Vol. 552: 177–193, 2016
doi: 10.3354/meps11770

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published June 23

Measuring physiological similarity of closely related littorinid species: a proteomic insight

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ABSTRACT: During recent years, the role of proteomics in ecology, population biology and evolutionary studies has been growing. It has been used effectively to resolve taxonomic ambiguity in closely related species and for detection of environmental effects on populations. The family Littorinidae (Gastropoda; periwinkles) includes a set of widely distributed species, which are important members of intertidal communities of the coasts around the world. Taxonomic relationships among several species, e.g. within the subgenus *Neritrema*, are still poorly understood. We explored proteomes of 3 closely related littorinid species (the so called '*saxatilis*'-group: *Littorina arcana*, *L. compressa* and *L. saxatilis*) from different tidal levels of one geographic area. The proteome data were obtained using 2-dimensional difference gel electrophoresis (2D-DIGE) followed by mass spectrometry for protein identification. We showed that *L. compressa* formed a well-supported clade within the '*saxatilis*' group, while *L. arcana* and *L. saxatilis* had similar proteomes. In contrast to *L. compressa*, the variable elements in the proteomes of the 2 latter species shifted similarly along the vertical shore gradient. Such proteomic divergence suggests that closely related species may function differently at the physiological level under very similar conditions.

KEY WORDS: *Littorina* · '*Littorina saxatilis*' species complex · Cryptic species · Proteomics · 2D difference gel electrophoresis · 2D-DIGE · Speciation

INTRODUCTION

The proteome is the set of proteins expressed in a certain cell type at a certain stage under given conditions. Keeping in mind that 'the fabric of life is protein-based' (Karr 2008), one can reasonably conclude that proteomics can provide insight into the mechanisms of adaptation and speciation. Although the earliest taxonomic applications were published in the beginning of the 1980s (Aquadro & Avise 1981, Ohnishi et al. 1983), the application of proteomics has intensified only in the last decade in the fields of ecology, population biology, taxonomy, and evolutionary studies (López 2005, 2007, Biron et al. 2006, Karr 2008, Kim et al. 2008, Cash 2009, Diz et al. 2012b). In contrast to discrete molecular markers, a proteomic approach to phylogenetic reconstruction has the advantage of integral analysis considering the complex system of working molecular machinery (Navas & Albar 2004). In addition, different phenomena of non-genetic inheritance clearly affect the proteome pattern, but they remain undetected by the genomic approach. These phenomena might be among the crucial factors of speciation and maintenance of species individuality (Mameli 2004, Bonduriansky & Day 2009, Danchin et al. 2011). Thus, although classical molecular phylogeny is very informative, proteomic (as well as transcriptomic) data provide valuable complementary information.

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Proteomic data describe a 'molecular phenotype'. Parallel, ecology-driven appearance of similar phenotypic variants along similar environmental gradients has been described many times for different species, including littorinids (reviewed in e.g. Galindo & Grahame 2014, Rolán-Alvarez et al. 2015, Johannesson 2016). Such phenotypic heterogeneity (i.e. plasticity) in response to environmental heterogeneity can be accepted as a species attribute, regardless of the mechanism that stands behind it — phenotypic plasticity or direct genomic determination. Both characteristics (a proteomic pattern per se and its plasticity within environmental heterogeneity) are very informative for exploring key traits of closely related species, especially when common DNA-based approaches lead to ambiguous results.

Marine littoral gastropod mollusks of the genus *Littorina* are widely distributed along the North Atlantic shores. They are characterized by high population densities, relatively low mobility (which predisposes populations to subdivide) and different modes of reproduction. During the last decades, *Littorina* spp. have become model organisms for the study of population biology, physiological adaptations, genetics, behavior and parasitology (Sokolova et al. 2000, Sokolova & Pörtner 2001a,b, Conde-Padín et al. 2009, Granovitch et al. 2009, Canbäck et al. 2012, Storey et al. 2013). Sympatric populations of closely related *Littorina* spp. inhabit specific microhabitats in the littoral zone and therefore may be viewed as possible products of recent speciation (Quesada et al. 2007, Johannesson et al. 2010, Rolán-Alvarez et al. 2015, Johannesson 2016). Several species of the genus exhibit substantial morphological variability (Reid 1996). The most prominent example of this is the formation of ecotypes by *L. saxatilis* in several locations across its range (Rolán-Alvarez et al. 2004, Panova et al. 2006, Johannesson et al. 2010, Butlin et al. 2014, Galindo & Grahame 2014).

The subgenus *Neritrema* within the genus *Littorina* comprises the *L. saxatilis* species complex (*L. saxatilis* (Olivi 1792), *L. arcana* Hannaford Ellis 1978 and *L. compressa* Jeffreys 1865). The phylogenetic relationships within this species group are still poorly understood. Morphology clearly separates *L. compressa* and brings *L. arcana* and *L. saxatilis* close together (Reid 1996). The males of the latter 2 species are barely distinguishable from each other, whereas males of *L. compressa* are easily recognizable. Penial characteristics (the only basis for identification) vary considerably within both species (Granovitch et al. 2008). The possibility of interspecific hybridization was demonstrated under laboratory conditions (one-sided hybridization; Warwick et al. 1990) and in wild populations (Mikhailova et al. 2009). No such hybridization between *L. compressa* and either of the other 2 species has been observed. Nevertheless, *L. compressa* and *L. arcana* are both oviparous, whereas *L. saxatilis* is ovoviviparous.

A variety of molecular markers has been used to clarify phylogenetic relationships within the *L. saxatilis* complex, but the results were inconsistent. In a series of studies, the 3 *Littorina* spp. grouped differently depending on the molecular approach used (e.g. Backeljau & Warmoes 1992, Crossland et al. 1996, Small & Gosling 2000, Wilding et al. 2000a,b, Reid et al. 2012, Panova et al. 2014). This controversy reflects the evolutionary closeness of these species and prompts the use of alternative approaches, such as proteomics.

Proteomic assays have been done on *L. saxatilis* and other littorinids to characterize the biochemical background of ecological adaptation along the vertical shore gradient (Martinez-Fernandez et al. 2008, 2010, Diz et al. 2012a, García et al. 2013, Wang 2013). In the present study, we applied a proteomic approach to compare 3 sympatric sister species of the '*saxatilis*'-group with respect to 3 characteristics: (1) the similarity of 2D-detectable proteomes, (2) changes in proteome along the vertical shore gradient and (3) similarity of proteins responsible for any observed variability associated with tidal elevation. We considered different somatic and reproductive body parts for both sexes at different tidal levels to evaluate which of these factors (species, tidal level, sex, body part) had the strongest effect on the proteome, and finally discussed the potential biological roles of observed proteomic differences among the species and groups of individuals from different tidal levels.

MATERIALS AND METHODS

Sample collection and preparation

Adult snails of the '*saxatilis*' group (*Littorina arcana*, *L. compressa*, *L. saxatilis*) were collected during the same low tide from a wild population in Cancale, France (48° 70′ N, 1° 84′ W), on 6 May 2014. The intertidal area was divided into 3 zones based on the position of the fucoid belt and tidal height: the lower level was between mean high water springs and mean low water neaps and below the fucoid belt, the middle level was between mean low water springs and mean high water neaps within the belt of fucoids, and the

upper level was above the mean high water neaps in the rocky zone with occasional fucoids. The absence of fucoids leads to greater desiccation and temperature stress. Thus, the boundaries of the fucoid belt mark the profound change in the biotope conditions along with the tidal height (see Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/m552p177_supp.pdf). Snails were collected from a 50 m stretch of coast, gathered within the same tidal level and mixed to eliminate the possible bunching-effect, coming from familial clumping. The sampling scheme followed zonal patterns of species-specific distribution, with *L. compressa* population tending to inhabit the lower part and *L. arcana* the upper part of the tidal zone (Table 1). Thus, only *L. saxatilis* was sampled from all 3 tidal levels, while *L. compressa* was found only in lower and middle levels, and *L. arcana* was found only in middle and upper tidal levels. Therefore, the following species samples were used: (1) lower level — *L. compressa* and *L. saxatilis*; (2) middle level — *L. arcana*, *L. compressa* and *L. saxatilis*; (3) upper level — *L. arcana* and *L. saxatilis*.

The animals were transported to the laboratory in aerated moist containers with a cooling agent within 24 h of collection. In the laboratory, animals were kept in the same containers at 8°C and washed with marine water once a day, for no longer than 1 wk. Samples from all shore levels were handled in parallel to avoid bias due to handling-time. Just before sample preparation, living snails (dead or frozen snails are poorly identifiable) were dissected under an MBI-10 binocular microscope at 79 to 849× magnification for identification of species, sex and possible trematode or other heavy parasitic infection. The identification was conducted according to the species-specific characteristics of the male and female reproductive system (Reid 1996, Granovitch et al. 2008). Females were identified according to the morphology of the distal part of their reproductive system — the complex of pallial glands (primarily bursa copulatrix and jelly gland). Males were identified based on penis morphology (Reid 1996): 2 or more rows of small numerous mamilliform penial glands and triangular filament (*L. arcana*); 1 row of distal large glands (≤6 in number) and short filament (*L. compressa*); 1 row of small, numerous (>6 in number) mamilliform penial glands and triangular filament (*L. saxatilis*). Only reliably identified mature individuals of different ages with a well-developed reproductive system and free of trematode or other obvious infection were used for further analysis.

We sampled the soft tissues of head and foot of every male and female and penises of males. Tissues of up to 20 animals were pooled (the body parts of males and females were pooled separately). We used pooled samples as they are representative of the group of individuals, still allow assessment of subpopulation variability and reduce the number of runs and consequently the loss of analytical efficiency due to spot mismatch and other technical difficulties (Karp et al. 2005, Karp & Lilley 2007, Diz et al. 2009). Consequently, 3 types of pooled samples were obtained from males (heads, feet, penises) and 2 types from females (heads and feet). For pooling, pieces of corresponding body parts of approximately equal volume (determined visually) were placed into the same tube and homogenized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 25 mM tris, pH 8.2) using a Mixer Mill MM 400 (Retsch); particles were sedimented by centrifugation at 12 000 × *g* for 15 min at 4°C, and supernatants were frozen at −80°C until use.

2D-DIGE electrophoresis

In total, 35 different samples representing different snail body parts, sexes and tidal levels were subjected to gel-based proteomic analysis (Table 1). Every sample was analyzed by 2D-DIGE at least twice in combination with different counter samples for comparison. Repeated runs of the same sample were considered as technical replicates (n = 2–4).

Prior to electrophoretic separation, proteins of each sample were conjugated with the fluorophores Cy2, Cy3 or Cy5 (Luminoprobe, BioDye) in a proportion

Table 1. Distribution of analyzed samples among 3 *Littorina* spp. and tidal levels. Numbers: no. of pooled individuals. fo: foot; he: head; pe: penis; (–) no sample

Tidal level	*L. arcana*		*L. saxatilis*		*L. compressa*	
	Males	Females	Males	Females	Males	Females
Upper	20: pe, he, fo	20: he, fo	5: pe, he, fo	16: he, fo	–	–
Middle	10: pe, he, fo	5: he, fo	14: pe, he, fo	20: he, fo	11: pe, he, fo	15: he, fo
Lower	–	–	14: pe, he, fo	20: he, fo	20: pe, he, fo	11: he, fo

of 400 pmol of Cy per 50 µg of total protein. The protein concentration in a sample was evaluated by absorbance at 280 nm with NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies). Samples mixed with Cy-flourophores were incubated for 30 min on ice in the dark, the reaction was then stopped by the addition of 10 µmol of L-lysin, and the samples were further incubated for 10 min under the same conditions. The samples conjugated with the different Cy-flourophores were pooled and loaded into an IPG ReadyStrip (7 cm, pH 3–10, BioRad) during passive rehydration (overnight at room temperature, in the dark). Separation in the first direction was carried out in a Protean IEF Cell (BioRad) using the method recommended by the manufacturer: 10 000 Vh, end voltage 4000 V, rapid ramp, 20°C.

Before separation in the second direction, IPG-strips with focused proteins were incubated in equilibration buffers (6 M urea, 2% SDS, 20% glycerin, 0.375 M tris, pH 8.8) for 15 min: first with 2% dithiothreitol and then with 2.5% iodoacetamide. Electrophoretic separation in the second direction was performed in a MiniProtean TetraCell (BioRad) in 15% PAAG in tris/glycine/SDS buffer (BioRad). A GE Typhoon 9500 FLA laser scanner (GE Healthcare) was used for visualization. Merging of the electrophoregrams was done using ImageJ 1.48 v (http://imagej.nih.gov/ij). For spots, excision gels were stained by Coomassie Brilliant Blue R250 (BioRad).

Protein identification

Protein identification was done in accordance with a 'bottom up' approach, which means using tandem mass spectrometry (MS/MS) identification of tryptic peptides with a database search. For trypsinization, spots of interest were excised from the gel with a scalpel and cut into pieces. Gel pieces of each spot were destained with 50% acetonitrile in 25 mM Tris (pH 8.2), dehydrated in 100% acetonitrile and rehydrated in bovine trypsin solution (20 ng μl^{-1}, 25 mM Tris, pH 8.2) on ice for 60 min. After rehydration, any excessive trypsin solution was removed, and gel pieces were covered with 25 mM Tris (pH 8.2) and incubated at 37°C overnight. Tryptic peptides were eluted and analyzed using liquid chromatography (LC; Agilent 1260) coupled with MS/MS (QTOF UHD 5238, Agilent Technologies). The gradient elution method was 10%B to 60%B for 25 min and further to 100%B (with corresponding decreasing of A) for 5 min, where B was 90% acetonitrile with 0.1% formic acid, A was 5% acetonitrile with 0.1% formic acid, and flow rate was 15 µl min^{-1}. We used a Zorbax SB-C18 column (5 µm grain, 80 Å pores, 150 × 0.5 mm; Agilent Technologies). An MS/MS search was carried out in the mode 'Identity' using Agilent Technologies Spectrum Mill MS Proteomics Workbench Rev B.04.00.127 software against 2 free databases: the *L. saxatilis* EST database (LSD; Canbäck et al. 2012; http://mbio-serv2.mbioekol.lu.se/Littorina) or SwissProt (ftp://www.expasy.ch/databases/uniprot/); the precursor mass tolerance was set to ±20 ppm. The validation procedure of identified proteins was performed with a minimum protein score of 20 and a peptide false discovery rate (FDR) for validated proteins of 1%. Qualitative and quantitative gel analysis was carried out using PDQuest Advanced 8.0.1 software (BioRad). Normalization for the spot intensity estimates was done on total gel density. Spots with intensity values below the detection limit (i.e. not exceeding 0 relative to the background) were considered as absent for qualitative analysis. Spots were considered as reliably detected if they were detected in at least 2 technical replicates of the same sample, or in 1 technical replicate in at least 2 different samples. We classified potential post-translational modifications and/or splice variants as independent spot signals, because every particular modification and/or splice form possesses its own features and functions. While in some cases we know that these independent spot signals represent different forms of the same enzyme (like arginine-kinase or aldolase, see Fig. S2 in Supplement 1, Table S2 in Supplement 2 at www.int-res.com/articles/suppl/m552p177_supp.xlsx), in other cases we suspect this, because of similar molecular weight and subtle differences in isoelectric point (like spots 49 and 53, see Fig. 1).

Statistical analysis

Missing protein expression values in technical repeats were filled using k-nearest neighbor averaging procedure with k = 10 (Troyanskaya et al. 2001) performed using the *impute* package (Hastie et al. 2011) in R (R Core Team 2015). After the imputation, the technical replicates were averaged.

We described the similarities in proteome composition among the samples in 2 different ways. To measure resemblance of proteome compositions, we computed a Jaccard dissimilarities coefficient based on presence/absence data. The Jaccard index is a pairwise dissimilarity measure, which accounts only for

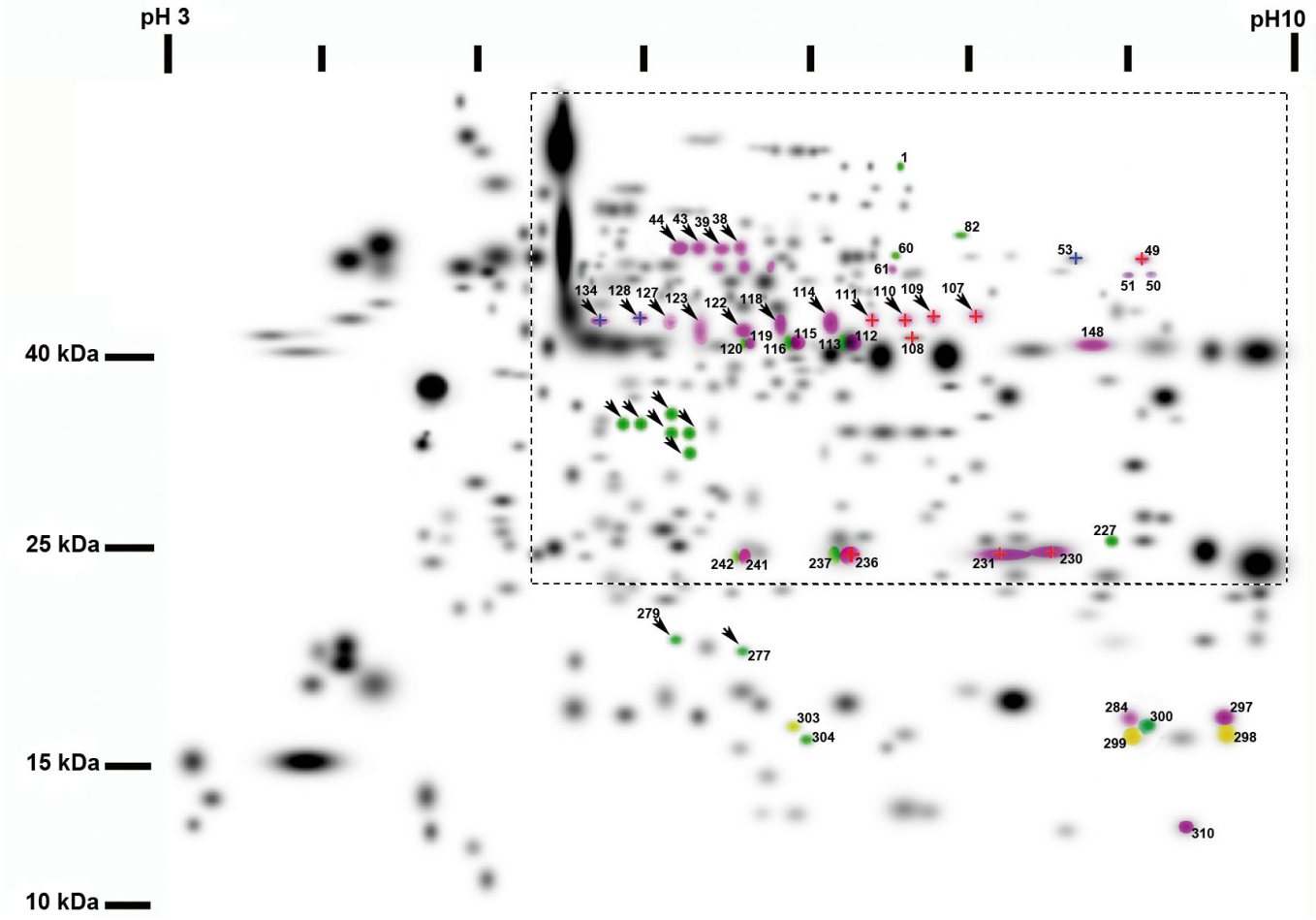

Fig. 1. Master gel (generated in PDQuest software) showing the generalized scheme of spots based on 2D-DIGE results. Numbers indicate spot IDs. Spot colour indicates strong species-specificity: yellow = *Littorina arcana*; purple = *L. arcana*/*L. saxatilis*; green = *L. compressa*. Arrows: spots specific to penial tissue. Red or blue pluses: tidal-level-variable proteins more abundant in the upper or middle/lower tidal level, respectively. Rectangle: area on the master gel corresponding to the gel fragments shown in Fig. 8. Examples of original DIGE-gels are shown in Fig. S2 in Supplement 1 at www.int-res.com/articles/suppl/m552p177_supp.pdf

the spots present in at least one of the 2 samples being compared; therefore, it is robust when samples lack a particular spot for different reasons (absence of expression or expression below the detection level; Duarte et al. 1999, Bonin et al. 2007, Meudt & Clarke 2007). However, it does not differentiate between the situations when the same spot is present in the 2 samples due to different reasons (which is a rather unlikely case).

To assess similarity of expression profiles we used Euclidean distance on standardized, untransformed, quantitative data. Results were clustered using UPGMA (unweighted pair group method with arithmetic mean). As this aggregation method is susceptible to inversions, we computed approximately unbiased (AU) p-values to assess stability of clustering using multiscale bootstrap resampling (Shimodaira 2004) in the package *pvclust* in R (Suzuki & Shimodaira 2015). We performed the analysis with 10 000 iterations to ensure accurate estimation of AU p-values (SE of AU p-values were < 0.01).

For visual comparison of the 2 alternatives we created tanglegrams using the *dendextend* package (Galili 2015). Penial samples clustered in a distinct manner on dendrograms, therefore, for clarity, we further analyzed them separately from somatic samples.

Prior to analysis, the data on normalized spot intensities were $\log_2(x + 1)$ transformed. To analyze the differential expression of proteins we used a moderated *t*-statistic, which is similar to ordinary *t*-statistics, but the variance estimator is shrunk (moderated) across proteins (*limma* package) (Smyth 2004). Specific comparisons were extracted from the models as sets of linear contrasts. The p-values were corrected

for false discovery rate in multiple tests using Benjamini-Hochberg correction (Benjamini & Hochberg 1995). Proteins with adjusted p-values ≤0.05 were considered differentially expressed.

We modelled the variation of somatic proteomes among species (*L. compressa*, *L. arcana* and *L. saxatilis*), body parts (head, foot) and interaction of these factors (to account for possible interspecific proteome differences of body parts). Male and female samples from different tidal levels served as replicates in this analysis. Differences in somatic proteomes along the vertical shore gradient were tested for all species/level combinations; sexes and body parts served as 4 biological replicates.

The penial samples were not replicated within tidal level; thus, interspecific expression variation could be tested with moderated *t*-test, only using the samples from different tidal levels as replicates. For the same reason, the differences between levels for each species were tested using fold-change (the ratio of protein expression levels, which is commonly used for analysis of unreplicated experimental designs in proteomics). Often, the proteins are considered differentially expressed when their expression level differs more than an arbitrary chosen constant factor (e.g. 2-fold change). The fold-change method is not very precise, because the significance depends not only on absolute expression differences but also on its natural variability (Mariani et al. 2003, Breitling et al. 2004). Taking into account that the 2D-DIGE method is suitable for detection of a minimum 2-fold expression change (Karp et al. 2004), we have chosen a slightly higher cut-off value (2.5-fold).

Heatmaps and dendrograms of differentially expressed proteins were produced using the *gplots* package (Warnes et al. 2015). For the dendrograms in Figs. 4, 6, 9 and 10, the samples and proteins were clustered using UPGMA on Euclidean distances computed on non-scaled spot intensities.

RESULTS

The visualized proteome

The 320 most reliable spots were used for the proteome comparison (Fig. 1, Table S1 in Supplement 2). Among them, 81 spots were successfully identified by MS/MS-search against LSD or SwissProt databases. Fig. S2 in Supplement 1 and Table S2 in Supplement 2 show the list of identified proteins and related information.

Presence/absence data analysis

Jaccard dissimilarity coefficients on qualitative data were calculated and used for sample clustering (Fig. S3 in Supplement 1). Two main trends were distinguished using this approach. (1) Species was the factor most strongly affecting the clustering pattern. There was a well-supported branch of *L. compressa*, but a mixed cluster of *L. saxatilis*/*L. arcana*, where somatic samples formed species-specific clusters, but penial samples clustered according to tidal level, not species. Several classes of house-keeping proteins matched between *L. arcana* and *L. saxatilis* (like arginine-kinase, peptidyl-prolyl cis-trans isomerase and aldolase; Fig. S2, Table S2). There were no opposite cases, when forms were common in pair '*L. compressa*/*L. saxatilis*' or '*L. compressa*/*L. arcana*' but different or absent in *L. arcana* or *L. saxatilis*, respectively (Fig. S2). (2) Somatic tissues (head and foot) of both males and females did not form distinct groups, unlike penial samples (Figs. 2 & 3). We considered somatic samples of both sexes as biological replicates, and their mixed pattern of clustering confirmed this assumption.

Somatic and penial proteomes based on Jaccard's dissimilarity coefficient clustered in different manners (Figs. 2 & 3). In both cases, *L. compressa* formed a well-supported individual branch. In the somatic proteome, however, the next division separated the other 2 species from each other (with one exception), whereas *L. saxatilis* and *L. arcana* clustered together within the same tidal level (upper and middle/lower) based on the penial proteome. This implies that expression of penial proteins (based on presence/absence data) is more influenced by tidal level than by species identity.

Quantitative data analysis

In the UPGMA clustering of Euclidian distances based on quantitative data, all 3 species grouped very closely together (Figs. 2 & 3). This grouping pattern indicates that the majority of proteins was identical for the 3 species and that those proteins were expressed in a very similar manner. In both somatic and penial samples, quantitative analysis of the full spot-set revealed different patterns of clustering than in the presence/absence analysis (Figs. 2 & 3), driven by the strong effect of the abundant proteins (such as actin, tubulin, troponin T and tropomyosin) masking otherwise minor differences among samples. Such an effect was reduced by the standardization procedure,

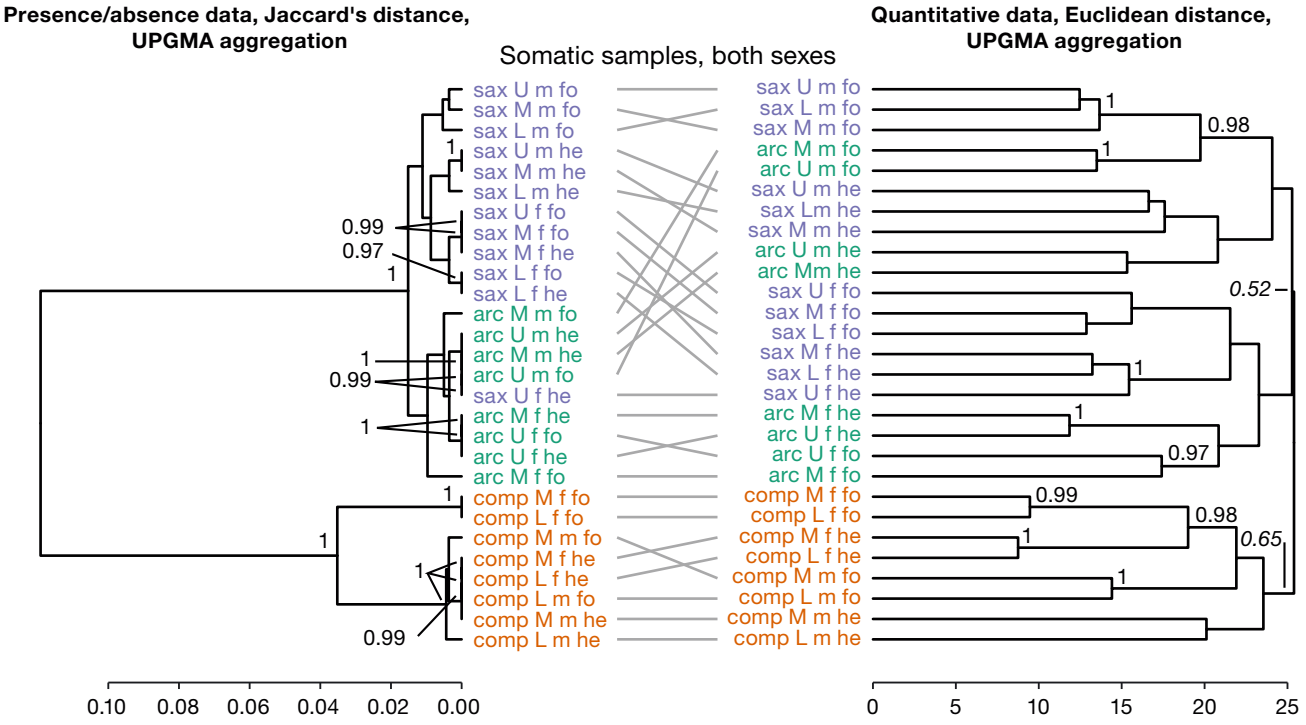

Fig. 2. Similarity trees for all somatic samples from 3 *Littorina* spp. Clustering was based on Jaccard dissimilarity coefficients for binary data (left tree) or Euclidean distance for quantitative data (right tree). Sample labels indicate species (arc: *L. arcana*; comp: *L. compressa*; sax: *L. saxatilis*), tidal level (L: lower; M: middle; U: upper), sex (f: female; m: male) and body part (fo: foot; he: head). Numbers at each node indicate the approximately unbiased bootstrap support values larger than 0.95. Low support values for the first 2 divisions on the right tree are shown in italics

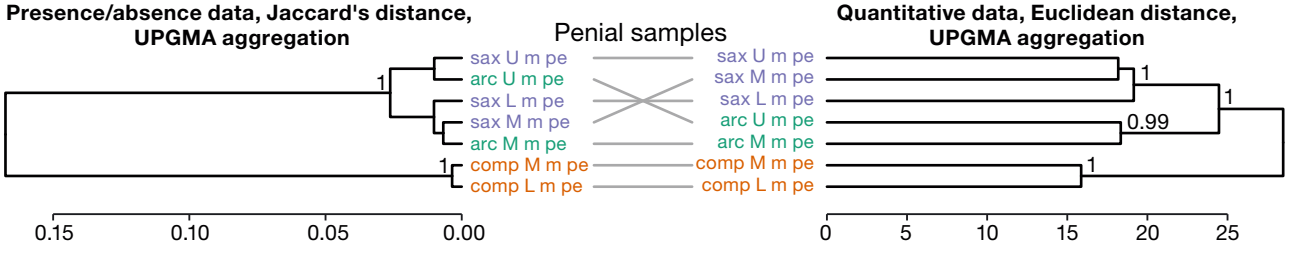

Fig. 3. Similarity trees for all penial samples from 3 *Littorina* spp. Clustering was based on Jaccard dissimilarity coefficients from binary data (left tree) or Euclidean distance from quantitative data (right tree). Sample labels indicate species (arc: *L. arcana*; comp: *L. compressa*; sax: *L. saxatilis*), tidal level (L: lower; M: middle; U: upper), sex (m: male) and body part (pe: penis). Numbers at each node indicate the approximately unbiased bootstrap support values larger than 0.95

but not fully eliminated. Those abundant proteins are expressed constitutively in different tissues under different conditions, and they are highly conservative among the 3 species. To avoid this masking effect, we constructed dendrograms based only on the proteins that were differentially expressed between any 2 types of samples — body part, tidal level or species.

In somatic samples, the proteins that differentiate species from each other dominated among differentially expressed proteins in terms of number and effect strength (Fig. 4). As in the presence/absence analysis, *L. compressa* diverged as a separate branch from the 2 other species. The expression pattern of 49 and 61 proteins separated this species from *L. saxatilis* and *L. arcana,* respectively (36 of these proteins were the same, Fig. 5). At the same time, expression of 15 proteins neatly separated *L. saxatilis* and *L. arcana* (Fig. 5). A full list of somatic proteins separating the species is presented in Table S3 in Supplement 2.

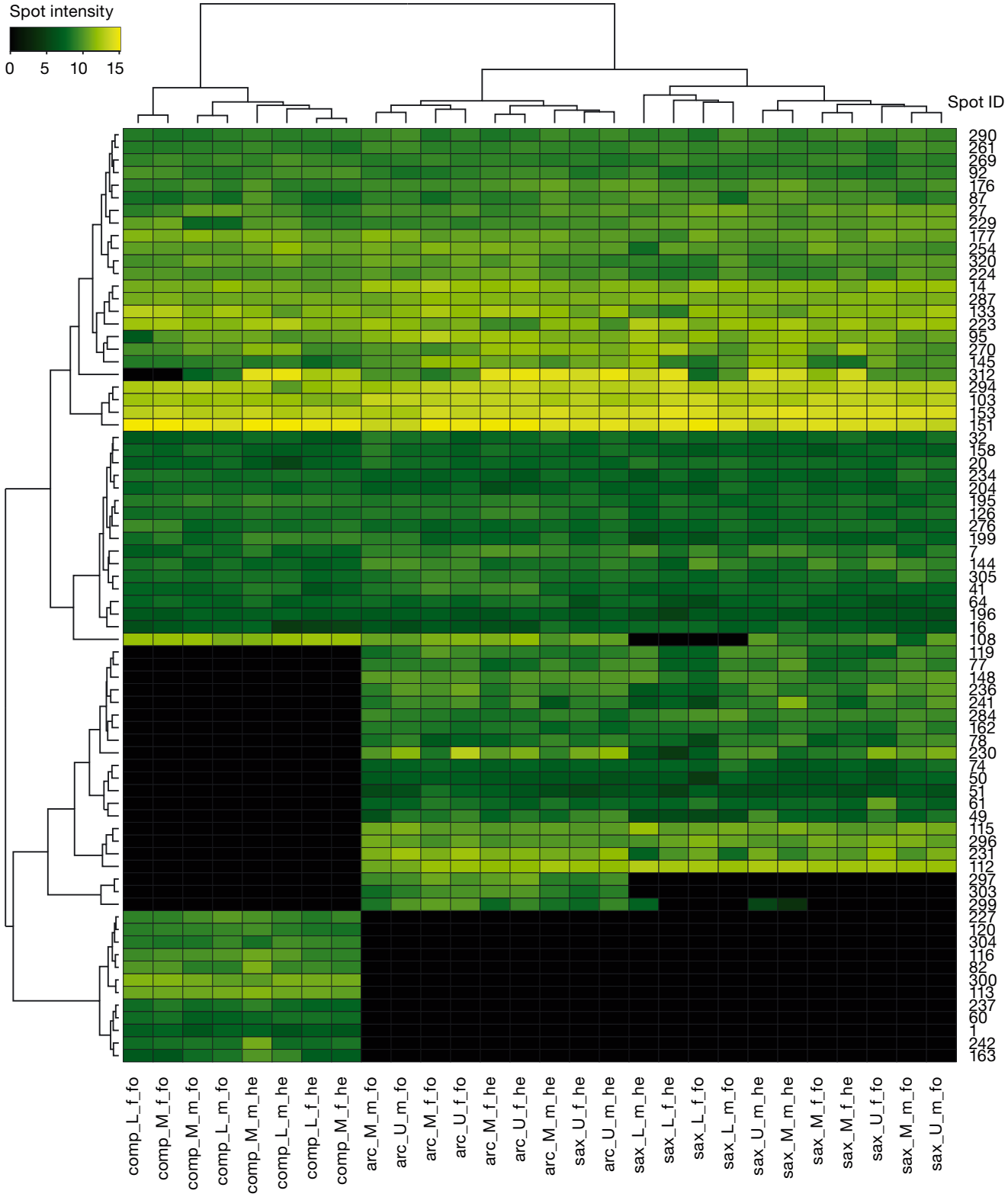

Fig. 4. Clustering of somatic samples from 3 *Littorina* spp. based on the proteins with differential expression pattern. Vertical tree on left: similarity of expression patterns of proteins; horizontal tree at top: similarity of samples containing those proteins. Sample labels along the bottom indicate species (arc: *L. arcana*; comp: *L. compressa*; sax: *L. saxatilis*), tidal level (L: lower; M: middle; U: upper), sex (f: female; m: male) and body part (fo: foot; he: head). Colour bar: unstandardized spot intensities

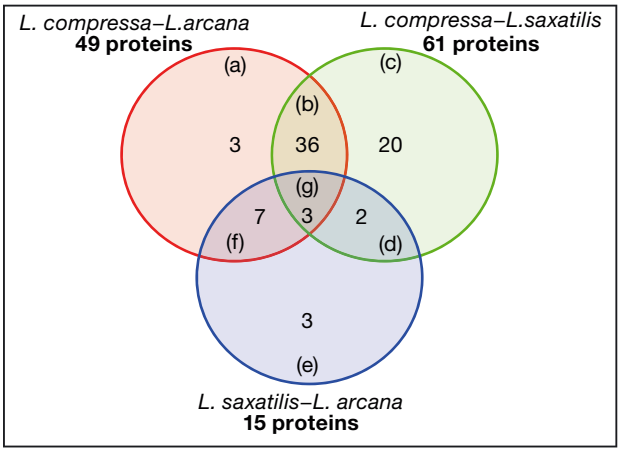

Fig. 5. Venn diagram of the distribution of the somatic proteins that differentiate *Littorina* spp. based on their expression pattern. Numbers indicate how many proteins differentiate the sets: (a) *L. compressa* from *L. arcana*, but not from *L. saxatilis*; (b) *L. compressa* from the pair *L. saxatilis*/*L. arcana*; (c) *L. saxatilis* from *L. compressa*, but not from *L. arcana*; (d) *L. saxatilis* from the pair *L. compressa*/*L. arcana*; (e) *L. arcana* from *L. saxatilis*, but not from *L. compressa*; (f) *L. arcana* from the pair *L. compressa*/*L. saxatilis*; (g) all 3 species from each other

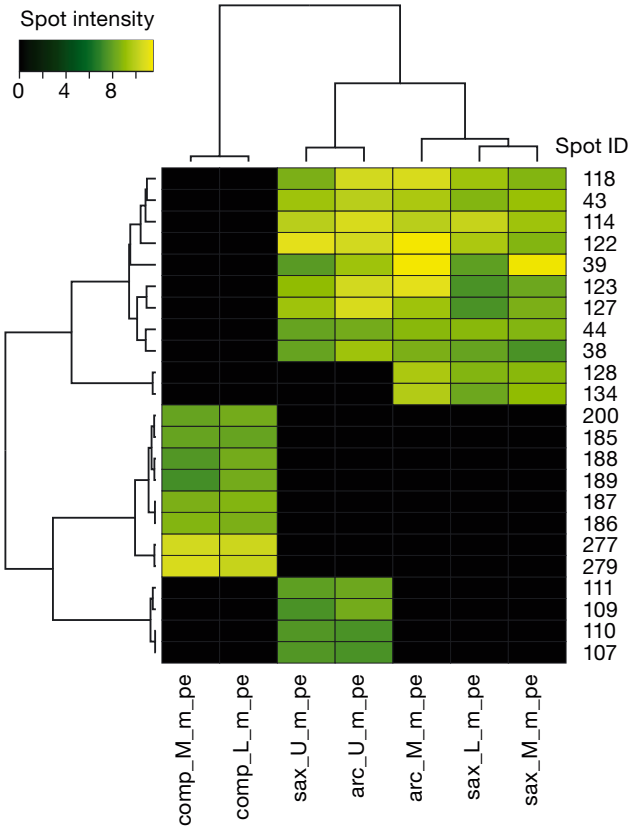

Fig. 6. Clustering of the penial samples from 3 *Littorina* spp. based on penial-specific proteins with differential expression pattern. Vertical tree on left: similarity of expression patterns of proteins; horizontal tree at top: similarity of samples containing those proteins. Sample labels indicate species (arc: *L. arcana*; comp: *L. compressa*; sax: *L. saxatilis*), tidal level (L: lower; M: middle; U: upper), sex (m: male) and body part (pe: penis)

Clustering of the penial proteomes based on expression levels confirmed the high similarity between *L. arcana* and *L. saxatilis* and their difference from *L. compressa*. Tidal level also affected the penial proteomes of *L. arcana* and *L. saxatilis* more strongly than did species identity (Fig. 6). This was also shown for the presence/absence data for penial samples (Fig. 3). Importantly, the number of proteins discriminating *L. arcana* and *L. saxatilis* (34) was far lower, than that separating *L. compressa* from either *L. arcana* (116) or *L. saxatilis* (116). As many as 69 proteins separated *L. compressa* from each of the other 2 species (Fig. 7). A full list of penial proteins differentially expressed between species is shown in Table S4 in Supplement 2.

Comparison along the vertical tidal gradient

This analysis aimed to evaluate (1) the variability in each species' proteome between different levels of the intertidal zone and (2) the similarity of the proteins responsible for this variability (if any was detected) in different species. We searched for proteins that varied in quantity among tidal levels in every species, in somatic and penial samples separately.

Multiple comparisons revealed 1 tidal-level-dependent protein in the somatic tissues of *L. com-*

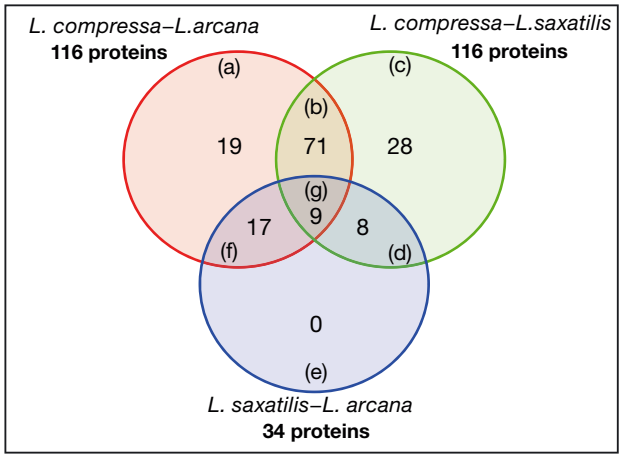

Fig. 7. Venn diagram of the distribution of the proteins from penial samples that differentiate *Littorina* spp. based on their expression pattern. Numbers indicate how many proteins differentiate the sets (see Fig. 5 for explanation)

pressa; 1 protein in *L. arcana* and 6 proteins in *L. saxatilis* (Table 2). The tidal-level-dependent protein of *L. arcana* was 1 of the 6 found to vary for *L. saxatilis*. Although all the proteins identified as tidal-level-sensitive varied in a similar manner for *L. arcana* (Fig. 8), these differences were not statistically significant, perhaps due to the lack of statistical power. This similarity is supported by a clustering pattern based on tidal-level-sensitive proteins: *L. arcana* and *L. saxatilis* grouped together within the same tidal level. In both species, the abundance of the tidal-level-dependent proteins gradually changed, increasing (spots 49, 108, 230, 231, 236) or decreasing (spot 53) from the lower to the upper tidal levels (Fig. 9). *L. compressa,* formed a separate branch. This implies that if any tidal-level-related proteomic variability exists in this species (which could remain undetected by statistical analysis, possibly also

Table 2. Tidal-level-sensitive proteins detected in 3 *Littorina* spp. arc: *L. arcana*; comp: *L. compressa*; sax: *L. saxatilis*; (–) protein does not vary in this compartment; LSD: *L. saxatilis* EST database (http://mbio-serv2.mbioekol.lu.se/Littorina)

Spot ID	Species, where variability was detected		Species-specificity	Tissue-specificity	Level of max. abundance	Protein, accession number (database)
	Somatic tissue	Penial tissue				
15	–	sax	Ubiquitous	Ubiquitous	Upper	Unidentified
18	–	sax	Ubiquitous	Ubiquitous	Upper	Unidentified
39	–	arc	arc/sax	Penial	Middle	Unidentified
49	arc/sax	arc/sax	arc/sax	Ubiquitous	Upper	Unidentified
53	sax	arc	Ubiquitous	Ubiquitous	Lower (sax) Middle (arc)	Unidentified
78	–	sax	arc/sax	Ubiquitous	Upper	Unidentified
100	–	arc	Ubiquitous	Ubiquitous	Middle	Unidentified
107	–	arc/sax	arc/sax	Penial	Upper	Unidentified
108	sax	arc/sax	Ubiquitous	Ubiquitous	Upper	Fructose-bisphosphate aldolase, c1637 (LSD)
109	–	arc/sax	arc/sax	Penial	Upper	Unidentified
110	–	arc/sax	arc/sax	Penial	Upper	Unidentified
111	–	arc/sax	arc/sax	Penial	Upper	Unidentified
122	–	sax	arc/sax	Penial	Upper	Unidentified
123	–	sax	arc/sax	Penial	Upper	Unidentified
127	–	arc/sax	arc/sax	Penial	Upper	Unidentified
128	–	arc/sax	arc/sax	Penial	Lower (sax) Middle (arc)	Unidentified
134	–	arc/sax	arc/sax	Penial	Lower (sax) Middle (arc)	Unidentified
136	–	sax	Ubiquitous	Ubiquitous	Lower	Unidentified
229	–	arc	Ubiquitous	Ubiquitous	Middle	Unidentified
230	sax	sax	arc/sax	Ubiquitous	Upper	Sigma class glutathione-S-transferase 3, c1445 (LSD)
231	sax	sax	arc/sax	Ubiquitous	Upper	Sigma class glutathione-S-transferase 3, c1445 (LSD)
236	sax	sax	arc/sax	Ubiquitous	Upper	Sigma class glutathione-S-transferase 3, c1445 (LSD)
241	–	sax	arc/sax	Ubiquitous	Upper	Unidentified
265	–	arec	Ubiquitous	Ubiquitous	Middle	Unidentified
278	–	sax	Ubiquitous	Ubiquitous	Lower	Small heat shock protein, c4901 (LSD)
304	comp	–	comp	Ubiquitous	Lower	Unidentified
310	–	sax	Ubiquitous	Ubiquitous	Lower	Globin (radular muscle), c5863 (LSD)
311	–	comp	Ubiquitous	Ubiquitous	Lower	Globin (radular muscle), c18425 (LSD)

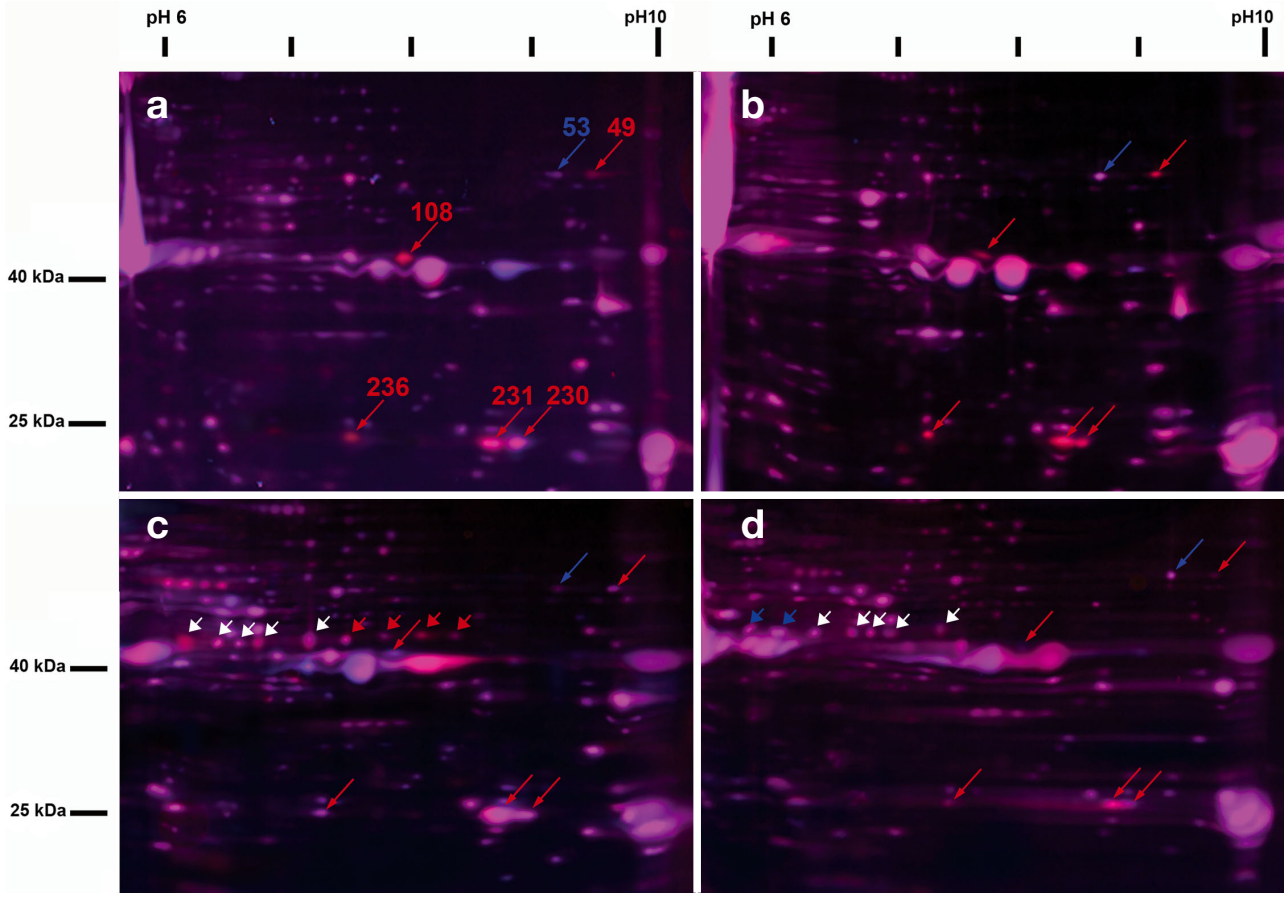

Fig. 8. Effect of tidal level on proteome of *Littorina arcana* and *L. saxatilis*, visualized via 2D-DIGE. (a,b) Comparison of tidal levels based on female foot tissues of (a) *L. arcana* and (b) *L. saxatilis* (blue: middle level; red: upper level). (c,d) Comparison of species based on penial samples in the (c) upper level and (d) middle level (blue: *L. saxatilis*; red: *L. arcana*). Long arrows: ubiquitous tidal-level-variable proteins; short arrows: specific penial proteins. Proteins variable in both species are marked by colored arrows (red: specific for upper level; blue: specific for middle and lower levels; white: penial proteins present in samples of both tidal levels). Numbers in (a) indicate spot IDs

because of low statistical power), it is due to completely different classes of proteins than those expressed in *L. arcana* and *L. saxatilis*.

Some of the tidal-gradient-related changes in the proteome were not simply the result of proteins being switched on or off, but resulted from one gradually displacing another. Spots 49 and 53 have an identical molecular weight, but slightly different isoelectric points, and the increasing abundance of spot 49 in the samples from the upper level relative to the middle level was accompanied by weakening of spot 53. Their concurrent abundance change and equality of molecular weight suggests that these spots are isoenzymes or 2 modifications of the same enzyme. Among all the proteins with level-dependent abundance, only 2 groups of proteins were successfully identified — fructose-bisphosphate-aldolase (enzyme of glycolysis) and glutathione-S-transferase (detoxifying enzyme responsible for the inactivation of free radicals). Most of the detected level-sensitive proteins were not identified by MS/MS analysis, however, because of low intensity of spots (preventing accurate dissection from Coomassie gel, like spots 49 and 53), absence from the available databases, or high degree of variability (like penial proteins) (Table 2).

In the penial tissue, the number of proteins detected as varying with tidal level was higher than in somatic samples, but those proteins were less reliable, as the analysis was based only on fold-change due to lack of replication. One level-dependent protein was found in penial tissues of *L. compressa*, 14 in *L. arcana* and 27 in *L. saxatilis*. The latter 2 species shared 10 of those proteins, 8 of which were specific for penial samples (Table 2). Among other tidal-level-variable proteins of *L. arcana* and *L. saxatilis*, 3 and 5 (respectively) matched those revealed in the somatic tissue. The single tidal-level-variable protein in the penial tissue of *L. compressa* did not match any

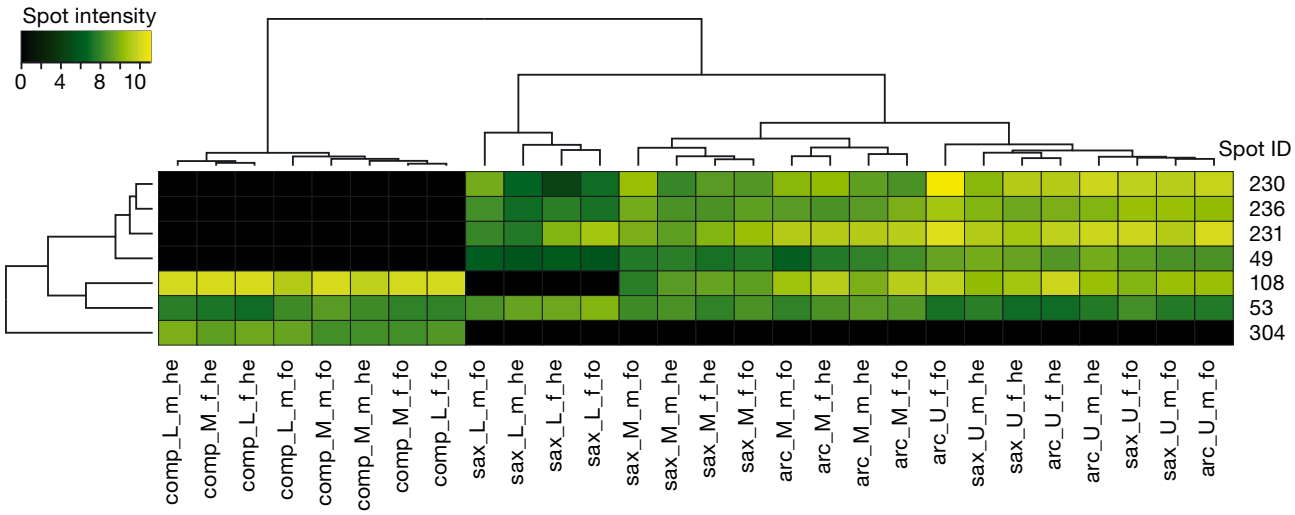

Fig. 9. Clustering of somatic samples from 3 *Littorina* spp. based on tidal-level-variable proteins. Vertical tree on left: similarity of expression patterns of proteins; horizontal tree at top: similarity of samples containing those proteins. Sample labels along the bottom indicate species (arc: *L. arcana*; comp: *L. compressa*; sax: *L. saxatilis*), tidal level (L: lower; M: middle; U: upper), sex (f: female; m: male) and body part (fo: foot; he: head)

variable proteins of either *L. arcana, L. saxatilis*, or its own somatic tissue.

The grouping of penial samples based on tidal-level-dependent proteins confirmed results from the somatic samples: the distinctness of the *L. compressa* proteome and a stronger effect of tidal level than species identity on the *L. arcana* and *L. saxatilis* proteomes (Fig. 10). Together, these results suggest that (1) *L. arcana* and *L. saxatilis* show proteomic plasticity along the vertical shore gradient; (2) in both these species, a similar set of proteins undergoes an analogous shift; and (3) the *L. compressa* proteome reacts differently to ecological change between tidal levels than *L. arcana* and *L. saxatilis*. This prominent difference between *L. compressa* and the *L. arcana*/*L. saxatilis* species pair is also illustrated by the intense overlap between the tidal-level-sensitive protein sets of *L. arcana* and *L. saxatilis* and those proteins differentiating these 2 species from *L. compressa* (Table 2).

DISCUSSION

Our study showed that 3 *Neritrema* spp. of the '*saxatilis*' species group possessed similar proteomes as evidenced by their close grouping on the dendrogram based on quantitative data. At the qualitative level, *Littorina compressa* formed a well-supported branch, outside the *L. arcana*/*L. saxatilis* cluster. The proteomes of the 2 latter species varied in composition similarly to each other along the vertical tidal gradient.

The approach we used in the present study has several advantages and limitations. We used 2D-DIGE to characterize the proteomes of the chosen periwinkle species, as this approach has the advantage of allowing the loading of up to 3 samples onto the same gel and revealing even minor changes in protein structure (Ünlü et al. 1997, Lilley & Friedman 2004, Arentz et al. 2015). We used pooled samples, as they are more representative of the proteome on the population level, because they contain a kind of 'averaged proteome' (Karp et al. 2005, Karp & Lilley 2007, Diz et al. 2009). We considered the proteomes of different body parts separately. This provides replicates for ubiquitously expressed proteins, increases the reliability of the detected spot set and allows the detection of body-part-specific level-driven changes, which could be overlooked in whole-organism samples. We analyzed both qualitative and quantitative data as they reveal different aspects of similarity and variability. The qualitative analysis emphasizes differences based on proteins specific for a certain type of sample (and absent in other ones). The quantitative analysis characterizes the differences in the expression patterns of proteins that are expressed in compared samples. While interpreting within-species proteomic changes in response to ecological shift, we did not discern whether it is a physiological adaptation due to phenotypic plasticity (acclimation) or a result of action of particular alleles (evolutionary adaptation). Our approach does not allow discrimination between these 2 categories.

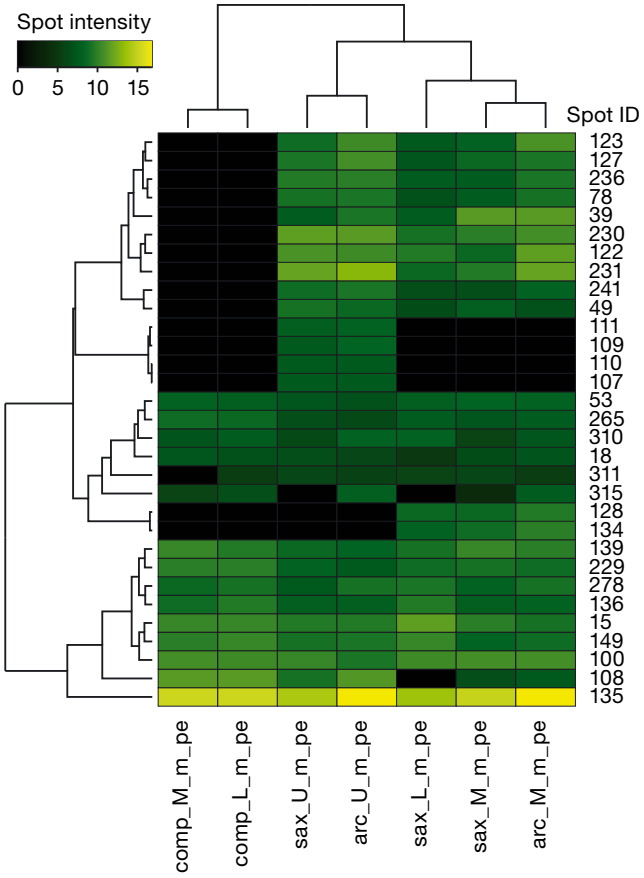

Fig. 10. Clustering of penial samples from 3 *Littorina* spp. based on tidal-level-variable proteins. Vertical tree on left: similarity of expression patterns of proteins; horizontal tree at top: similarity of samples containing those proteins. Sample labels indicate species (arc: *L. arcana*; comp: *L. compressa*; sax: *L. saxatilis*), tidal level (L: lower; M: middle; U: upper), sex (m: male) and body part (pe: penis)

Analysis of proteomes, both qualitatively and quantitatively, distinguished *L. compressa* from the 2 other species, a result that is in line with morphological data (Reid 1989, 1996). Molecular systematics data, however, are inconclusive regarding species relationships. Different molecular markers group the 3 species of the '*saxatilis*'-group in all possible combinations. Some studies of allozymes (Knight & Ward 1991), as well as RFLP-analyses on mtDNA or nuclear loci (Wilding et al. 2000a,b) suggest that *L. compressa* is the sister taxon to the *L. saxatilis*/*L. arcana* pair. Another allozyme analysis (Backeljau & Warmoes 1992), sequence clustering on cytochrome b (Small & Gosling 2000) or ribosomal 28S, 12S and COX showed *L. arcana*/*L. compressa* grouping together as sister taxa, separated from *L. saxatilis* (Reid et al. 2012). A complex array comparative genomic hybridization approach on the number of strongly differing genes separated *L. arcana* from the *L. compressa*/*L. saxatilis* complex, but overall hybridization success had been highest between *L. saxatilis* and *L. arcana* (Panova et al. 2014). These contradicting results illustrate the close relationship among these 3 species on the genomic level and their recent divergence. Alternative approaches such as proteomics can help resolve such a controversy.

The 3 species from the '*saxatilis*'-group can often be found on the same shore with partially differing microhabitat preferences (Reid 1996, Granovitch et al. 2013). Both *L. arcana* and *L. compressa* have a more limited geographic range than *L. saxatilis* (Reid 1996). There are still no statistical data indicating that either of them co-occurs with *L. saxatilis* more frequently than the other. None of them has been described in allopatric populations without *L. saxatilis*. So, their ecological preferences can hardly explain why *L. arcana* more strongly resembles *L. saxatilis* than *L. compressa* at the proteomic level. Not only is there a match in house-keeping proteins between *L. arcana* and *L. saxatilis*, there is also a similar change in the proteome in response to tidal elevation. This indicates the high level of physiological similarity, at least for adult life stages.

L. arcana differs from *L. saxatilis* in its reproductive strategy. It is oviparous, and this feature alone might strongly limit its distribution in stressful habitats such as estuaries, which are also occupied by the ovoviviparous *L. saxatilis* (Reid 1996). It is important to keep in mind that we sampled only adults. The early stages of *L. saxatilis* and *L. arcana* occupy different biotopes — the former live within a parent, while the latter develop in mucus (Reid 1996). The possibility of more prominent interspecific differences between *L. arcana* and *L. saxatilis* during juvenile stages cannot be excluded, but was not addressed here.

In quantitative analyses, we detected a similar shift in proteome composition along the vertical shore gradient for *L. arcana* and *L. saxatilis*, but we found no such shift for *L. compressa*. Considerable differences in a set of physiological features (characterized as thermal resistance ranges) along the vertical shore gradient had been previously described for *L. saxatilis* (Sokolova et al. 2000, Sokolova & Pörtner 2001a,b). We found specific forms of fructose-bisphosphate aldolase and glutathione-S-transferase that appeared in both *L. arcana* and *L. saxatilis* in the upper intertidal, which likely represents the most stressful biotope. These enzymes take part in anaerobic metabolism or antioxidant responses. The appearance and disappearance of signals on 2D elec-

trophoresis can be interpreted as strong changes in the expression pattern or posttranslational modification of a protein. Our data might indicate a potentially adaptive shift in energy consumption and distribution in snails inhabiting different shore levels. Higher abundances of particular forms of fructose-bisphosphate aldolase have been linked to lower or upper tidal levels and interpreted as adaptation of energetic consumption processes to ecological conditions (wave activity vs. heat and desiccation) (Martinez-Fernandez et al. 2008, Diz et al. 2012a). We also found specific forms of fructose-bisphosphate up-regulated in the upper tidal level conditions; however, the particular form we found was not shown to be regulated in the previous studies (based on comparison of 2D-pictures).

Hypoxia is one of the most dangerous stressors in the intertidal zone (McMahon 1988). Hypoxic stress may increase the level of the generation of toxic reactive oxygen species (Lushchak 2011). The appearance of an additional form of glutathione-S-transferase can represent an adaptive response to that change. Enzymes of the same classes (metabolic and antioxidant) were also affected by hypoxic stress in the shrimp *Fenneropenaeus chinensis* (Jiang et al. 2009), Pacific oyster *Crassostrea gigas* (David et al. 2005) and other marine invertebrates (reviewed in e.g. Sheehan & McDonagh 2008, Tomanek 2011, Sokolova et al. 2012), suggesting this response may be widespread in stressful habitats.

Temperature, desiccation and osmotic stress are the other stress factors on the upper shore (Rolán-Alvarez 2007). The proteomic approach has been applied to uncover the biochemical background of the response to thermal stress in other littorinid snails (*Echinolittorina malaccana* and *E. virida* (Wang 2013)), 2 *Mytilus* spp. (Tomanek & Zuzow 2010) and in littorinids and other mollusks living in the upper shore environment in general (not only affected by extreme temperature, but also by other dangerous factors such as osmotic stress, desiccation and hypoxia; Martinez-Fernandez et al. 2008, Diz et al. 2012a, García et al. 2013). These studies revealed changes in expression of a complex set of proteins, such as metabolic enzymes, chaperones, cytoskeletal proteins and antioxidant response proteins. Our approach was not able to detect the changes in these highly variable proteins (possibly due to the short maintenance of snails in standard laboratory conditions). Instead, it was directed at the stable tidal-level-driven proteomic changes. In addition to changes in the metabolic and antioxidant enzymes (which are consistent with published data), several unidentified proteins also were differentially expressed along the vertical shore gradient in 2 of our study species. The existence of similar tidal-level-driven proteomic shifts in both *L. arcana* and *L. saxatilis* are striking, since previous work has shown that these stressors cause differential alterations in the proteomes of even closely related species (Tomanek & Zuzow 2010, Wang 2013).

One more class of proteins varying between the tidal levels in *L. arcana* and *L. saxatilis* includes unknown penis-specific components. Those proteins could be components of the residual sperm, present in low abundance within the ejaculatory duct. The only sperm protein identified in littorinids so far is LOSP of *L. obtusata* (Lobov et al. 2015). But there is a significant difference in molecular weight between LOSP and these penial proteins. Most probably they are components of the mamilliform gland secretion. These components are expressed exclusively in the penis and are most likely involved in reproduction (conditioning of sperm, sealing of a copulating pair, etc). To our knowledge, there are no data that suggest any differences in reproductive biology among snails inhabiting different tidal levels. Consequently, these proteins might be neutral markers. The proteins are polymorphic, with a unique pattern of forms at every tidal level tested, and were very similar between *L. arcana* and *L. saxatilis*. We can exclude misidentification of males as a cause for our result, since proteomes from somatic tissues of those males clustered in the same manner as those from females. The interspecific similarity between *L. arcana* and *L. saxatilis* within the same tidal level (where they co-occur and could copulate), together with intraspecific differences among tidal levels (partially reproductively isolated because of limited mobility of individuals), are compatible with data on the possibility of interspecies hybridization in the wild (Mikhailova et al. 2009), but not conclusive evidence.

We found a considerable overlap between the sets of proteins responsible for tidal-level-driven proteomic shifts and those separating *L. compressa* from the *L. arcana*/*L. saxatilis* pair. Among 6 ubiquitously expressed tidal-level-sensitive proteins in the *L. arcana*/*L. saxatilis* pair, 4 were not detected in the proteome of *L. compressa*. In the penial tissues, there were 8 additional, penis-specific proteins strongly specific for the *L. arcana*/*L. saxatilis* pair. The only level-sensitive protein identified in the somatic samples of *L. compressa* was also strongly species-specific. This confirms the 2 general conclusions of the present proteomic analysis: the proteomic separation of *L. compressa* from the 2 other littorinids studied

here and the difference in how proteomes of *L. compressa* and the *L. arcana*/*L. saxatilis* pair react to environmental variability.

The 3 species of littorinids in the present study are phylogenetically close and genomically similar, as evidenced by variable clustering patterns resulting from different molecular approaches (e.g. Backeljau & Warmoes 1992, Crossland et al. 1996, Small & Gosling 2000, Wilding et al. 2000a,b, Reid et al. 2012). The most comprehensive existing study — an array comparative genomic hybridization analysis — suggested a closer phylogenetic relationship between *L. saxatilis* and *L. compressa* (Panova et al. 2014). In our study of the 'molecular phenotype', the proteome of *L. compressa* grouped outside of the tight cluster of *L. saxatilis* and *L. arcana*, and also had distinct responses to the vertical tidal gradient. While these 3 species appear to inhabit the same environment (they co-occur in overlapping biotopes), the proteomes of *L. compressa* differed from that of *L. saxatilis* and *L. arcana*. This poses several additional questions for future studies: (1) Is this proteomic distinctness of *L. compressa* also observed in other geographical locations? (2) What factor (or factors) determines this segregation? Why is *L. compressa* functionally different from 2 other species? (3) What is the mechanism behind the proteomic difference of *L. compressa*?

Acknowledgements. This project was partially supported by grants 14-04-93083 and 15-04-08210 from the Russian Foundation for Basic Research, grant 1.42.1493.2015 from St. Petersburg State University, and by the Research Council of Norway (EMAP project #233635). Opportunities for 2D-DIGE, LC-MS/MS and corresponding software for analysis were provided by the Resource Center 'Molecular and Cell Technologies' of St. Petersburg State University. We specially thank Pavel Zykin for encouraging the research in collaboration with the Resource Center 'Molecular and Cell Technologies'. We thank the anonymous reviewers for their thorough reading of the manuscript and their extensive and helpful comments.

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Editorial responsibility: Inna Sokolova, Charlotte, North Carolina, USA

Submitted: September 15, 2015; Accepted: May 11, 2016
Proofs received from author(s): June 14, 2016