PHYSIOLOGY

Mild osmotic stress in intertidal gastropods *Littorina saxatilis* and *Littorina obtusata* (Mollusca: Caenogastropoda): a proteomic analysis

Olga Muraeva[1], Arina Maltseva[1], Marina Varfolomeeva[1], Natalia Mikhailova[1,2], and Andrey Granovitch[1]

[1] Department of Invertebrate Zoology, Faculty of Biology, Saint Petersburg State University, Universitetskaya nab. 7–9, St. Petersburg, 199034, Russian Federation;
[2] Center of Cell Technologies, Institute of Cytology RAS, Tikhoretsky prospect, 4, St. Petersburg, 194064, Russian Federation

Address correspondence and requests for materials to Arina Maltseva, arina.maltseva@spbu.ru

Abstract

Salinity is a crucial abiotic environmental factor for marine animals, affecting their physiology and geographic ranges. Deviation of environmental salinity from the organismal optimum range results in an osmotic stress in osmoconformers, which keep their fluids isotonic to the environment. The ability to overcome such stress is critical for animals inhabiting areas with considerable salinity variation, such as intertidal areas. In this study, we compared the reaction to mild water freshening (from 24 to 14 ‰) in two related species of intertidal snails, *Littorina saxatilis* and *L. obtusata*, with respect to several aspects: survival, behavior and proteomic changes. Among these species, *L. saxatilis* is more tolerant to low salinity and survives in estuaries. We found out that the response of these species was much milder (with no mortality or isolation reaction observed) and involved weaker proteomic changes than during acute stress (freshening from 24 to 10 ‰), characterized earlier. The proteomic response of the second species, *L. obtusata*, was weaker (6 % vs 10 % of regulated proteins) than that of *L. saxatilis* and engaged a mostly different set of proteins. Among proteins potentially involved in adaptation to low salinity, we identified enzymes of energetic metabolism and antioxidant response, chaperones, proteins of extracellular matrix and cytoskeleton, ion channels and regulators of cell growth and proliferation.

Keywords: salinity adaptation, osmotic stress, proteomic analysis, intertidal molluscs, periwinkles, *Littorina*, 2D-DIGE

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Author's information: Olga Muraeva, student, orcid.org/0000-0003-3932-2624, Researcher ID: B-6284-2018 Arina Maltseva, Associate Professor, orcid.org/0000-0003-1973-4728, Researcher ID: I-3631-2014; Marina Varfolomeeva, Assistant Professor, orcid.org/0000-0003-0887-8486, Researcher ID: D-1024-2013; Natalia Mikhailova, Associate Professor, orcid.org/0000-0003-1650-9330, Researcher ID: H-1811-2013; Andrey Granovitch, Dr. Sci., Professor, orcid.org/0000-0002-5203-104X, Researcher ID: B-1264-2013

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Introduction

Salinity is one of the most important factors for marine organisms, as it determines the geography of their ranges (Gunter, 1961). Salinity reflects the concentration of inorganic ions in water — primarily, ions of sodium, potassium, calcium, magnesium, chloride and sulfate (Robertson, 1941). These concentrations are more or less stable in the open ocean waters, but vary significantly in coastal area and estuaries among and within seasons (Redfield, 1958; Tabata, 1961; Hansen and Rattray, 1966; Berger, 1986).

Among marine invertebrate species, osmoconformers are most common — these are organisms which maintain their inner fluids isotonic to the surrounding seawater. They retain their normal activities within a salinity tolerance range, while within resistance range they survive due to isolation reaction (Berger, 1986; Khlebovitch and Aladin, 2010). A profound deviation of salinity from the optimum range causes an osmotic stress in these animals, which adversly affects

their functioning. At the cellular level, the stress boosts osmolyte concentration imbalance between cells and their environment, followed by a lesion of electrochemical potential of cellular membranes (Pierce, 1982; Stucchi-Zucchi and Salomao, 1998). Osmotic stress deeply affects various physiological processes, including those in nervous, immune and other systems (Stucchi-Zucchi and Salomao, 1998; Cheng et al., 2004; Bussell et al., 2008; Jauzein et al., 2013; Honorato et al., 2017). Considerable progress has been made in recent years in our understanding of the molecular background of salinity adaptation (An and Choi, 2010; Hoy et al., 2012; Lockwood and Somero, 2011; Zhao et al., 2012; Tomanek et al., 2012; Meng et al., 2013; Seveso et al., 2013; Carregosa et al., 2014; Muraeva et al., 2016; Gharbi et al., 2016; Yang et al., 2016; Yan et al., 2017). Nevertheless, greater knowledge about the physiological mechanisms of adaptation to salinity variation is still needed, especially in regard to intertidal invertebrates, which deal with osmotic stress throughout their lives.

The family Littorinidae includes a set of widely distributed species which are important members of intertidal communities of seacoasts around the world. During the last decades, *Littorina* species have become model organisms for the study of population biology, physiological adaptations, genetics, behavior, and parasitology (Johannesson et al., 1997; Sokolova et al. 2000, Sokolova and Pörtner 2001a,b; Panova and Johannesson, 2004; Panova et al., 2006; Quesada et al., 2007; Mikhailova et al., 2009; Conde-Padín et al. 2009, Granovitch et al. 2009; Galindo et al., 2010; Granovitch and Maximovich, 2013; Granovitch et al., 2013; Storey et al. 2013; Butlin et al., 2014; Lobov et al., 2015; Rolan-Alvarez et al., 2015; Maltseva et al., 2016; Westram et al., 2014; Ravinet et al., 2016, Granovitch, 2016). Along the North Atlantic shore, *Littorina saxatilis* (Olivi, 1872) and *L. obtusata* (Linnaeus, 1758) are most common (Reid, 1996). *L. saxatilis* is characterized by a wide geographic range, a considerable interspecies variability, and an ability to survive in brackish waters of estuaries (Berger, 1986; Reid, 1996; Granovitch et al., 2004). This species was chosen earlier for analysis of adaptation to acute osmotic stress (freshening down to 10 ‰) using proteomic methodology (Muraeva et al., 2016). The second species (*L. obtusata*) has been shown to be less tolerant to low salinity (Berger, 1986) but had not previously been studied on a proteomic level. In this study, we modeled a mild osmotic stress (a decrease of salinity from 24 to 14 ‰) to compare the response of proteomes (1) to salinity stress of different magnitude in one species and (2) to mild salinity stress in two related species. Our study demonstrated that the effect of mild stress was expectedly weaker in comparison to the effect of acute stress; and that changes in the proteomes of two closely related species differed under stress conditions.

Material and methods

Animals. Snails *L. saxatilis* and *L. obtusata* were collected from wild populations in the vicinity of the White Sea Biological Station "Kartesh" (the Chupa Inlet of the Kandalaksha Bay, the White Sea) on October 15–16, 2014. Salinity in this region varies within the range of ~20–28 ‰ depending on the season and weather. The snails were transported to St. Petersburg in wet containers in a thermostatic chamber at 4 °C. Before the experimental exposure, adult molluscs with shell height exceeding 7 mm were acclimated to the standard laboratory conditions (24 ‰, 4 °C, aeration) for 7 days. Once a day, water was removed for 3–4 h from the containers where the snails were kept to imitate tides. No feeding was provided.

Low salinity stress. Two groups of six animals each (3 males and 3 females, healthy mature individuals without trematode infection) were fixed to obtain tissue lysate before the start of the experiment as an "intact" control ("zero–point"). The rest of the animals were separated into two groups and put into containers with seawater of salinity either 14 ‰ (mild salinity stress) or 24 ‰ (dynamic control). The seawater used in the experiment was drawn near the Kartesh Cape at 5 m depth; it was diluted with distilled water to salinity 14 ‰. Tide imitation was performed daily in both experimental groups. Throughout the experiment (6 days) we examined the presence/absence of the isolation reaction. On the second, fourth and sixth days of the experiment, the molluscs from both experimental groups were sampled in two replicates to obtain tissue lysates (3 male and 3 females in each sample).

Sample preparation. Tissue lysates were prepared and analyzed as described previously (Muraeva et al., 2016; Maltseva at al., 2016). Here we present only a short summary of the protocol. The soft body was withdrawn from the shell and put into a cold lytic buffer (25 mM tris pH 8.5, 7 M urea, 2 M thiourea, 4 % CHAPS). Several individuals (3 males and 3 females) were pooled in 1 ml of the lytic buffer (to improve reliability of samples, Diz et al., 2009). Two replicates were made for every experimental time point. Tissues were homogenized in the lysis buffer using a Mixer Mill 400 (Retsch, Germany); 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. Samples were analyzed using 2-dimensional difference gel electrophoresis (Ünlü et al., 1997). Total protein concentration was determined following the Bradford method with a 96-well plate spectrophotometer Epoch 2 (Epoch Microplate Spectrophotometer, BioTek Instruments, USA). Proteins of the tissue lysates were conjugated with fluorescent dyes Cy2, Cy3 or Cy5 (LuminoProbe, BioDye, Russia) with 400 pmol of a

fluorophore per ~50 μg of total protein, and then incubated for 30 min on ice in the dark. Dye swap was done when conjugating a corresponding sample of biological replicates. The reaction was stopped by adding 10 μmol of L-lysine in a buffer (25 mM tris, pH 8.5), followed by incubation for 10 min under the same conditions. Three samples for comparison were pooled then; dithiothreitol (Sigma-Aldrich, USA; up to concentration 100 mM) and biolytes (BioRad, USA; up to concentration 0.4 %) were added. The mixed samples were loaded into an IPG ReadyStrip (7 cm, pH 3–10, BioRad, USA) via passive rehydration (overnight at room temperature, in the dark). Separation in the first direction was carried out in a Protean IEF Cell (BioRad, USA) using the method recommended by the manufacturer: 10 000 Vh, end voltage 4000 V, rapid ramp, 20 °C. Before the separation in the second direction, IPG-strips with focused proteins were incubated in the equilibration buffers (6 M urea, 2 % SDS, 20 % glycerol, 0.375 M tris, pH 8.8) for 15 min: first with 2 % dithiothreitol; and then with 2.5 % iodoacetamide. Electrophoresis in the second direction was performed in a MiniProtean TetraCell (BioRad, USA) in 15 % PAAG in tris/glycine/SDS buffer (BioRad, USA). A GE Typhoon 9500 FLA laser scanner (GE Healthcare, USA) was used for visualization.

Images processing. Qualitative and quantitative gel analysis was carried out using PDQuest Advanced 8.0.1 software (BioRad, USA). A spot was included into the analysis if it was detected in all groups of comparison (control "zero-point", 14 ‰ and 24 ‰ at 2nd, 4th and 5th days) in at least one replicate or in at least one group in both replicates. A total of 267 significant spots were identified during analysis of *L. saxatilis* samples, and 281 spots were identified in the analysis of *L. obtusata*. Normalization for the spots intensities estimates was done on total gel density using PDQuest embedded algorithm. Merging of the electrophoregrams was performed using ImageJ 1.48v (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov.ij).

Protein identification. Mass-spectrometry identification of proteins was carried out in accordance with a "bottom up" methodology — via MS/MS-analysis of tryptic peptides with a database search. For trypsinization, spots of interest were excised from the Coomassie Brilliant Blue G250 stained gel. Gel pieces were destained with 50 % acetonitrile/25 mM tris, pH 8.2, dehydrated in 100 % acetonitrile and rehydrated in bovine trypsin solution (20 ng/μL/25 mM tris, pH 8.2) on ice for 60 min. After rehydration, any excessive trypsin solution was removed, and the gel pieces were covered with 25 mM tris, pH 8.2 and incubated at 37 °C overnight. Tryptic peptides were eluted with 50 % acetonitrile, 0.1 % formic acid, and were vacuum dried to 10 μl volume. Samples were analyzed using LC (Agilent 1260)-MS/MS (QTOF UHD 5238, Agilent Technologies, USA). The elution method was 10 % B to 60 % B for 25 min and further to 100 % B for 5 min, where B was 90 % acetonitrile/0.1 % formic acid; A was 5 % acetonitrile/0.1 % formic acid; flow rate 15 μL/min; and column Zorbax SB-C18 (Agilent Technologies, USA), 5 μm grain, 80 A pores, 150 × 0.5 mm. Mass analysis was carried out with constant reference signal; detection frequency 3 spectra/sec, automatic MS/MS, mother ions 2+, 3+, n+. An MS/MS search was carried out in the mode "Identity" using Agilent Technologies Spectrum Mill software. For protein identification, we used the *L. saxatilis* EST database http://mbio-serv2.mbioekol.lu.se/Littorina1/ (Canbäck et al., 2012).

Statistical analysis. All analyses were performed in R (R Core Team, 2017). Missing spot intensities were imputed using Bayesian principal component analysis (Bishop, 1999) in pcaMethods package (Stacklies et al., 2007). Afterwards, the data were normalized using quantile normalization, log2-transformed, and the technical replicates were averaged.

To visualize the overall similarity of proteomes, we performed nonmetric multidimensional scaling (nMDS) on a matrix of Bray-Curtis dissimilarities among samples in vegan package (Oksanen et al., 2017).

To construct expression profiles of individual proteins, we averaged biological replicates and standardized expression values by treatment and exposure group. The protein expression profiles were clustered based on matrix of Euclidean distances with unweighted pair-group average method (UPGMA). Bootstrap support values were obtained using multiscale bootstrap with 1000 iterations in package pvclust (Suzuki and Shimodaira, 2015). The dendrograms were plotted with the help of dendextend package (Galili, 2015). The trees of protein profiles were divided into significant clusters based on bootstrap support values. The proteins that grouped together with high support (AU p-value > 95) were considered a significant cluster. For *L. obtusata* the whole process was automated using the function pvpick from pvclust package. For *L. saxatilis*, the largest of the two significant clusters was additionally manually subdivided into smaller significant subclusters. The proteins which grouped with low support, but within a significant subcluster, were included into that cluster. The proteins which did not form significant clusters were grouped into an artificial subcluster. The expression profiles for each cluster were plotted with the help of ggplot2 package (Wickham, 2009) and visually screened for asymmetry. The proteins from the most asymmetric clusters, in which expression dynamics differed at 14 ‰ and 24 ‰, were considered as potentially regulated under mild salinity stress.

Results

1. Mild osmotic stress does not affect snail survival and motility. The behavior and vitality of the molluscs were checked throughout the experiment in both incubated groups (14 ‰ and 24 ‰) of both species (*L. saxatilis* and *L. obtusata*) with no differences detected. There was no mortality; snails did not isolate either under or without the water and demonstrated usual locomotor and feeding (scratching by radula) activity.

2. Laboratory maintenance under normal conditions causes physiological changes. Multidimensional scaling, which was used for visualization of similarity between proteomes, showed that distances were greater among different time points than between different salinities within the same time point (Fig. 2 and 3). This was true for both species, and it implies that laboratory maintenance without feeding during acclimation and exposure periods affects proteomes stronger than mild osmotic stress (a change of salinity from 24 to 14 ‰).

3. Salinity drop affects expression of several functional groups of proteins. To reveal the proteins which changed abundance with a drop in salinity, we assembled "hybrid" expression profiles, reflecting abundance dynamics during both 14 ‰ and 24 ‰ for each protein (Fig. 1). These expression profiles were clustered to distinguish characteristic patterns of expression change (Supplement 1 and 2). Among the clusters of expression profiles (Supplement 3 and 4), we have selected the proteins with the most asymmetric temporal patterns of expression at 14 ‰ and 24 ‰ relative to the initial state (Fig. 4 and 5). In total, 28 proteins of *L. saxatilis* (~ 10%) and 18 proteins of *L. obtusata* (~ 6%) were recognized as regulated during mild salinity stress. Among these proteins there were members of several functional groups (Table 1): antioxidant proteins (glutathione-S-transferase), chaperons (small heat shock protein), ion channels (Vdac-2), metabolic enzymes (arginine-kinase; ATP-synthase), cytoskeleton proteins (light chain of myosin, alpha-tubulin, troponin T), extracellular matrix proteins (matrilin) and regulatory proteins (tyrosine 3-monooxygenase, translationally-controlled tumor protein). The full list of the significant clusters' content and master-gels of analyzed proteomes are presented in Supplement 5.

4. Stress response in two related species differs. The proteomic response was slightly stronger in *L. saxatilis* compared to *L. obtusata* (see above): the abundance of approximately 10 % and 6 % of detected proteins experienced regulation, respectively. Nevertheless, only two of those proteins were common for the two species (small heat shock protein and translationally-controlled tumor protein). Other differentially expressed proteins did not match between two species as can be concluded from their positions on the gel (the majority of them were not identified), but some of them represented the same functional groups (e.g., tubulin in *L. obtusata* vs myosin and troponin in *L. saxatilis* are all cytoskeletal proteins).

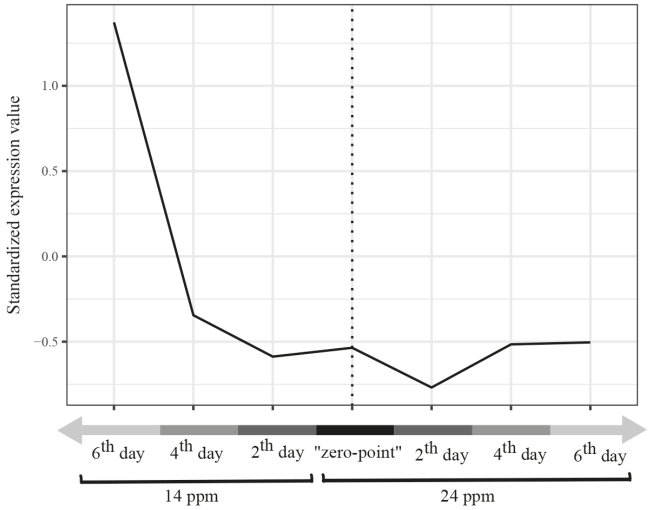

Fig. 1. An example of a protein expression profile, which reflects dynamics of abundance during both 14 ‰ and 24 ‰ starting at "zero-point", corresponding to intact animals at the start of experiment. This particular profile belongs to *L. saxatilis* gel-spot 2605, corresponding to Vdac-2.

Discussion

The response to low salinity. In this study, we exposed the intertidal snails of two species to mild osmotic stress (salinity change from 24 to 14 ‰) under laboratory conditions and evaluated changes in proteomes using a 2D gel-based approach, followed by MS-based proteins identification. There were no effects on behavior or survival of experimental animals, unlike in the previously studied acute stress in *L. saxatilis*, with salinity drop to 10 ‰ (Muraeva et al., 2016). This indicates that salinity of 14 ‰ falls within the tolerance range of both species, while 10 ‰ is already a part of the resistance range of *L. saxatilis* and apparently of *L. obtusata*, which is less tolerant to low salinity (Berger, 1986).

The general scale of proteomic changes in *L. saxatilis* was expectedly weaker under mild stress conditions compared to acute ones: although in both cases ~ 10 % of proteins were found regulated, the sensitivity of the methodology was higher in the analysis of the "mild" experiment results. Another mark of relative weakness of response to mild stress was the fact that the strength of salinity-related changes was similar to the strength of changes related to laboratory maintenance. In contrast, this was not the case in the acute experiment, partially due to continuous isolation reaction (up to 4th day inclusively): consequent hypoxia and acidosis prominently affect organisms in addition to low salinity influence per se. The composition of differentially expressed proteins under two types of stress partially overlapped. Thus, in

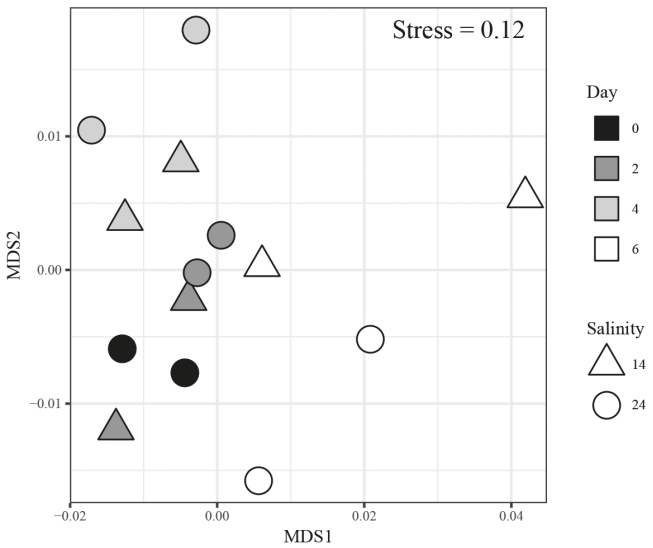

Fig. 2. Nonmetric multidimensional scaling of *L. saxatilis* proteomes. Exposure duration is indicated by different hues of gray; triangles correspond to low salinity (14 ‰) and circles to control (24 ‰).

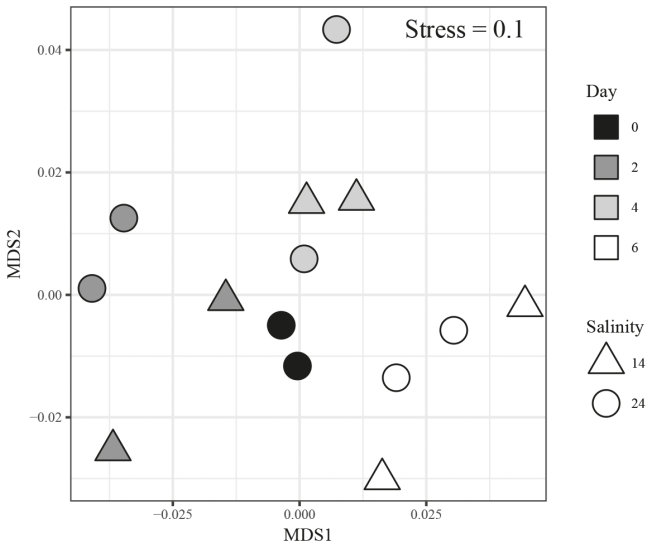

Fig. 3. Nonmetric multidimensional scaling of *L. obtusata* proteomes. Exposure duration is indicated by different hues of gray; triangles correspond to low salinity (14 ‰) and circles to control (24 ‰).

Table 1. Identified differentially expressed proteins. LSD: *L. saxatilis* database; MW: molecular weight of a protein in accordance to database. MH+: molecular weight of a single positively charged ion of an identified peptide

Spot N	Cluster N	Type of regulation	Protein, accesion number (database), peptide sequences	MH+, Da	Theoretical MW (kDa)	Species
X2605	0	up	**Vdac2 protein, c819 (LSD)** (K)GYNYGFFK(L) (K)LAFDTSFAPQTGK(K) (K)LAFDTSFAPQTGKK(S) (K)LTLSALIEGK(S) (K)VNNSSQIGLGYSQK(L) (R)VNPDLETAVNLSWTSGTNATR(F) (K)WSEYGLNFTEK(W)	 995.4621 1382.6951 1510.7900 1044.6299 1494.7547 2246.1048 1373.6371	30.3 kDa	*L. saxatilis*
X2702	0	down	**Arginin kinase, c5132 (LSD)** (K)LAATPEFK(E) (R)ASVHIKIPK(L) (R)LVSAIETMEK(K) (R)LGLSEFDAIMENK(N)	 876.4825 992.6251 1120.5918 1483.7171	39.3 kDa	*L. saxatilis*
X7506	0	down	**Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide, c9110 (LSD)** (K)AAFDDAIAELDTLSEESYK(D) (K)AAFDDAIAELDTLSEESYKDSTLIMQLLR(D)	 2087.9655 3258.6086	29.2 kDa	*L. saxatilis*
X8002	4	up	**Ribosomal protein, c16990 (LSD)** (K)ILSSVGIEAEADKVK(K)	 1558.8687	11.5 kDa	*L. saxatilis*
X9903	4	up	**Matrilin, c1810 (LSD)** (K)DLVDTLAGIDWR(H) (R)VVIVLTDGNSQETALTK(X)	 1373.7060 1787.9749	45.5 kDa	*L. saxatilis*
X7502	6	down	unknown			*L. saxatilis*
X8205	6	down	**Myosin regulatory light chain, c1759 (LSD)** (K)GKFNYNEFVSILK(G) (K)GKFNYNEFVSILKGKQEDEGQPA(-) (R)ATSNVFAMFR(Q)	 1558.8264 2598.2835 1143.5615	13.9 kDa	*L. saxatilis*
X4902	7	up	unknown			*L. saxatilis*
X6901	7	up	unknown			*L. saxatilis*
X3901	15	up	**ATP synthase subunit alpha, c131 (LSD)** (K)APGIIPR(T) (K)AVDSLVPIGR(G) (R)STVAQLVK(R) (R)TSVKEPMQTGIK(A) **ATP synthase subunit alpha, c2055 (LSD)** (R)TGAIVDVPVGR(E) (R)VVDALGNPIDGKGPLGSSER(L)	 723.4512 1026.5942 845.5091 1318.7035 1081.6157 1981.0349	59.7 kDa	*L. saxatilis*

X6005	15	up	unknown			*L. saxatilis*
X3203	19	down	unknown			*L. saxatilis*
X6402	19	down	unknown			*L. saxatilis*
X6603	19	down	**Tyrosine-3-monooxygenase, c2612** (K)LAEQAERYDDMAEAMR(N) (R)NLLSVAYK(N)	1898.8371 907.5247	29.2 kDa	*L. saxatilis*
X1502	20	up	**Sigma class glutathione-s-transferase 3, c1445 (LSD)** (R)LLLHATGQPFEDVR(V) (K)YGQSLAIGPFLAR(K)	1595.8540 1392.7634	23.0 kDa	*L. saxatilis*
X2401	20	up	**Small heat shock protein, c4901 (LSD)** (R)VNVDVQQFKPEEINVK(T)	1886.0018	18.9 kDa	*L. saxatilis*
X9004	20	up	unknown			*L. saxatilis*
X3503	24	down	unknown			*L. saxatilis*
X1402	24	down	unknown			*L. saxatilis*
X3201	24	down	unknown			*L. saxatilis*
X3202	24	down	unknown			*L. saxatilis*
X3502	24	down	unknown			*L. saxatilis*
X7302	24	down	**Translationally-controlled tumor protein, c287 (LSD)** (K)DLSFYGAEPR(E) (K)FTVAWAPK(A)	1154.5477 919.5036		*L. saxatilis*
X9502	29	up	unknown			*L. saxatilis*
X3704	29	up	**Troponin T, c420 (LSD)** (K)GATVMVDESFDR(L) (R)LVGDKYDLEQR(F) (R)QQYDMIELAER(A) (R)RLVGDKYDLEQR(F) (R)TYVER(K)	1326.5994 1335.6903 1395.6573 1491.7914 667.3410	38.0 kDa	*L. saxatilis*
X9401	29	up	unknown			L. saxatilis
X1505	33	up	unknown			*L. saxatilis*
X5702	33	up	**Troponin T, c420 (LSD)** (K)GATVMVDESFDR(L) (K)KGATVMVDESFDR(L) (R)LVGDKYDLEQR(F) (R)QQYDMIELAER(A) (R)RLVGDKYDLEQR(F)	1326.5994 1454.6944 1335.6903 1395.6573 1491.7914	38.0 kDa	*L. saxatilis*
X5706	11	up	**Tubulin alpha1, c2217 (LSD)** (R)AVFVDLEPTVIDEVR(T) (K)DVNAAIATIK(T) (K)EIVDLVLDR(I) **Tubulin alpha1, c1018 (LSD)** (R)AVFVDLEPTVIDEVR(T) (K)EIVDLVLDR(I)	1701.9058 1015.5782 1071.6045 1701.9058 1071.6045	39.3 kDa	*L. obtusata*
X7905	11	up	unknown			*L. obtusata*
X8308	24	down	unknown			*L. obtusata*
X4404	24	down	unknown			*L. obtusata*
X6906	24	down	unknown			*L. obtusata*
X7608	40	down	unknown			*L. obtusata*
X7230	40	down	**Unknown protein Crassostrea/Aplysia, c8291 (LSD)** (K)VVLPTGEDVQQEK(T)	1441.7533		*L. obtusata*
X7607	40	down	unknown			*L. obtusata*
X8411	44	down	**Translationally-controlled tumor protein, c287 (LSD)** (K)FTVAWAPK(A)	919.5036		*L. obtusata*
X3815	44	down	unknown			*L. obtusata*
X5609	44	down	unknown			*L. obtusata*
X5409	48	up	unknown			*L. obtusata*
X8605	48	up	unknown			*L. obtusata*
X1204	48	up	**Small heat shock protein, c4901 (LSD)** (R)VNVDVQQFKPEEINVK(T)	1886.0018	18.9 kDa	*L. obtusata*
X1904	48	up	unknown			*L. obtusata*
X1905	48	up	unknown			*L. obtusata*
X1912	48	up	unknown			*L. obtusata*
X4410	48	up	unknown			L. obtusata

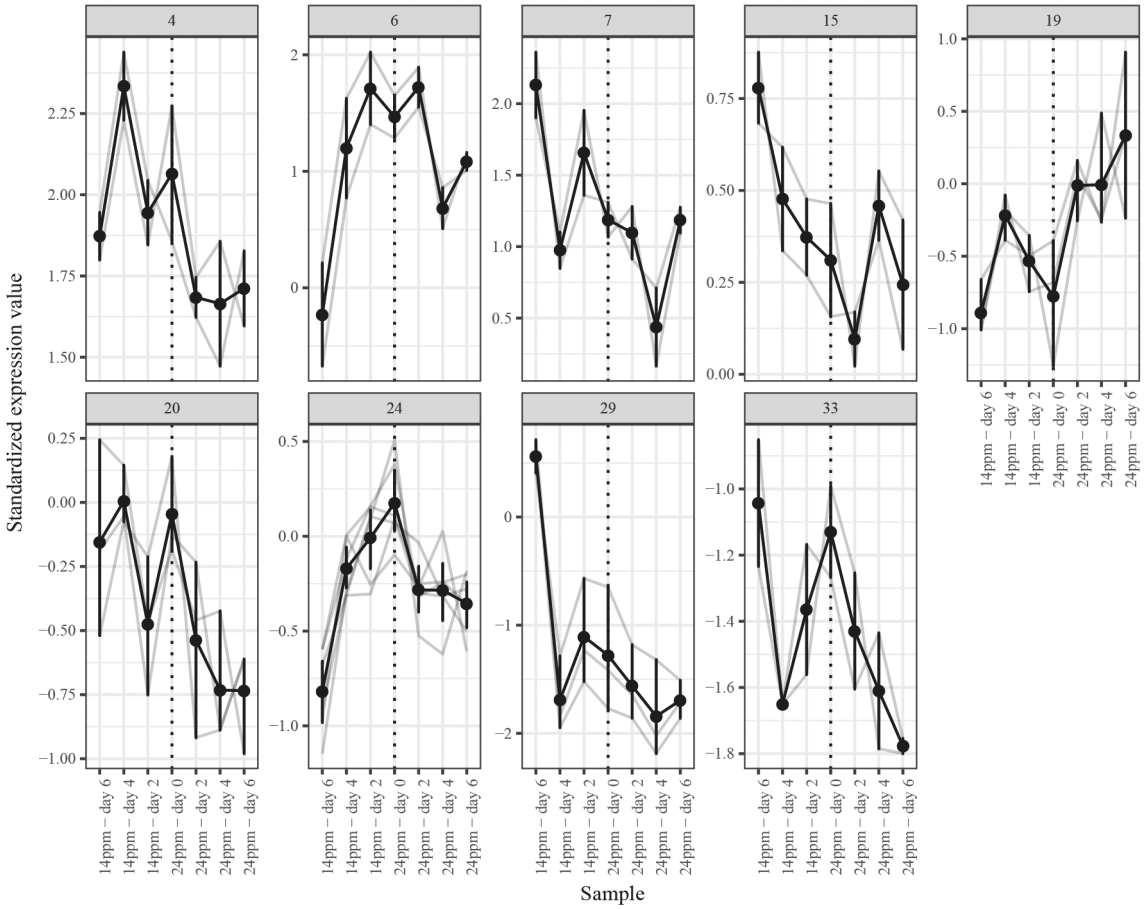

Fig. 4. Clusters of proteins of *L. saxatilis* with asymmetric temporal patterns of expression at 14 ‰ and 24 ‰ during the experiment relative to the initial state.

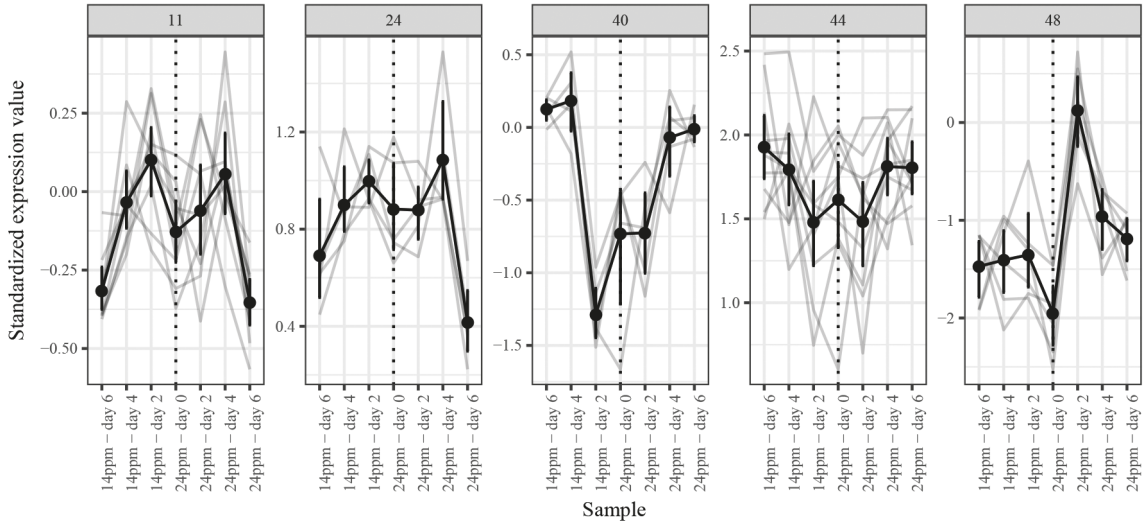

Fig. 5. Clusters of proteins of *L. obtusata* with asymmetric temporal patterns of expression at 14 ‰ and 24 ‰ during the experiment relative to the initial state.

both cases, forms of small heat shock protein, arginine-kinase, ATP-synthase (even different subunits), matrilin, tyrosine-3-monooxygenase/tryptophan 5-monooxygenase activation protein were shown to undergo abundance regulation. Glutathione-S-transferase was detected as regulated during mild stress, while thioredoxin-peroxidase differentially expressed during acute stress, both being antioxidant proteins (Muraeva et al., 2016).

The proteomic changes in *L. obtusata* were slightly weaker than in *L. saxatilis*. Only three proteins (small

heat shock protein, unknown protein *Crassostrea*/*Aplysia* and translationally-controlled tumor protein) were identified among 18 proteins with low-salinity-related abundance changes; two of them were also detected in *L. saxatilis*. Unidentified regulated proteins do not correspond to those of *L. saxatilis*, though they could belong to the same functional groups, like identified cytoskeletal proteins. Thus, although the scale of response of these species was roughly comparable, the sets of executive proteins matched only partially, which may be related to differences in their tolerant ranges (Berger, 1986).

Differentially expressed proteins. Arginine-kinase (AK) is a functional analogue of vertebrates' creatine-kinase. These enzymes are responsible for an alternative pathway of ATP synthesis: they catalyze the transfer of phosphate from phosphoarginine to ADP under conditions of insufficient oxidative phosphorylation within mitochondria (Morrison, 1973). Thus, phosphoarginine is actively consumed by tissues under oxygen deficiency conditions. The up-regulation of AK in the acute salinity stress experiment was most likely related to isolation-caused hypoxia. Similarly, compensatory upregulation of AK during thermal/hypoxic stress was shown in other invertebrates: blue mussels (Tomanek and Zuzow, 2010), snails *Echinolittorina* (Wang, 2013), shrimps *Fenneropenaeus* (Jiang et al., 2009) and *Marsupenaeus japonicas* (Abe et al., 2007). Under mild stress conditions, there was no isolation-related hypoxia, and AK was temporally down-regulated. Down-regulation of proteins not urgently needed during stress has been detected previously, and may demonstrate a kind of cellular economy reaction (Warner, 1999; Weber et al., 2006; Muraeva et al., 2016). Interestingly, the abundance of another enzyme of energetic metabolism, ATP-synthase, which is involved in the oxidative phosphorylation process, increased in both experiments. During mild stress response, the up-regulation was quite fast, while during acute stress it was delayed until the 5th day, when snails began to open their shells during tide-imitation after continuous isolation (Muraeva et al., 2016). This implies that the metabolic intensification may be involved in any type of salinity stress response, but it was delayed under acute stress conditions due to long isolation reaction.

Vdac-2 is a voltage-dependent anion channel, a very conservative member of the porin-family, located in the outer membrane of mitochondria. It plays an important role in regulation of metabolic and energetic-related transport between cytosol and mitochondrion (Lemasters et al., 2006). This protein is also known to be a regulator of apoptosis (Tsujimoto and Shimizu, 2002). In agreement with this, Vdac-2 inhibited apoptosis in oysters *Crassostrea gigas* during viral infection (Li et al., 2016). The regulation of this protein was also established during thermal stress in bivalve *Geukensia demissa* (Fields et al., 2016) and during osmotic stress in plants (Kumari et al., 2009; Wen et al., 2011). Thus, Vdac-2 protein can be involved in cell protection under different stress conditions in different organisms.

A translationally-controlled tumor protein (TCTP) is another putative regulator of apoptosis with detected changes in abundance within cells. This house-keeping protein interacts with several cytosolic targets, e.g., tubulin, TSAP6, etc., and is involved in regulation of cell cycle, growth and proliferation (Bommer and Thiele, 2004; Tuynder et al., 2004). TCTP is also an important participant of the TOR-pathway, a nutrition sensor and growth regulator in different organisms (Deprost et al., 2007; Brioudes et al., 2010). Its regulation was detected during salinity stress in plants and bacteria (Vincent et al., 2007; Santa Brígida et al., 2014). TCTP proved to be down-regulated during mild salinity stress in both *Littorina* species. This may be interpreted as a sign of repartition of energy between stress reaction and growth processes in organism.

Chaperones are responsible for renaturation of damaged proteins and folding of de novo synthesized ones, and the prevention of apoptosis by these proteins also was reported (Hartl, 1996; Kamradt et al., 2005; Hartl et al., 1996). The participation of heat shock proteins in salinity adaptation has been demonstrated many times in different taxa: protists *Amoeba proteus* and *Paramecium jenningsi* (Plekhanov et al., 2006); sponges *Tetilla mutabilis* (Kültz et al., 2007); corals *Seriatopora caliendrum* (Seveso et al., 2013); sea cucumbers *Apostichopus japonicas* (Meng et al., 2011); oysters *Crassostrea gigas* (Zhao et al., 2012); and mussels *Mytilus galloprovincialis*, *M. trossulus* (Tomanek et al., 2012) and *M. edulis* (Podlipaeva et al., 2012, 2016). In our study, heat shock proteins were the only protein group which we revealed as important for low salinity adaptation in both stress types and in both species studied.

Different types of stress (thermal, osmotic or hypoxic) are accompanied by an appearance of toxic reactive forms of oxygen, damaging cellular structures (Lushchak, 2011). Glutathione-S-transferase (GST) belongs to the diverse group of cytosolic and mitochondrial enzymes which defend the cell against various harmful agents produced endogenously or acquired from the environment. GST catalyzes conjugation of reduced glutathione with electrophilic targets, both environmental toxicants or numerous by-products of oxidative stress causing their inactivation (Hayes and Strange, 2000; Strange et al, 2001). The importance of GST for cell defense during osmotic stress has been demonstrated in several marine invertebrates, e.g., in bivalve species of the Veneridae family (Carregosa et al., 2014). Other antioxidant proteins also were involved in overcoming salinity and hypoxic stress: superoxide-anion dismutase and catalase in holothuria *Apostichopus japonicus* (Wang et al., 2008); catalase, thioredoxin peroxidase and glutathione peroxidase in snails

Haliotis discus discus (De Zoysa et al., 2010); a diverse set of antioxidant enzymes in mussels *M. galloprovincialis* and *M. trossulus* (Lockwood and Somero, 2011). We detected up-regulation of GST in *L. saxatilis* in this study, while thioredoxin peroxidase was regulated in the same species during acute salinity stress (Muraeva et al., 2016). Thus, activation of the antioxidant system is an essential part of adaptation to low salinity in this species as well as in other organisms.

Tyrosine-3-monooxygenase (Tyr-3-m) is the first enzyme in a pathway of catecholamine synthesis from tyrosine. Catecholamines (e.g., adrenaline and noradrenaline) are hormones and neurotransmitters with many effects on different cellular and organismal functions; in particular, those mediators participate in regulation of the stress response. Up-regulation of Tyr-3-m was described as a part of thermal stress response in corals *Scleronephthya gracillimum* (Woo et al., 2006). Unexpectedly, in this study we detected down-regulation of this enzyme and its activator, which agrees well with previous results (down regulation of Tyr-3-m/tryptophan 5-monooxygenase activation protein) in the same species under acute salinity stress (Muraeva et al., 2016). The biological meanings of this down-regulation remain to be elucidated.

The detected increase of the relative abundances of matrilin (extracellular matrix protein) corroborates our previous results. Together with revealed regulation of several cytoskeletal proteins, controlling cell-shape, it implies the possible tissue remodeling under salinity stress, also inferred earlier. Up- and down-regulation of genes, coding for proteins of cytoskeleton, cell adhesion and extracellular matrix, was also shown to be part of the response to salinity stress in oysters *C. gigas* and other marine invertebrates (Tomanek et al., 2012; Zhao et al., 2012).

Conclusions

Increasing the stressful salinity value from 10‰ to 14‰ drastically alters the response of intertidal molluscs *L. saxatilis* due to transition from resistance to tolerance range of this species. The response to mild water freshening (14‰) in this species was expectedly weaker (compared to 10‰) in terms of survival, behavior and characteristics of the proteome. The general scale of stress response of the related species *L. obtusata* was a bit weaker than that of *L. saxatilis* and was mainly fulfilled via other proteins (possibly belonging to the same functional groups). This difference might represent a background for the differences in salinity tolerance ranges between the two species studied. The main functional groups of proteins involved in an organism's response to low salinity in the studied species are antioxidant proteins, chaperones, metabolic enzymes and regulators, ion channels and cytoskeletal and extracellular matrix proteins. This fits well with data obtained in other organisms.

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