Results

**3.1. Two-Dimensional Protein Profiles of *L. saxatilis*.** For each of the ecotypes, we obtained 6 samples with 10 individuals each (5 males and 5 females), so altogether, we analyzed 12 gels representative of 120 specimens (see Figure 1). Protein quantity was determined in each sample and expressed as the weight of protein (in mg) in 100 mg of the snail. For the RB ecotype, the mean value and its standard deviation was 6.23 $\pm$ 1.07 mg per 100 mg of tissue, whereas in the SU ecotype, it was 5.59 $\pm$ 0.87 mg per 100 mg of tissue. Applying a one-way ANOVA, we concluded that there was no significant difference ($p > 0.05$) in the protein amount between ecotypes. The samples were then submitted to 2-DE and compared as indicated in Figure 1, detecting an average of 726 spots per gel (although only 136 well-defined spots were present in all the gels; see below). The reproducibility was calculated, obtaining a correlation coefficient of 0.88, and this value increased to 0.96 when only those spots showing significant differences between ecotypes were considered (see below).

**3.2. Qualitative Analysis.** The purpose of the qualitative analysis was to find proteins that appeared exclusively in one of the ecotypes studied, either because they were uniquely expressed in one of the ecotypes or expressed below the threshold of stain detection. Therefore, this data set included all the spots detected in the analytical gels, both common and unique to each group, accounting for a total number of 1498 distinct spots. The fact that we used 10 individuals per gel (5 males and 5 females) in addition to the 6 replicates for each ecotype allowed us to disregard individual allelic variations, ensuring that the differences found in our analyses were not due to individual or sexual characteristics.

The maps of the RB ecotype versus those of the SU ecotype were compared. We counted those particular spots which appeared in the 6 gels of one of the ecotypes and were not present in any gel of the other, a situation that would only appear by chance with a two-tail probability of 0.002 using the Fisher's exact test of independence. We found 12 spots that appeared exclusively in the RB samples and 9 that were only present in the SU specimens (Figure 2) (Supplementary Table 1 in Supporting Information). Notice that none of these would be significant after the conservative sequential Bonferroni multitest correction. However, these 21 significant spots represent 1.4% of the 1498 spots studied, which could hardly be explained by chance as we only expect 0.2% of these cases. In

**Table 1.** Relative Volume (in ppm), p*I* and Estimated Mass ($M_r$) for Each Spot Showing Differences of Expression between Ecotypes[a]

| spot number | RB average (std) | SU average (std) | experimental p*I* | experimental $M_r$ (kDa) | variation | $F_{ECO}$ | $F_{DIS}$ | $F_{INT}$ |
|---|---|---|---|---|---|---|---|---|
| 13 | 273.78 (110.52) | 66.59 (66.53) | 5.6 | 32.9 | +4.1 | 13.9** | 0.4 | 0.6 |
| 14 | 688.84 (220.14) | 239.77 (233.51) | 5.9 | 34.1 | +2.9 | 15.1** | 4.8 | 0.1 |
| 15 | 2614.95 (992.02) | 1068.61 (941.23) | 5.9 | 32.6 | +2.5 | 9.1* | 0.9 | 2.9 |
| 16 | 194.90 (57.25) | 100.01 (44.21) | 6.5 | 31.3 | +1.9 | 8.8* | 0.3 | 0.2 |
| 17 | 138.67 (58.65) | 59.23 (25.87) | 7.0 | 34.7 | +2.3 | 11.2** | 0.2 | 3.9 |
| 18 | 464.76 (141.14) | 227.74 (162.46) | 7.3 | 33.5 | +2.0 | 10.3** | 5.1 | 1.1 |
| 19 | 563.35 (220.78) | 242.13 (178.62) | 7.3 | 38.7 | +2.3 | 9.1* | 1.9 | 1.9 |
| 20 | 222.82 (135.27) | 43.93 (57.83) | 7.3 | 59.8 | +5.1 | 8.6* | 1.1 | 0.5 |
| 21 | 437,25 (196.79) | 232.69 (117.83) | 5.9 | 37.6 | +1.88 | 7.3* | 2.6 | 4.7 |
| 22 | 71.6 (20.04) | 28.27 (33.19) | 6.2 | 37.7 | +2.53 | 8.2* | 2.3 | 0.6 |
| 23 | 3199.43 (2235.88) | 1062.74 (1368.77) | 7.3 | 28 | +3.01 | 12.6** | 6.8* | 16.8** |
| 24 | 286.75 (152.45) | 117.49 (125.37) | 7.2 | 33.5 | +2.44 | 7.2* | 0.4 | 7.9** |
| 34 | 387.75 (441.24) | 895.10 (257.51) | 5.8 | 36.9 | −2.3 | 15.5** | 15.1** | 0.1 |
| 35 | 1411.72 (685.07) | 2713.18 (1238.55) | 6.5 | 31.9 | −1.92 | 5.6* | 2.6 | 0.4 |
| 36 | 79.49 (86.69) | 226.94 (127.00) | 6.5 | 60.9 | −2.9 | 5.9* | 0.8 | 1.9 |
| 37 | 587.81 (197.76) | 1162.22 (571.66) | 6.8 | 41.4 | −1.9 | 7.2* | 3.2 | 2.2 |
| 38 | 418.92 (523.59) | 1474.60 (639.37) | 6.8 | 47.3 | −3.5 | 11.1** | 1.8 | 1.5 |
| 39 | 25.84 (56.11) | 1830.88 (1147.19) | 7.5 | 39.0 | −70.0 | 59.6*** | 16.8** | 15.4** |
| 40 | 3195.88 (2139.95) | 8146.47 (3079.67) | 7.6 | 39.8 | −2.6 | 8.4* | 0.1 | 0.1 |
| 41 | 1902.15 (645.72) | 3087.58 (716.89) | 7.3 | 49.0 | −1.6 | 11.0* | 0.1 | 4.0 |
| 42 | 327.91 (214.41) | 1079.14 (956.47) | 5.9 | 50.0 | −3.29 | 10.3* | 6.5* | 14.9** |
| 43 | 46.68 (74.90) | 674.92 (587.53) | 7.5 | 39 | −14.46 | 5.5* | 0.1 | 0.1 |

[a] Results of the *F*-test from the two-way ANOVA are provided. Std, standard deviation; p*I*, isoelectric point; $M_r$, relative molecular mass. Variation is given as the ratio of the spot relative volume in the RB ecotype over the SU ecotype for increases in RB (+) or as the SU/RB ratio for decreases in RB (-). *: $P \leq 0.05$. **: $P \leq 0.01$. ***: $P \leq 0.001$. $F_{ECO}$, between ecotypes; $F_{DIS}$, between ecotype distribution; $F_{INT}$, interaction.

**Table 2.** Partial Amino Acid Sequences[a] of the Up-and-Down-Regulated Proteins analyzed by MS/MS

| peptide no. | spot number | peptide sequence | homologous proteins (sources)[b] | accession number (nrNCBI) | score (sequence coverage) |
|---|---|---|---|---|---|
| 1a | 4 | TTSNLLRAR | No | - | - |
| 1b | | TTSNLLARR | No | - | - |
| 1c | | TTSNLLQVR | No | - | - |
| 1d | | TTSNLLLNR | No | - | - |
| 1e | | TTSNLLVQR | No | - | - |
| 2a | 15 | LYYDSVNQR | No | - | - |
| 2b | | LYYDSVNRAG | No | - | - |
| 2c | | LYYDSVNAGR | No | - | - |
| 2d | | LYYDSVNRGA | No | - | - |
| 2e | | LYYDSVNARG | No | - | - |
| 3a | 39 | FGGTLADCIR | Arginine kinase (*Octopus vulgaris*)<br>Arginine kinase (*Sepioteuthis lessoniana*) | gil7770085<br>gil7770087 | 63 (2%) |
| 3b | | FLVWVNEEDHLR | Arginine kinase (*Cellana grata*) | gil13647113 | 52 (3%) |
| 4a | 40 | LQGIGVENTEENR | Fructose-bisphosphate aldolase (*Crepidula fornicata*) | gil71370900 | 110 (7%) |
| 4b | | GILAADESTGTMGK | Predicted protein (*Nematostella vectensis*) | gil156374279 | 101 (7%) |
| 4c | | GILAADESVGSMGK | Unnamed protein product (*Tetraodon nigroviridis*) | gil47227795 | 97 (7%) |

[a] Peptide sequences were obtained by *de novo* sequence analysis with the Protein Global Server (v.1.5). [b] Protein identification was achieved through searches performed by MASCOT and Blast against the nrNCBI database.

fact, a G goodness of fit-test showed that the differences between observed and expected frequencies of significant cases were not due to chance ($G = 46.0$, d.f. $= 1$; $p < 0.001$). If the ratio is corrected for the random expectation (0.2% of spot differences due to chance), our best estimate of the ecotype differences in qualitative protein expression is about 1.2%.

**3.3. Quantitative Analysis.** Protein patterns obtained after 2-DE were compared with the aim of finding proteins differentially expressed between ecotypes. The 6 samples (10 individuals/sample) from each ecotype were processed and compared. All the saturated, overlapped and streaked spots were discarded. Of all the spots detected in the gels (average: 726), 136 well-defined spots were shared by the 12 gels and therefore were chosen for the statistical comparison. These restrictive criteria have been used in previous proteomic studies[21,22,25] and ensure a better performance, although it means that potentially interesting proteins will be missed.

To detect statistically significant differences between ecotypes, the two-way ANOVA was applied to the 136 common spots. Considering a 95%-confidence level ($p \leq 0.05$), there were 22 spots (16%) which significantly differed in relative volume between ecotypes, most of which did not show significant distribution or interaction factors (Table 1). Only one out of 22, the spot number 39 (arginine kinase), remained significant after the conservative sequential Bonferroni multitest correction. The remaining 21 significant spots, however, hardly could be explained by chance as we only expect 6.8 due to the *a priori* type-I error. The *G* goodness of fit-test showed significant differences between the observed and expected frequencies of those significant cases ($G = 20.6$, d.f. $= 1$; $p < 0.001$). After correcting for the expected type-I error (5%), this represents an 11% of the proteins studied.

**3.4. Identification of Protein Spots.** The identification process was first accomplished using Coomassie-stained preparative gels. However, not many spots could be visualized with this stain. When we increased the number of visible spots by concentrating the samples and bathing the gel with warmed Coomassie (R-350) for several days, the number of significant

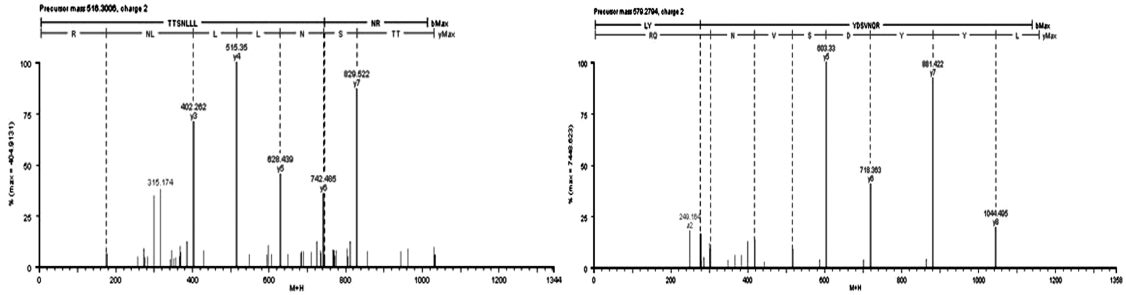


**Figure 3.** Representative peptide sequences obtained from silver-stained gels after fragmentation through ESI-Q-TOF. Diagrams show the spectra corresponding to a peptide sequenced as TTSNLLLNR from spot 4 (left), and a peptide corresponding to the sequence LYYDSVNQR, obtained from spot 15 (right).

spots detected was still very low and only 6 spots (15, 29, 31, 35, 39 and 40) of the 43 previously found could be picked and submitted to MS. Although some of these spots yielded proper MS/MS spectra, none of the peptides sequenced provided a significant homology with the proteins contained in the nrNCBI database (Supplementary Figure 1 in Supporting Information).

Next, we tried to visualize as many spots as possible using a silver-staining protocol compatible with protein digestion and MS analysis, although less sensitive than the silver-staining method employed for the analytical gels. Comparing the protein maps obtained in this study with the maps previously published for other molluscs, one protein appears clearly differentiated in all maps: the actin spot (see Figure 2). Although its extremely irregular shape makes it very difficult to analyze its volume among maps, in order to determine the applicability and efficiency of the MS method, we picked actin as a control sample. We submitted the protein to MS, identifying it as actin with high certainty (scoring 592 over a threshold of 50). Therefore, the silver-staining protocol did not affect the subsequent MS analysis, obtaining spectra with adequate quality.

Then, we picked all the altered spots that could be located without ambiguity with the new staining protocol (24 out of 43) and performed MS/MS as stated before. Some spots presented low signal intensities (probably due to insufficient amount of protein), with spectra that only showed the ions corresponding to the autotryptic peptides, whereas for other spots (5, 9, 14 and 16), the low amount of protein yielded no significant results or incomplete spectra, and therefore, we could not gather enough information for database searching.

Spots 4 and 15 allowed good fragmentation, at least of one peptide each ([M + 2H] = 516.30 Da and [M + 2H] = 579.28 Da, respectively). *De novo* peptide sequencing was performed, (Table 2) finding 5 possible peptide sequences (Figure 3) for spot 4 (1a, 1b, 1c, 1d, 1e) and another 5 for spot 15 (2a, 2b, 2c, 2d, 2e). However, when the sequences were searched against the nrNCBI database, neither protein identity nor even homology was obtained, both against entries from molluscs and from all species. New searches were carried out using Peaks and Phenyx search engines but the same results were obtained.

Finally, from protein spot 39, 2 partial amino acid sequences (Table 2: 3a, 3b) were obtained and were assigned to the arginine kinase (E.C. 2.7.3.3) found in molluscs. The first sequence (3a) was assigned to the arginine kinase of the common octopus *Octopus vulgaris*, the squid *Sepioteuthis lessoniana* and the abalone *Haliotis madaka*, whereas the second amino acid sequence (3b) was assigned to the arginine kinase of the limpet *Cellana grata*. From protein spot 40, 3 partial amino acid sequences (Table 2: 4a, 4b, 4c) were obtained. The first sequence (4a) was assigned to the fructose-bisphosphate aldolase (E.C. 4.1.2.13) of the slipper limpet *Crepidula fornicata*, the second one (4b) was assigned to a predicted protein of the sea anemone *Nematostella vectensis* and the last one (4c) was assigned to an unnamed protein product of the spotted green pufferfish *Tetraodon nigroviridis*.

## 4. Discussion

In this work, we report the proteomic comparison of two ecotypes of the marine snail *L. saxatilis*. Two-dimensional electrophoresis was chosen since this technique is excellently suited for the detection of proteins with altered expression in various conditions, as in distinct species or cell types, in response to different stimuli, and so forth.[22] As with other methods employed for quantification, it is necessary to keep technical variation low when performing 2-DE studies. However, the reproducibility still constitutes the major limitation of this technique.[58] The sources of gel-to-gel and sample-to-sample variability in 2-DE-based proteomics cannot be removed completely, and because of this, multiple samples must be analyzed to ensure statistical significance.[59] In this work, the reproducibility observed was high enough, in good agreement with previous reports.[21,25,59−61]

We found differences in protein expression in a subset of the spots analyzed between the two ecotypes of *L. saxatilis*. A small proportion (1.2%) of the proteins analyzed were detected only in one of the ecotypes. Such pattern could be caused by some genes being expressed (or being detected) uniquely in one of the ecotypes, or genes showing fixed allele differences[62] between ecotypes that would produce spots located at different positions in the 2-D maps. Independently of the mechanism, they could be directly involved in adaptation. That would correspond to the first and second strategies of biochemical adaptation mentioned in the Introduction.[2] In addition, around 11% of the proteins studied differed in their level of expression between ecotypes (second strategy of biochemical adaptation).[2] Therefore, we can preliminarily conclude that, in the Galician *L. saxatilis*, a change in protein expression seems to be more frequently involved in adaptation than changes related to the protein sequence (a maximum of 1.2%). In a previous study, none of eight allozyme loci showed systematic differences in allele frequencies between these two ecotypes.[33]

The differences in protein expression found in this work could originate in environmental changes, since intertidal organisms live in a rapidly fluctuating environment in which conditions can determine their survival. However, in our study, half of the samples of each ecotype belong to the middle shore

of the intertidal region, where environmental conditions are similar,[26] indicating that they are not likely to be the unique source of the differences observed. Thus, the change in expression of certain proteins between the ecotypes might be intrinsically due to the morph characteristics and genetic background, not dependent on habitat. In fact, previous analyses found that the majority of observed morphological variation was inherited rather than plastic.[26,27,35] However, future studies will need to precisely quantify the amount of plastic and inherited protein expression between ecotypes.

The present study suggests that the RB and SU ecotypes differ in about 12% (including qualitative and quantitative differences) of their proteome in the wild. Such estimate is based on a relatively small number of spots (136), but there is no reason to think that they are not representative of the whole proteome. The difference could be caused by a few regulatory genes being divergently selected in each habitat or by differential evolution of a large part of the genome. In this sense, some new data support a large genetic differentiation: an AFLP genome scan comparing these ecotypes has revealed that they differ in at least 3−4% of the loci studied (Galindo, J., 2007, unpublished observations). Both the genomic and proteomic differences could be due to regulatory genes affecting the relative expression of some loci (which could be structurally identical between ecotypes), again pointing toward the quantitative importance of the second strategy of gene regulation.[2] These results are relevant to the discussion of how speciation is produced. There is some controversy about how many genes are involved in speciation and the way they affect it.[63] Such discussion is difficult to conclude when comparing genetic or proteomic differentiation of true species, since it is hard to know if a particular allelic difference was fixed during speciation or after it. The advantage of the *L. saxatilis* model system is that these ecotypes still maintain gene flow, and the reproductive isolation is only about 70% of the maximum possible.[26] At least in this species it seems clear that strong adaptation and emergence of reproductive isolation can be achieved *in situ* by affecting relatively large genome areas (at least more than 3% of the genome).

To identify the proteins of interest, we found two main methodological problems: the limitation in protein amount and the absence of *Littorina* sequences in protein/gene databases. The former is a typical limiting factor in spot identification after in-gel digestion because an insufficient amount of peptides generates low signal intensities.[64] In our case, this problem was due to the abundance of the protein actin, a fundamental component of the cellular cytoskeleton[13] that made the rest of the spots appear in relatively lower amounts in the protein maps. To solve the problem of the overwhelming quantity of actin, also reported for *Mytilus galloprovincialis* and *Chamaelea gallina*,[13,21] prefractionation or affinity-capture methods, similar to that already implemented in the case of albumin in human serum, could be used.[60]

The second problem regarding protein identification is due to the relatively few complete genomes available for eukaryotes in databases. This problem has already been addressed in previous reports.[12,13,65,66] One possible solution would be employing *de novo* sequencing, although in this case it could be challenging due to the requirement of a high number of ions for MS, and therefore a higher amount in the spot samples.

MS analysis of the 43 spots differentially expressed between ecotypes allowed the identification of two interesting proteins. The protein spot 39 was assigned to arginine kinase. Several isoforms have been reported for invertebrate arginine kinases with cytosolic and mitochondrial forms identified in muscle.[67] In fact, several invertebrates have also been reported to have three or more genes coding for the arginine kinase.[68] Arginine kinase is a phosphotransferase that catalyzes the interconversion between phosphoarginine and ATP. This enzyme is widely distributed among invertebrates[69] and it is an analogous system to the creatine kinase found in vertebrates.[70] Phosphoarginine acts as an energy reservoir because its high-energy phosphate can be rapidly transferred to ADP when ATP is required.[71] Moreover, this enzyme has already been proposed as a constituent of an adaptative response to nutritional stress conditions.[72] When SU specimens are exposed to extreme wave action, they could require a fast supply of energy for contracting the foot muscle, which could be supplied by phosphagens such as phosphate arginine as an indirect source of ATP. Thus, it is reasonable to find an increase of the arginine kinase, an enzyme able to produce ATP quickly. Regarding this enzyme in *L. saxatilis*, its polymorphisms have been studied in both RB and SU ecotypes at a geographical scale, and interestingly, no consistent differences between ecotypes were found in its allelic frequency,[33] further supporting that, in this case, adaptation is achieved by controlling the relative amount of protein produced (as detected here by 2-DE) rather than by selecting different alleles.

Fast energy could be also obtained from anaerobic glycolysis. This is consistent with the increase of the spot 40, which was assigned to fructose-bisphosphate aldolase. This is one of the most important proteins in the glycolytic pathway, catalyzing the cleavage of fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Therefore, its increase or decrease is correlated with the level of glycolysis.[73] It is reasonable to think that arginine kinase increases its level more than the fructose-bisphosphate aldolase because energy from phosphate arginine may be "cleaner" than when supplied from anaerobic glycolysis, which produces lactate and causes acidosis.

One issue that should be considered is that the SU individuals present a muscle 1.4 times bigger than their RB counterparts, so as to be able to hold to the rocks and avoid being pulled out by the swell. However, the differences of expression found in the identified proteins (nearly triple in the fructose-bisphosphate aldolase and higher in the arginine kinase, see entries for spots 40 and 39, respectively, in Table 1) indicate they are not only due to this anatomical reason. Therefore, their up-regulation in the SU ecotype is consistent with the need of a high supply of energy in a short time, to stand up to the wave action typical of its habitat by means of quickly rising the ATP levels and accelerating glycolysis. Future experiments should confirm the pattern of expression of these two proteins between ecotypes in this and other shores, in specific tissues or organs, as well as quantify their differences in expression more precisely in order to have a better understanding of this speciation process.

The patterns observed here suggest that adaptation across strong environmental gradients of a few meters could still result in extensive changes in the genome and proteome of the sympatric subpopulations of *L. saxatilis*, in spite of maintaining a considerable gene flow between them.[33] Moreover, the study of this ecological model system requires additional efforts in identifying more of the differently expressed proteins detected here, possibly using *de novo* sequencing in a larger subset of spots, in order to interpret protein function in biological and

ecological terms. In addition, it could be extremely interesting to compare these results at the genomic or transcriptomic levels, as combining research approaches targeting different functional levels helps to understand phenotypic evolution in ecological or evolutionary contexts.[74,75]

## 5. Conclusions

The present report shows the utility of proteomic techniques to study differences in protein expression between two ecotypes with different ecological adaptations. Qualitative and quantitative differences were found when comparing the RB and SU ecotypes of *L. saxatilis*, and the spots with differential expression were analyzed by mass spectrometry (MS). These two sympatric ecotypes of the same species showed differential expression in about 12% of the detected proteome, implicating extensive changes (plastic or genetic) during adaptation before speciation. Fructose-bisphosphate aldolase and arginine kinase were up-regulated in the SU ecotype, suggesting the need of a quick supply of energy by increasing the level of glycolysis and, thus, the ATP generated. On the other hand, our results highlight the necessity of increasing the number of proteomic studies from the marine realm to allow an increased representation of marine species in databases and the successful identification of the most relevant proteins from an evolutionary point of view.

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**Supporting Information Available:** Supplementary Figure 1, representative MALDI-TOF spectra obtained from Coomassie-stained gels for spots 39 and 40; Supplementary Table 1, spots expressed only by one of the ecotypes included in the study. This material is available free of charge via the Internet at http://pubs.acs.org.

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