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Differences in the expression of soluble proteins in freshwater and brackish-water ecotypes of the snail *Theodoxus fluviatilis*

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Abstract

The neritid snail *Theodoxus fluviatilis* has formed regional subgroups in northern Europe, where it appears in both freshwater (FW) and brackish water (BW) in coastal areas of the Baltic Sea. These ecotypes show clear differences in osmotolerance and in the modes of accumulating organic osmolytes under hyperosmotic stress. We reasoned that the expression patterns of soluble proteins in the two ecotypes may differ as well. BW snails have to deal with a higher salinity (up to 20‰) than FW snails (0.5‰) and also cope with frequent fluctuations in environmental salinity that occur after heavy rains or evaporation caused by extended periods of intense sunshine. Therefore, the protein expression patterns of specimens collected at five different FW and BW sites were analyzed using 2D SDS-PAGE, mass spectrometry, and sequence comparisons based on a transcriptome database for *Theodoxus fluviatilis*. We identified 89 differentially expressed proteins. The differences in the expression between FW and BW snails may be due to phenotypic plasticity, but may also be determined by local genetic adaptations. Among the differentially expressed proteins, 19 proteins seem to be of special interest as they may be involved in mediating the higher tolerance of BW animals towards environmental change compared with FW animals.

KEYWORDS
genetic adaptation, osmotolerance, phenotypic plasticity, protein expression, salinity acclimation

1 | INTRODUCTION

Coping with environmental stress is a key feature in organisms to survive in their ever-changing habitat. Phenotypic plasticity allows organisms to adjust to changes within their genetically defined limits (local adaptation) (Hildebrandt et al., 2018; Rowiński & Rogell, 2017). The underlying mechanisms of phenotypic plasticity or phenotypic elasticity include environmentally driven alterations in gene expression, epigenetic processes, post-translational protein modification, regulation of enzyme activity, and redirection of metabolic processes (Burggren, 2015; Lockwood & Somero, 2011; Woods, 2014).

The neritid snail *Theodoxus fluviatilis* (Linnaeus 1758) has formed regional subgroups in Europe, where it appears in both freshwater (FW) and brackish water (BW) habitats. These ecotypes show a clear difference in osmotolerance (Symanowski & Hildebrandt, 2010; Wiesenthal et al., 2018) and in the mode of accumulating organic

osmolytes under hyperosmotic stress (Wiesenthal et al., 2019). Animals of the BW ecotype have a wider range of tolerable environmental salinities (up to 24‰, Zettler et al., 2004) than animals of the FW ecotype who show signs of stress at salinities of 16‰ (Wiesenthal et al., 2018). Even when specimens of both ecotypes are gradually acclimated to extreme salinities (FW to 21‰ and BW to 28‰), their responses to salinity change do not match, indicating that there may be genetic differences between the two ecotypes that limit their plastic responses in different ways. The metabolic pathways underlying the accumulation of organic osmolytes, which balances cell volume under hyperosmotic stress, are different in the two ecotypes (Wiesenthal et al., 2019). FW animals seem to generate large amounts of free amino acids from the hydrolysis of storage proteins, whereas BW animals seem to newly synthesize amino acids, mostly alanine and proline as well as urea (Wiesenthal et al., 2019). These differences indicate that the metabolic responses to osmotic stress in animals of the different ecotypes seem to be different, raising the question of whether such differences may be mirrored in the expression patterns of soluble cellular proteins. We therefore set out to use a proteomic approach to compare the protein expression in foot muscle tissue of specimens from BW and FW ecotypes. The foot muscle was chosen because it is the most prominent homogenous tissue within the animal and has the largest contribution to osmotic swelling or shrinking. Thus, the foot muscle is the main contributor to potential volume changes in the animal by changing osmotic gradients between internal space and external medium. We expected that volume regulatory measures such as accumulation of organic osmolytes under hyperosmotic challenges would be most pronounced in foot muscle tissue.

2 | METHODS

2.1 | Animal collection and transfer experiments

Adult individuals of *Theodoxus fluviatilis* were collected from three freshwater (FW) and two brackish water (BW) sites in northern Germany between late May and September 2016. The FW collection sites were situated in the lake Schmaler Luzin (S1 and S2) and in the lake Carwitzer See (S3), which are both part of the Feldberger Seenlandschaft in the Mecklenburg Lake District. The BW collection sites were situated along the coast of the Baltic Sea close to Greifswald (S5) and to the island of Hiddensee (S6). Between 4 and 26 days after the respective collection, during which the snails were held in 60-L tanks with water of their respective salinity and rocks and substrate from their collection site, snails were transferred to small tanks, where they were held at their original salinities (S1–S3, 0.5‰; S5, 7.5‰; S6, 9‰) under controlled conditions at room temperature (21°C) for 48 h. A sample size of 15 was used. Details on the collection and storage of the snails in the laboratory until the beginning of the experiments were given previously (Wiesenthal et al., 2018).

2.2 | Sample preparation

After 48 h at their natural salinity, snails were cooled down in a refrigerator at 4°C for 10–15 min, and the foot muscle was dissected with a needle and a razor blade. The foot muscle tissue was blotted dry, immediately weighed, and placed in liquid nitrogen. The fresh weight of the muscle tissue was determined with a precision scale (Quintix, Sartorius AG, Göttingen, Germany) to the nearest 0.001 g. Frozen tissue samples were then homogenized on ice in 500 µl lysis buffer (7 mol/L urea, 2 mol/L thiourea, 65 mmol/L DTT, 33 mmol/L CHAPS, 0.8% v/v Biolyte pH 3–10, 5 mmol/L Pefabloc, redistilled water) with a T8-Ultraturrax (IKA Labortechnik, Staufen, Germany), stored on ice for 3 min, and centrifuged (Heraeus Fresco 21, ThermoFisher Scientific, Waltham, MA, USA) at 16,000 *g* and 4°C for 3 min, leaving the intracellular proteins in the supernatant. The supernatant was stored at −86°C.

The protein concentration in the supernatant was determined using the Bradford method (Bradford, 1976), an infinite F200 PRO plate reader (Tecan, Männedorf, Switzerland), and the software Magellan 7.2 SP1 (Tecan, Männedorf, Switzerland). For the quantification, 250 µl of diluted (1:5) 5× Bradford reagent (0.6 mmol/L CBB G 250, 24% v/v pure ethanol, 42.5% v/v orthophosphoric acid, redistilled water) were thoroughly mixed with 5 µl of sample, and extinctions were measured in a double determination along with calibration standards prepared using bovine serum albumin (BSA).

2.3 | 2D SDS-PAGE

For isoelectrical focusing of the extracted proteins, appropriate amounts of sample and rehydration buffer (9 mol/L urea, 33 mmol/L CHAPS, 50 mmol/L DTT, 0.2% v/v, and redistilled water) were mixed to reach a total volume of 400 µl containing 100 µg of protein. The respective volumes of sample and rehydration buffer depended on the individual protein concentrations of the samples that were previously determined with the Bradford method. The mixtures were each applied to individual slots of a focusing tray (Bio-Rad, Munich, Germany) and 17 cm IPG (immobilized pH gradient) strips with a pH gradient from 5 to 8 (Bio-Rad, Munich, Germany) were carefully placed on top. These were then covered with 750 µl of mineral oil (Bio-Rad, Munich, Germany). The rehydration took place in a PROTEAN IEF Cell (Bio-Rad, Munich, Germany) for 12 h at 20°C and was immediately followed by the focusing for 5.5 h. For subsequent equilibration, strips were moved from the focusing tray into slots of an incubation tray, where they were consecutively incubated in equilibration solution (6 mol/L urea, 3 mol/L glycerol, 2% w/v SDS, 3% v/v resolving gel buffer [1.5 mol/L Tris calibrated to a pH of 8.8 with hydrochloric acid, deionized water]) containing DTT (65 mmol/L) and iodoacetamide (260 mmol/L) for 10 min each. The equilibrated strips were then incubated in SDS sample buffer (99 mmol/L, calibrated to a pH of 6.8 with hydrochloric acid, 40% v/v glycerin, 8% v/v β-mercaptoethanol, 2% w/v SDS, 6 mmol/L bromophenol blue, deionized water) for 5 min before being placed in the pockets of 20 × 20 cm

12% gels (12% w/v acrylamide, 25% v/v resolving buffer, 33.9% v/v redistilled water, 0.1% w/v SDS, 0.1% v/v TEMED, 0.1% w/v APS) that were filled with diluted (1:5) 5× running buffer (125 mmol/L Tris, 960 mmol/L glycine, deionized, water, 0.5% w/v SDS). As a size reference, 10 µl Roti®-Mark STANDARD (Carl Roth, Karlsruhe, Germany) was placed in a small pocket next to the IPG strip. The vertical electrophoresis was carried out using a PROTEAN II xi multi-cell (Biorad, Munich, Germany) and started by applying 50 V for 30 min and then increased to 250 V for 4–5 h. Gels were washed in deionized water for 10–15 min (twice) and then transferred to sensitive colloidal Coomassie staining solution (5% w/v aluminum sulfate, 10% v/v ethanol, 0.02 mmol/L CBB G250, 2% v/v orthophosphoric acid) (modified from Kang et al., 2002) overnight. The next morning, gels were repeatedly (four times) washed in deionized water for 1–2 h, followed by scanning with an Xfinity Pro42 scanner (Quatographic, Kiel, Germany), and images were saved as tiff files with 300 dpi.

2.4 | Spot pattern analysis and statistical analysis

The spot patterns of the gel images were analyzed using the software Delta 2D (Decodon, Greifswald, Germany). Background speckles were removed (settings: black, 3; white, 5) from all uploaded images, but no further changes were applied. We collected three samples per collection site, with two replicates per sample. For two samples, however, the sample volume was limited and only one replicate could be produced (Table S1). To carry out a quantitative comparison of the spots across all collection sites, the individual spots on the uploaded images were linked using a group warping strategy. Spots were detected by the software using the following settings: local background = 127, average spot size = 42, sensitivity = 25%; the creation of model spots was checked and the spot attributes were kept (example image in Figure S1). The detected spots were manually edited, and spots most likely representing artifacts (those with spot values <0.032) were deleted. The analysis was carried out using the spot volume, which is a combination of the spot size and staining intensity. For each gel the individual spot volumes were normalized, so that the final spot volume quantities used for the analysis were relative to the total spot volume of the respective gels. The sum of all detected spot volumes on a gel added up to 100%. For samples in which two replicates per sample were available, the mean spot volume of individual spots was calculated for that sample.

The statistical analysis that was carried out included a principal component analysis (PCA) to show how spot patterns from snails from five different collection sites grouped under control conditions. These groups described all samples from the same collection site and were independent of the warping groups mentioned previously. For the PCA, the assumption of data linearity was confirmed and the volumes were standardized by a *z* transformation to achieve a clearer differentiated visualization of the grouping result.

Spot volumes were compared between FW and BW snails using a Welch t-test. The FW group contained all samples from snails collected at sites S1 and S2, and the BW group contained the samples from snails collected at sites S5 and S6. For the statistical analysis the mean spot volume of the respective samples was determined for each spot. S3 snails were excluded from this ecotype comparison because they showed an exceptional grouping in this study and have shown this for several other parameters (Wiesenthal et al., 2018, 2019).

All statistical analyses were carried out with R 3.3.2. Images were generated with the software Delta 2D (Decodon, Greifswald, Germany). Any additional editing of images (i.e., addition of circles around groupings or spots) was carried out with the software Adobe Photoshop CS4 Version 11.0.2 and Microsoft Power Point.

2.5 | Mass spectrometry analysis and homology searches

There were 43 spots of interest that showed the greatest difference between FW and BW snails or were of interest to be actively or passively involved in the higher osmotic tolerance range of BW snails (Wiesenthal et al., 2018). These were picked from 2 SDS-PAGEs with the Ettan spot picker (AmershamTM Pharmacia Biotech, Amersham, UK), with a picker head of 2 mm diameter, and transferred into 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). Peptides were extracted from picked spots by an automated *in gel* trypsin digestion with the Ettan Spot Handling workstation using a standard protocol previously described (Eymann et al., 2004; Wolf et al., 2008). Depending on the size and intensity of the spot, digestion was carried out with a 1:5 or 1:10 trypsin dilution. The mass spectrometric analysis was done using an AB SCIEX TOF/TOF™ 5800 Analyzer (AB Sciex/MDS Analytical Technologies, USA) and previously described parameters (Wolf et al., 2008). The MS and MS/MS spectra that were obtained via the 4,000 Explorer Software V3.5.3 (Applied Biosystems, Waltham, MA, USA) were matched to a transcriptome database for *Theodoxus fluviatilis* (GenBank, accession number: PRJNA750758) using the MASCOT Search Engine Version 2.4.1 (Matrix Science Ltd, London, UK) and the MASCOT MS/MS databank search function (details on the *Theodoxus fluviatilis* transcriptome database generation can be found in Appendix S1). The amino acid sequences derived from identified expressed sequence tags were then blasted to the NCBI database using default settings, but restricted to molluscs, in the search for annotated sequence homologs. Within the top 50 hits of the BLAST, annotated sequences were considered likely sequence homologs. To validate these considered BLAST hits, pI values and molecular sizes of the respective proteins were compared with the corresponding spot positions on the gel.

2.6 | Associated data

Transcriptome data have been deposited in the NCBI SRA database, bioproject accession number PRJNA750758. Mass spectrometry data are available in the supporting information of this article.

3 | RESULTS

Despite variances within the collection sites, the PCA analysis of the spot patterns from snails under control conditions resulted in a clear grouping of snails from the FW collection sites S1 and S2 and the BW collection sites S5 and S6. Interestingly, the spot pattern of snails from the FW collection site S3 grouped with those of the BW snails (Figure 1). These groupings are distinct, despite the first two principal components only explaining 33% of the variance (PC1 = 19%; PC2 = 14%) and thus do not clearly distinguish among all five collection sites. Nonetheless, protein expression patterns of S1 and S2 snails are evidently different from those of S5 and S6 snails, and represented the FW and BW ecotypes of snails, respectively, which is why these four collection sites were used as representatives of FW and BW snails for further analyses.

A total of 222 spots were detected and included in the spot volume analysis. Although a large number (81) of spots appeared different in their expression between FW and BW snails (which perhaps corresponded to different biological effects), only 49 of these spots were statistically significantly different between the two groups of snails. There were eight spots that visually did not appear different between FW and BW snails but were in fact statistically different. This led to a total of 89 spots that were analyzed in more depth. In Table S2, the results of the MS output of the analyzed spots with significant GenBank hits are listed.

Out of the 222 analyzed spots, 19.8% were downregulated, and 20.2% were upregulated in BW snails compared with FW snails, whereas 59.9% remained unchanged. Of the 27 spots that showed the greatest difference between FW and BW snails, those that represented proteins that may be actively or passively responsible for the difference in osmotolerance between FW and BW snails were of special interest (Wiesenthal et al., 2019). These proteins were identified by mass spectrometry analysis and sequence matching, and belonged to different functional groups (Table 1). FW snails had higher expression levels of proteins involved in intermediate metabolism and protein degradation, whereas BW snails showed increased expression levels of proteins involved in protein synthesis, avoidance of oxidative stress, and organization of the cytoskeleton (Table 1). All proteins listed in Table 1 were statistically significantly different in spot volumes between FW and BW animals, with only four exceptions: spots V5-315 ($p = 0.101$), V5-363 ($p = 0.068$), V5-383 ($p = 0.129$), and V5-397 ($p = 0.092$). Although not statistically significant, these spots showed an increase of 52% (V5-315) or decreases between 22% (V5-397) and 48% (V5-383), respectively,

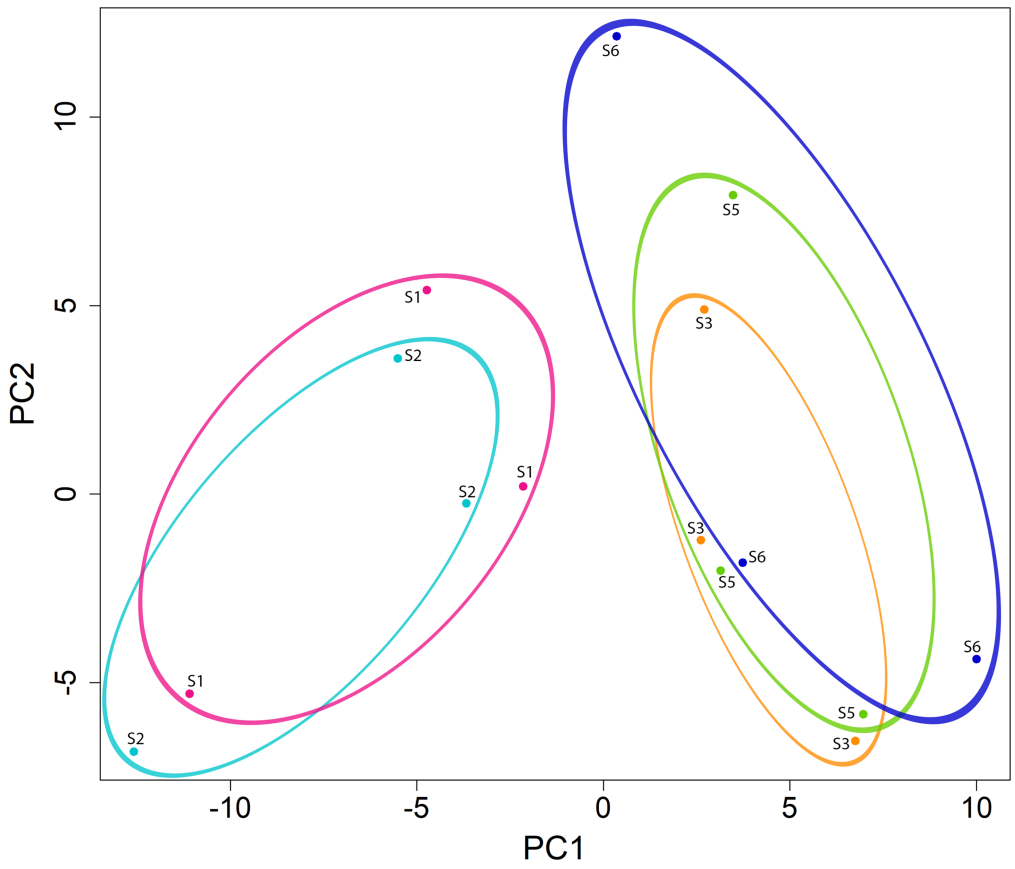

FIGURE 1 Principal component analysis (PCA) of protein expression patterns in the snail *Theodoxus fluviatilis* under control conditions, grouped by collection site. The *x* and *y* axes represent the first (PC1) and second (PC2) principal components, respectively. Samples of the same collection site are circled in either pink (S1, FW), turquoise (S2, FW), orange (S3, FW), green (S5, BW), or blue (S6, BW). FW, freshwater; BW, brackish water

TABLE 1 Identified and functionally grouped protein spots that showed expression differences between freshwater (FW) and brackish-water (BW) snails

Spot label	Protein name	Abundance	Fold difference in BW compared with FW snails
Stress response, heat shock proteins, chaperone function			
V5-361	Alpha-crystallin B chain-like **or** Heat shock protein 20	++	1.72 (↑)
V5-363	Heat shock cognate 71 kDa protein	+	0.76 (↓)
V5-357	Annexin A7-like	++	1.46 (↑)
V5-255	Retinal dehydrogenase	+	1.73 (↑)
Protein degradation, digestion, nutrient transport			
V5-79	Putative aminopeptidase W07G4.4	+	0.27 (↓)
Cytoskeleton			
V5-243	UDP-N-acetylhexosamine pyrophosphorylase-like **or** MACPF domain-containing protein **or** Coronin-1A-, B- **or** C-like	+	0.39 (↓)
V5-311	Transgelin-3-like protein (TAGLN3) **or** Rac guanine nucleotide exchange factor B-like	++	2.09 (↑)
V5-369	Gelsolin-like protein 2 **or** Severin	++	3.57 (↑)
V5-315	Coronin-1B-like isoform X2 **or** Coronin-1C-like isoform X2	+	1.51 (↑)
V5-357	Annexin A7-like	++	1.46 (↑)
V5-386	Rac guanine nucleotide exchange factor B-like **or** Transgelin-2-like **or** Transgelin-3-like protein (TAGLN3)	+++	1.79 (↑)
V5-383	Filament-like protein-2 **or** 70 kDa neurofilament protein (NF70)	+	0.68 (↓)
V5-403/V5-52	Lamin-L(I)-like **or** Lamin-B1-like **or** Lamin Dm0-like	+++/+	0.75/0.70 (↓)
Intermediate metabolism			
V5-393/V5-405	Phosphoenolpyruvate carboxykinase [GTP]-like	+/++	0.41/0.50 (↓)
V5-397	Arginine kinase	+++	0.82 (↓)
Protein synthesis			
V5-199	Glycine-rich RNA-binding protein GRP2A-like	+	1.83 (↑)
V5-377	Protein disulfide-isomerase A3	++	3.16 (↑)
Avoidance of oxidative stress			
V5-242	Cu/Zn super oxide dismutase	+	1.55 (↑)
V5-273	Peroxiredoxin-6-like	+	2.26 (↑)

Note: Abundance indicates the relative spot volumes and thus expression quantities of the respective proteins: +, low; ++, middle; +++, high. The fold difference is additionally visualized by arrows indicating whether proteins were upregulated (↑) or downregulated (↓) in BW snails compared with FW ones.

which could indicate biologically relevant changes in protein expression.

4 | DISCUSSION

Populations of the euryhaline snail *Theodoxus fluviatilis* inhabit water bodies with very different salinities (FW and BW ecotypes) despite belonging to the same species. They also show great variability in morphology, fecundity, tolerance to a range of stressful salinities, and different underlying metabolic mechanisms when coping with these stressful environmental conditions (Bunje, 2005; Claparede, 1857; Glöer & Pešić, 2015; Kangas & Skoog, 1978; Neumann, 1960; Poul Bondesen, 1940; Symanowski & Hildebrandt, 2010; Wiesenthal et al., 2018, 2019; Zettler et al., 2004). Whether these parameters can be used to clearly distinguish between the two ecotypes has been discussed in the literature, but thus far there has not been a conclusive decision. The main reason lies within the high variability of the FW group that are only distinguishable from BW animals when collection sites are analyzed individually and not grouped by ecotype (Wiesenthal et al., 2018). As shown in Figure 1, animals from the FW collection site S3 grouped closer to BW snails than to their geographically close FW neighbors from S1 and S2 with respect to shell size, tolerance of hyperosmotic

conditions, and mechanism of accumulating organic osmolytes as means to cope with hyperosmotic stress (Wiesenthal et al., 2018, 2019). Moreover, analysis of expressed sequence tags in FW or BW animals revealed clear differences in single nucleotide polymorphism (SNP) patterns in animals of the two ecotypes, with S3 animals grouping much closer to S5 or S6 animals than to their FW neighbors S1 and S2 (Wiesenthal, Hildebrandt, Müller, unpubl. data). This indicates that S3 animals may be more closely related to snails collected from the Baltic Sea and may have been transferred into the Carwitzer See from a brackish water habitat by means of birds or human activities. For this reason, we excluded the S3 animals from the study of comparative proteomics to focus on the clearly distinguishable FW- and BW-ecotypes.

Despite being genetically adapted, physiologically adjusted, and easily able to cope with the salinities in their environments, FW and BW snails need to osmoregulate to maintain the higher osmotic concentration of their body fluids with respect to the environmental medium (Symanowski & Hildebrandt, 2010). Since this gradient is even higher in FW snails compared with BW snails, we expected to find proteins related to energy metabolism, intermediate metabolism, and salt–water homeostasis expressed at higher levels in FW than in BW animals.

Snails of FW and BW ecotypes expressed proteins related to general stress responses, yet neither one of the groups did so in high quantities. The expression of stress proteins in both groups might be caused by a lack of food during the 48-h experimental period, by being held under laboratory conditions which do not perfectly match the conditions in their natural habitats, or by changes in the circadian rhythm, to name a few. These circumstances might explain the expression of stress proteins better than the difference in environmental salinities, considering that the animals of both groups should be acclimated to their respective environmental conditions. And yet, α-crystallin B chain-like heat shock protein 20, annexin A7, retinal dehydrogenase, superoxide dismutase, and peroxiredoxin-6, which are generally associated with stress responses in eukaryotic organisms (Freire et al., 2011; Moreira et al., 2018; Rivera-Ingraham et al., 2016; Tomanek, 2015; Wan et al., 2012), showed higher expression levels in BW than in FW animals (Table 1). Because BW animals have only minor osmotic gradients between body fluids and environment, it is unlikely that expression of these proteins is related to chronic osmotic stress. However, it seems possible that BW animals are generally better prepared for coping with environmental stress and thus express higher levels of protective proteins because their habitat generally undergoes more rapid and severe changes in abiotic parameters than that of FW animals. In *Anadara kagoshimensis* (identified as *Scapharca subcrenata*) heat shock protein 20 was found to be upregulated when exposed to a sudden drop in salinity as means for osmotic volume regulation (Mo et al., 2020), whereas in *Mytilus trossulus* and *M. galloprovincialis* superoxide dismutase was upregulated during hypoosmotic stress to cope with increased ROS production (Tomanek, 2014). Other than inland freshwater lakes, the shallow water coastal zones of the Baltic Sea also undergo rapid changes in water temperature or salinity due to the acute weather conditions (sunshine, rain, wind). The same explanation might apply to the finding that cellular proteins associated with structuring, stabilization, or rearrangement of the cytoskeleton were expressed at higher levels in BW than in FW animals (Table 1). These proteins help in stabilizing cell shape and function under changing osmotic conditions, as seen in *M. trossulus* (Tomanek, 2014), in which individuals are tolerant of hypoosmotic conditions. Nonetheless, further studies are needed to fully understand the role of these proteins in animals that are exposed to osmotically changing environments.

Freshwater snails, however, expressed higher amounts (32% reduction in BW snails) of the heat shock cognate 71 kDa protein, also known as HSPA8. It functions as a chaperone to stabilize protein conformation through stress events, is involved in the correct folding of newly synthesized polypeptides, and accelerates degradation of misfolded proteins (Sopha et al., 2012; Stricher et al., 2013). Because these cognate proteins are constitutively expressed, FW snails may experience a chronic state of osmotic stress (Boutet et al., 2003) because they have to maintain an internal osmolality that is about 45 mOsmol/kg higher (2.7-fold) than that of their freshwater habitat (Symanowski & Hildebrandt, 2010).

Because the integument of *T. fluviatilis* is water permeable, the snails have developed mechanisms to avoid detrimental shrinking under hyperosmotic or swelling under hypoosmotic conditions. Like other gastropods, they adjust the internal osmolality to the external conditions by either accumulating organic osmolytes in their cells under hyperosmotic stress or by releasing them when exposed to hypoosmotic conditions in the environment (Hawkins & Hilbish, 1992; Pierce, 1982; Wiesenthal et al., 2019; Yancey, 2005). Despite being osmoconformers in salinities above 8‰, both ecogroups of *T. fluviatilis* hyper-regulate at lower external salinities (i.e., they maintain a higher internal osmolality compared to their environmental medium) (Symanowski & Hildebrandt, 2010). When exposed to hyperosmotic stress, these snails accumulate free amino acids as means to increase their internal osmolality (Wiesenthal et al., 2019). Whereas FW snails rely on the hydrolysis of proteins to acquire these organic osmolytes, BW snails newly synthesize free amino acids (Wiesenthal et al., 2019). These conditions are mirrored by the patterns of protein expression in animals of the two ecotypes. Whereas FW snails show higher expression levels in an enzyme of the protein degrading machinery (putative aminopeptidase W07G4.4), BW snails have higher expression levels of proteins that are involved in protein synthesis such as the glycine-rich RNA-binding protein GRP2A-like or the disulfide-isomerase A3 (Table 1).

We did not find significant differences in the expression levels of regulatory proteins affecting salt–water homeostasis or of rate-limiting enzymes in energy metabolism. However, two enzymes related to energy metabolism and internal energy storage had a higher expression level in FW than in BW animals (Table 1). Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting key enzyme in gluconeogenesis (Matte et al., 1997), and arginine kinase mediates energy storage by elevating the cellular level of phosphoarginine, a phosphagen that supports fast ATP regeneration (Ellington, 2001). Expressing such enzymes at higher levels in their cells might prepare the FW

animals for higher basal rates of energy metabolism. Supporting this idea is a study in which protein changes associated with increased energy demand were detected in *M. galloprovincialis*, which is less tolerant of hyposaline stress, but not detected in the more tolerant *M. trossulus* (Tomanek et al., 2012).

In summary, snails of the BW and the FW ecotypes express slightly different patterns of soluble proteins in their foot muscle tissues. The known molecular functions of these proteins seem to correspond with potential biological roles of such molecules in coping with particular habitats and especially with unexpected changes in environmental conditions. To fully understand the relevance of the identified proteins in this study in FW and BW animals, and to clarify their role in the respective osmoregulatory processes, further investigations are needed.

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CONFLICT OF INTEREST

None of the authors have a financial or commercial conflict of interest to declare.

AUTHOR CONTRIBUTIONS

A.A.W. and J.-P.H. conceived the ideas and designed methodology; A.A.W. prepared and measured the tissue samples; A.A.W. analyzed the data; D.A. carried out the MS measurements; A.A.W., J.-P.H., and D.A. drafted the manuscript. All authors contributed to the preparation of the final manuscript and gave approval for publication.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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