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ORIGINAL PAPER

Differences in osmotolerance in freshwater and brackish water populations of *Theodoxus fluviatilis* (Gastropoda: Neritidae) are associated with differential protein expression

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Abstract The euryhaline gastropod *Theodoxus fluviatilis* is found in northern Germany in freshwater or in brackish water habitats in the Baltic Sea. Previous studies have revealed that individuals from both habitats are not distinguishable by morphological characters or by sequence comparison of DNA encoding 16S RNA or cytochrome C. As reported in this study, animals collected in the two habitats differ substantially in their physiological ability to adapt to different salinities. Comparison of accumulation rates of ninhydrin-positive substances (NPS) in foot muscle upon transfer of animals to higher medium salinities revealed that brackish water animals were perfectly able to mobilize NPS, while freshwater animals had only limited ability to do so. In an attempt to explore whether this difference in physiology may be caused by genetic differentiation, we compared protein expression patterns of soluble foot muscle proteins using 2D gel electrophoresis and silver staining. Of the 40 consistently detected protein spots, 27 showed similar levels in protein expression in animals collected from freshwater or brackish water habitats, respectively. In 12 spots, however, protein concentration was higher in brackish water than in freshwater animals. In four of these spots, expression levels followed increases or decreases in medium salinities. In a different set of 4 of these 12 spots, protein levels were always higher in brackish water as compared to freshwater animals, regardless of their physiological situation (14 days in artificial pond water or in medium with a salinity of 16‰). The remaining 4 of the 12 spots had complex expression patterns. Protein levels of the remaining single spot were generally higher in freshwater animals than in brackish water animals. These expression patterns may indicate that freshwater and brackish water animals of *T. fluviatilis* belong to different locally adapted populations with subtle genetic differentiation.

Keywords Salinity adaptation · Physiological adaptation · Osmoregulation · Euryhalinity · Foot muscle · Protein expression · *Theodoxus fluviatilis* · Neritidae

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Introduction

Animal species with wide geographical distribution show regional differentiation in specific characters. Successful colonization of different habitats requires physiological acclimation and local adaptation and may result, over many generations, in genetic differentiation (Ravigné et al. 2009). *Theodoxus fluviatilis* (Gastropoda: Neritidae) may be an example of such a species. Molecular phylogenetic studies using 16S rRNA- and cytochrome C-sequence data of snails collected at various locations throughout Europe and adjacent areas of Asia have revealed that *T. fluviatilis* has formed regional subgroups (Bunje 2005; Bunje and Lindberg 2007). Individuals of one of these extant subgroups, however, may have dispersed from brackish water habitats at the Black Sea (Butenko 2001) to freshwater

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habitats throughout northern Europe. Members of this subgroup are now found in freshwater habitats throughout Germany and have re-entered brackish water habitats of the Baltic Sea, probably following the receding ice sheets during the Holocene (Bunje 2005). According to their different ecological niches, these groups were assigned to the subspecies *T. fluviatilis littoralis* and *T. fluviatilis euxinus*, respectively, although individuals of *T. fluviatilis* from freshwater and brackish water habitats in northern Germany are morphologically identical (Zettler et al. 2004) and are genetically uniform with respect to DNA fragments encoding portions of 16S rRNA or cytochrome C (Bunje 2005; Bunje and Lindberg 2007).

In their responses to salinity changes, however, snails from freshwater and brackish water locations do differ (Neumann 1960; Kangas and Skoog 1978). Individuals from both the groups were found to be moderately euryhaline, sustainedly tolerating salinities in the range of 1.5–10‰ (Kangas and Skoog 1978). Only individuals from freshwater populations showed prolonged survival in media less concentrated than 1.5‰, and only individuals from brackish water locations survived for long periods (up to 70 days) in media with salinities up to 15‰ (Kangas and Skoog 1978). This pronounced salinity tolerance enable individuals of the brackish water population of *T. fluviatilis* to thrive in areas of the Baltic Sea with salinities up to 18‰ (Jaeckel 1950), but never invade freshwater lakes and streams with connections to the Baltic Sea (Zettler et al. 2004). These authors state that animals of the freshwater population, in turn, do not migrate into brackish water habitats. Interestingly, Zettler et al. (2004) observed a zone between the two types of habitats that was not occupied by *T. fluviatilis* at all.

These observations indicate that individuals of both brackish water and freshwater populations may still belong to one species, but may have developed physiological and biochemical adaptations that may be based on subtle genetic differences. We reasoned that in contrast to slowly evolving and/or selectively neutral genetic markers, such as cytochrome C or rRNA (Bunje 2005; Bunje and Lindberg 2007), the expression of proteins involved in osmoregulation may show such subtle differences between individuals of both populations as, for instance, was observed in the European flounder from different habitats by Larsen et al. (2008).

Therefore, the aim of this study was to compare the osmoregulatory capacities of individuals of *T. fluviatilis* from freshwater populations in northern Germany with those of individuals of brackish water populations collected at the southern coast of the Baltic Sea in terms of their abilities to respond to hyperosmotic stress. We first explored whether and, if yes, to what extent acute increases in medium salinity lead to mobilization of low molecular weight osmolytes such as free amino acids (FAA) in the tissues, the mechanism of choice to maintain volume homeostasis in euryhaline invertebrates (Gilles and Jeuniaux 1979; Pierce 1982). We then compared the expression patterns of soluble proteins in foot muscle of individual snails from freshwater and brackish water populations using 2D-electrophoretic separation of soluble proteins and quantification of silver-stained proteins. We looked for differences in basal expression in the two groups of animals (adaptation) as well as expression changes upon transfer of animals from their original medium to medium with different salinities (acclimation). Mismatches in protein expression in the two populations would indicate a genetic basis of physiological differences.

Materials and methods

Animals

Snails (*T. fluviatilis*) were collected from brackish water (salinity 8–10‰, osmolality 176–226 mOsmol/kg H_2O, pH 7.8, temperature 15–17°C) at the southern shore of the Baltic Sea near Wismar, Germany (Breitling, eastern shoreline of Poel, 53°58′35″N, 11°28′14″E) or at the beach of Ludwigsburg in the east of Greifswald (54°07′11″N, 13°28′52″E), respectively. Animals were also collected at two freshwater locations, Liepsee next to Prillwitz (53°27′06″N, 13°08′30″E) or Oberuckersee next to Warnitz (53°11′06″N, 13°52′20″E), respectively. Medium osmolalities at the freshwater collection sites were between 20 and 28 mOsmol/kg H_2O and temperature was between 16 and 20°C. Animal collection took 5 days. Animals were brought into the laboratory in the original medium. Animals from the freshwater populations (referred to as "freshwater animals") were maintained in aerated artificial pond water (APW; 0.5 g/l sea salt (Sera Premium, Sera, Heinsberg, Germany) in deionized water; 0.5‰ salinity). Animals from the brackish water populations (referred to as "brackish water animals") were maintained in aerated artificial brackish water (ABW; 10 g/l sea salt in deionized water; 10‰ salinity). Animals were maintained at 20°C and a 12-h dark-light cycle for 1 week prior to the experiments. The animals were not fed 2 days before and during an experiment.

In preliminary experiments, we could not detect any differences in osmoregulatory parameters between the freshwater animals collected at the two different sites. The same applies to the animals from the two brackish water collection sites. Because the total number of animals that we were allowed to collect was limited, we did not discriminate between animals from the two freshwater sites or the two brackish water sites, respectively, in further experiments.

Chemicals and reagents

Reagents for the measurements of FAAs (ninhydrin reagent) were obtained from Sigma-Aldrich (Munich, Germany). Reagents for isoelectric focussing of proteins were obtained from Biorad (Munich, Germany). All other chemicals were reagent grade and obtained from Roth (Karlsruhe, Germany).

Experimental design

To avoid problems in subsequent experiments with animals which had not reached a new steady state after transfer in "foreign" media, we used animals collected at freshwater sites and determined the time course of changes in hemolymph osmolality during the exposure of animals to media with a salinity of 16‰ (382 mOsmol/kg H_2O). Groups of 30 animals from brackish water or from freshwater populations were put in containers containing 3 l of aerated saline (20°C) with different salt concentrations (salinities of 0.5, 1.6, 3.0, 3.2, 8, 9.5, 16, 24 or 32‰ corresponding to 25, 43, 84, 88, 192, 216, 382, 600 or 769 mOsmol/kg H_2O as measured by vapor pressure osmometry; Vapro 5520, Wescor Inc., Logan, UT, USA). For measurements of time courses of hemolymph osmolality, soft tissue weight or FAAs in foot tissue, individual animals were removed from the containers in groups of 5–12 per time point after different incubation times up to 24 h or after 14 days (protein analyses). Different analyses were performed on different groups of animals. Media were changed every other day for acclimation periods longer than 24 h.

Sampling of hemolymph and measurement of osmotic concentration

Animals were quickly fixed with the operculum pointing upward on a wooden board using plasticine and blotted dry with a Kleenex tissue. Before the animal could withdraw into its shell and close the operculum, a heat-drawn glass capillary (tip opening approximately 50 µm) filled with paraffin oil and connected to an oil-filled glass syringe was quickly and deeply inserted into the foot muscle. At least, 12–15 µl hemolymph was obtained from each animal. Osmolality of the body fluid (in mOsmol/kg H_2O) was immediately determined using a vapor pressure osmometer (Vapro 5520, Wescor Inc., Logan, UT, USA) calibrated with standard salt solutions provided by the manufacturer.

Determination of animal fresh weight

To determine the animal's fresh weight, accessible parts of living snails were completely blotted dry using Kleenex tissues, the shells were individually labeled and the animals weighed (total weight). Snails were killed in boiling water and the bodies removed from the shells. Shells were air-dried for several days and weighed (shell weight). The difference between the total weight and shell weight was considered to be the animal's fresh weight or soft tissue weight.

Extraction and analysis of free amino acids

Foot muscle tissue was prepared by the removal of the operculum using forceps, peeling the animal's body out of the shell using a needle and cutting the foot from the rest of the animal's body using a scalpel under the stereo microscope. Tissue samples of individual animals were quickly blotted dry on Kleenex tissue, weighed and frozen in liquid nitrogen. Each tissue sample was homogenized on ice for 3× 30 s in 500 µl deionized water using a T8-Ultraturrax (IKA Labortechnik, Staufen, Germany) on full speed. The homogenate was heated to 95°C for 5 min to denature any enzymatic activity in the sample and centrifuged at 13,000 × *g* at 4°C to precipitate residual particulate material. The supernatants were transferred to Eppendorf tubes and stored on ice until use.

The content of FAA was determined using ninhydrin reagent and photometric quantitation of the colored reaction product (Ruhemann 1910). The assay kit (Sigma-Aldrich, Munich, Germany) was used according to the manufacturer's instructions. Standard curves were generated using different concentrations (0–10 mmol/l) of L-leucine in deionized water. Light absorption was measured at 570 nm using a DU530 spectrophotometer (Beckman Coulter, Wals, Austria). Data for ninhydrin-positive substances (NPS) in the samples were expressed in µmol/g fresh weight.

Extraction and 2D-analysis of soluble proteins in foot muscle

Individual samples were frozen in liquid nitrogen. Samples were mechanically pulverized in a porcelain mortar. The tissue powder was transferred into a homogenizing tube containing 1.5 ml lysis buffer solution (7 mol/l urea, 2 mol/l thiourea, 1% dithiothreitol, 2% CHAPS, 0.8% Biolyte pH 3–10, 5 mmol/l Pefablock). The samples were homogenized on ice for 30 s using a T8-Ultraturrax (IKA Labortechnik, Staufen, Germany) on full speed, cooled on ice for 3 min, and then centrifuged at 13,000 × *g* at 4°C to precipitate residual particulate material. The supernatant was stored in aliquots at −80°C until used. Total protein concentration was measured (Bradford 1976) in the remaining supernatant using bovine serum albumin (BSA), dissolved in lysis buffer solution, as a standard.

For the first dimension (isoelectric focussing) of protein separation, approximately 115 µg of protein was mixed with 330 µl rehydration buffer (9 mol/l urea, 30 mmol/l

CHAPS, 50 mmol/l dithiothreitol, 0.2% Biolyte pH3-10) and filled in one slot of a focussing tray. An IPG strip (pH 5-8, Biorad, Munich, Germany) was inserted into the slot and covered with 750 µl of mineral oil. Upon mounting the focussing tray into the focussing cell (IEF, Biorad, Munich, Germany), rehydration was performed for 12 h at 20°C and 40 V before the voltage was raised to 200 V during 1 h, then to 500 V for 1 h and finally to 10,000 V for 2.5 h. Strips containing the focussed proteins were stored at 4°C until processed in the second dimension.

In preparation for the separation of the focussed proteins according to their molecular masses, the strips were incubated in equilibration solution (6 mol/l urea, 30% glycerol, 2% sodium dodecyl sulfate, 1.65 mmol/l Tris, pH 8.8) for 10 min at room temperature, then transferred to another slot containing 325 mmol/l iodoacetamide in equilibration solution for another 10 min and finally incubated for 3 min in SDS sample buffer solution (50 mmol/l Tris, 1% sodium dodecyl sulfate, 7 mmol/l bromophenol blue, 4% mercaptoethanol and 40% glycerol, pH 6.8). Strips were then placed on top of the stacking gel in a water-cooled Protean IIxi (Biorad, Munich, Germany) electrophoresis cell. For the first 30 min, the cell was run at a constant current of 20 mA followed by 4 h of protein separation at a voltage of 300 V.

Protein spots in the gels were silver stained (Blum et al. 1987). Stained gels were scanned on a Quato digital scanner (Quatographic, Braunschweig, Germany) and qualitatively and quantitatively analyzed using Phoretix 2D analysis software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). Individual spot densities were normalized to the mean density measured across the entire series of spots to correct for differences in staining intensity.

Data presentation and statistics

Data of hemolymph osmolality, soft tissue weight, FAA concentration or normalized protein abundances are expressed as mean ± SD throughout the text and in the graphs. Two-way ANOVA was used to analyze expression levels of individual proteins in foot muscle preparations, before individual comparisons were made using one-way ANOVA. Individual means were routinely tested for significant differences to the appropriate controls using Student's *t* test or the Mann–Whitney rank test (in cases of uneven standard deviations as detected by the *F* test). Significant differences of means were assumed at $P < 0.05$.

Results

The time course of changes in body fluid osmolality upon exposure of freshwater animals to different salinities is indicated by the data shown in Fig. 1a. The initial hemolymph osmolality of 75.7 ± 12.6 mOsmol/kg H_2O in the freshwater animals increased rapidly upon transfer of the animals to brackish water (salinity of 16‰, 382 mOsmol/kg H_2O) and reached the osmolality of the medium at 5 h. No differences between the slightly hyperosmotic values of the hemolymph were observed between 8 and 24 h upon transfer of the animals. Therefore, an exposure period of 24 h was deemed sufficient to reach a new osmotic steady state upon changes of medium salinity. Within 2 h upon transfer of freshwater animals to medium with a salinity of 16‰, soft tissue body weight decreased by approximately 30% and remained unchanged up to 24 h (Fig. 1b).

Animals of both the freshwater and the brackish water populations regulated their body fluids at or slightly above the iso-osmotic line when transferred to different medium salinities (0.5–16‰) (Fig. 2), indicating the tendency in both the groups of animals to osmoconformity. While the animals from brackish water (Fig. 2b) tolerated media with a minimal salinity of 0.5‰ (25 mOsmol/kg H_2O) equally well as media with 32‰ salinity (769 mOsmol/kg H_2O) (all animals had their operculi open and were actively

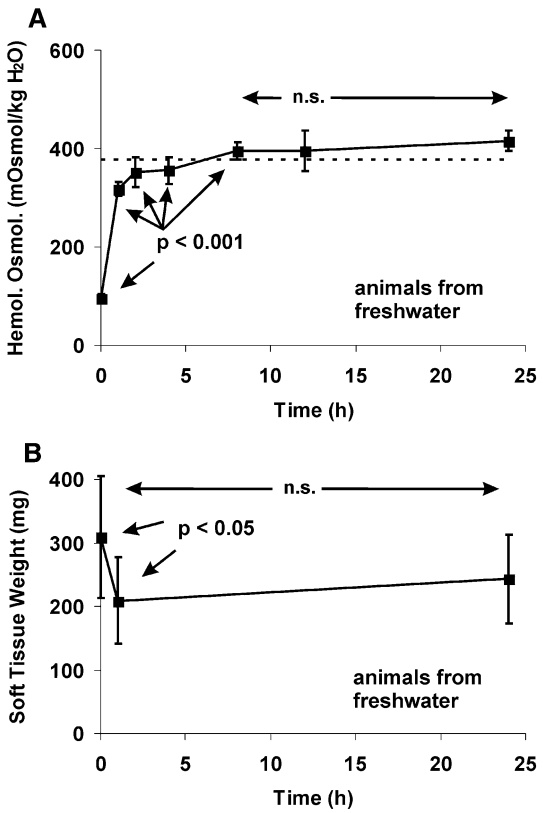

Fig. 1 Changes in hemolymph osmolality (mOsmol/kg H_2O) in *Theodoxus fluviatilis* (freshwater population) (**a** $n = 10$) or in soft body weight (**b** $n = 6$) during exposure of animals to a medium with a salinity of 16‰ (382 mOsmol/kg H_2O). *n.s.*—Differences of mean between *arrows* are statistically not significant (ANOVA)

moving around in their containers), animals of the freshwater population tolerated media with a salinity up to 16‰ (382 mOsmol/kg H_2O), but did not well in media with higher salt concentrations (Fig. 2a). When transferred to containers containing medium with a salinity of 32‰, the animals kept their operculi closed for approximately 8 h before they started to move around. During this time, body fluid osmolality increased to values close to that of the external medium. However, the animals did not raise their hemolymph osmolality to the isoosmotic line during the incubation period (Fig. 2). Up to 10% of these animals died within 48 h. The data show that these animals were obviously not reaching a new steady state within an incubation period of 24 h.

When freshwater animals were transferred from APW to medium with a salinity of 16‰ (382 mOsmol/kg H_2O), they lost approximately one-third of their soft tissue weight within 2 h (Fig. 1b), while shell weight remained constant (data not shown). Between 2 and 24 h after the transfer, however, these animals seemed to be able to limit further osmotic water loss, because their soft tissue weight did not decrease any further.

An established mechanism of maintaining body fluid homeostasis in marine invertebrates under hyperosmotic stress is the mobilization of amino acids from storage proteins (Gilles and Jeuniaux 1979; Pierce 1982). To test whether *T. fluviatilis* uses the same strategy, we measured the contents of NPS (as an approximation for osmotically relevant amino compounds) in freshwater animals at different times upon transfer from 0.5‰ (APW, 25 mOsmol/kg H_2O) to 16‰ (382 mOsmol/kg H_2O). Freshwater animals had a basal level of 14.3 ± 2.6 µmol/g fresh weight NPS in foot muscle tissue. Within 4 h after the transfer, NPS increased to levels well above twice the original concentration (31.1 ± 2.0 µmol/g fresh weight) and stabilized at that level between 4 and 12 h (Fig. 3).

Having established that *T. fluviatilis* accumulates NPS, we compared the performances of freshwater and brackish water animals when transferred to a wider range of salinities (between 0.5 and 32‰) for 24 h. As shown in Fig. 4, freshwater animals did accumulate NPS upon transfer to hyperosmotic media, but had a limited ability to do so. At the maximum medium salinity of 32‰ (769 mOsmol/kg H_2O), the accumulation of FAAs was even lower (25.6 ± 4.6 µmol/g fresh weight) than in animals transferred to medium with a salinity of 16‰ (382 mOsmol/kg H_2O). In additional experiments, individuals of freshwater animals were first acclimated to medium with a salinity of 16‰ for up to 10 days and then transferred to medium with a salinity of 32‰. Even in these animals, the concentration of NPS in foot tissue homogenates did not exceed 50 µmol/g fresh weight (data not shown). No such limitations were observed in animals collected at the brackish water locations. Our measurements in tissues of these animals revealed a linear relationship of medium salinity and NPS accumulation in the body fluids (Fig. 4b).

Such differences in physiology may be caused by genetic differences in animals of the freshwater or the brackish water populations. To address this question, we used a screening approach to detect possible differences in protein expression in foot muscle in individuals of the two populations using 2D gel electrophoresis of soluble proteins and silver staining. We also set out to differentiate between protein expression which was due to physiological

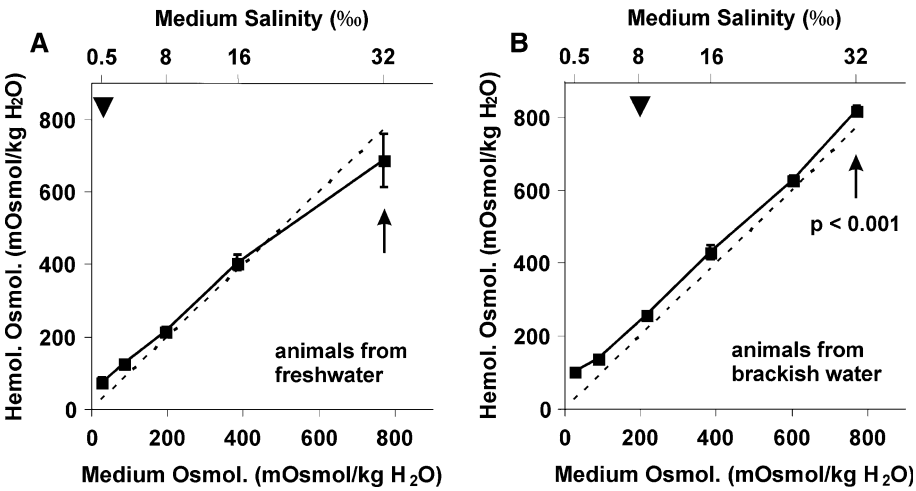

Fig. 2 Hemolymph osmolality (mOsmol/kg H_2O) in *Theodoxus fluviatilis* (**a** freshwater population, **b** brackish water population) upon 24 h exposure of animals to media with different salinities in the range of 0.5–32‰ (25–769 mOsmol/kg H_2O) (n = 6–10 each). The *broken line* is the line of isoosmolality of external media and hemolymph. *Arrowheads* mark the osmolalities of the original habitats of the animals. Differences of mean of data points marked by *arrows* (in **a**, **b**) are statistically significant, $P < 0.001$ (Mann–Whitney)

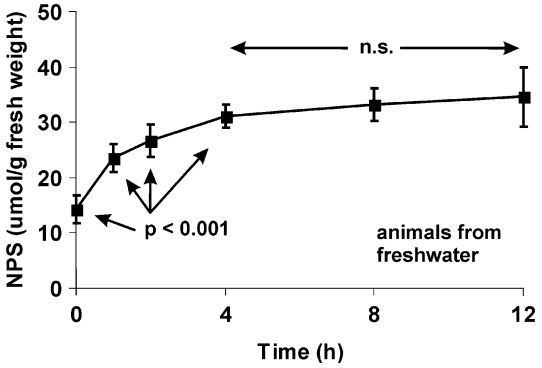

Fig. 3 Changes in ninhydrin-positive substances (*NPS*, mostly free amino acids) in foot muscle homogenates of *Theodoxus fluviatilis* (freshwater population) during exposure of animals to a medium with a salinity of 16‰ (382 mOsmol/kg H_2O) ($n = 5$). *n.s.*—Differences of mean between *arrows* are statistically not significant (ANOVA)

acclimation of animals to their respective media and protein expression which may have been genetically fixed. We transferred freshwater animals to medium with a salinity of 16‰ (382 mOsmol/kg H_2O) or brackish water animals to APW (0.5‰, 25 mOsmol/kg H_2O) for 14 days and analyzed foot muscle protein expression.

Approximately, 40 protein spots were consistently detected on 2D gels prepared using samples from freshwater animals or in those prepared using samples from brackish water animals (Fig. 5). These spots were matched and labeled in all individual gels, and the protein amounts in the spots were quantified using densitometry. For 27 of these spots, no differences in protein abundance were found in samples of freshwater or brackish water animals (Table 1). However, in 12 spots (Fig. 6, all spots except no. 36), protein amounts were higher in samples from brackish water animals ("bw") as compared to those from freshwater animals ("fw"). In 7 of these 12 cases, the respective proteins were not detected in gels obtained using samples of freshwater animals. Only one spot (no. 36) was detected in samples of freshwater animals, but not in samples obtained from brackish water animals.

As shown in Fig. 6, spot intensities in samples of freshwater animals ("fw") and samples of freshwater animals acclimated to 16‰ salinity for 14 days ("fw to bw") revealed 4 spots (no. 6, 25, 26 and 35) which indicated the presence of higher amounts of protein in brackish water-acclimated freshwater animals ("fw to bw") compared with freshwater water animals in APW ("fw") (Fig. 6, panel a). Moreover, the spot intensity in brackish water-acclimated freshwater animals ("fw to bw") resembled that in brackish water animals maintained in their original medium ("bw"). When brackish water animals which had been kept in medium with a salinity of 16‰ were acclimated to APW for 14 days ("bw to fw"), proteins in these four spots were as low as observed in samples of freshwater animals in APW ("fw"). We conclude that the proteins represented by the spots 6, 25, 26 and 35 were upregulated in animals exposed to media with higher salinities and downregulated in animals exposed to freshwater, regardless whether the animals belong to the freshwater population or to the brackish water population.

In five other cases (no. 4, 18, 23, 32 and 36), the regulation of protein abundance differed. Figure 6 (panel b) shows that protein expression correlated well with the animal's original habitat (freshwater population, brackish water population), but not with the incubation conditions. Protein abundance in spots 4, 18, 23 and 32 was higher in brackish than in freshwater animals regardless of their physiological situation [transfer of freshwater animals from 0.5 to 16‰ ("fw to bw") or of brackish water animals from 16 to 0.5‰ ("bw to fw") for 24 h]. In spot 36, protein abundance was lower in samples of brackish water animals when compared with those of freshwater animals and was independent of the incubation condition of the animals.

The regulation of proteins represented by the spots 5, 15, 22 and 30 was more complex. All four proteins were more

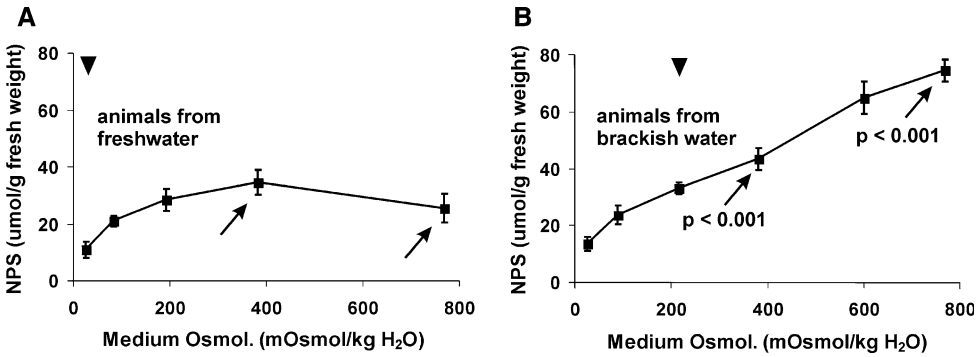

Fig. 4 Ninhydrin-positive substances (*NPS*) in foot muscle homogenates of *Theodoxus fluviatilis* (**a** freshwater population, **b** brackish water population) after 24 h exposure of animals to media of different salinities in the range of 0.5 and 32‰ (25–769 mOsmol/kg H_2O) ($n = 5$, each). The *arrowheads* point to the respective osmolality values of the original media of the experimental animals. Differences of mean of the respective data points marked by *arrows* (in **a** and **b**) are statistically significant, $P < 0.001$ (Mann–Whitney)

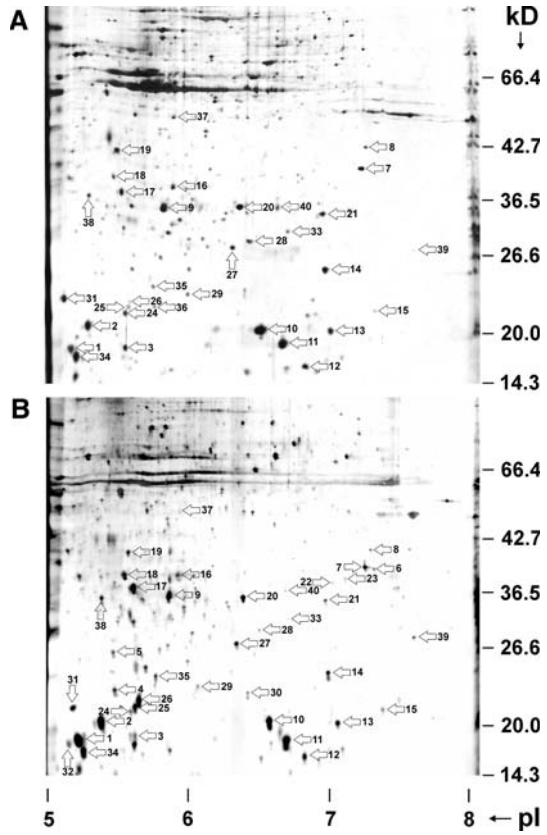

Fig. 5 Reference 2D-gels of soluble proteins extracted from foot muscle tissue of individuals of *Theodoxus fluviatilis* (**a** freshwater population, **b** brackish water population). Protein spots which were consistently detected in all gels prepared with samples of freshwater animals or protein spots which were consistently detected in all gels prepared with samples from brackish water animals or in both are labeled and numbered. The *scales on the right* indicate the positions of molecular mass standards (kDa), the *scale at the bottom* indicates isoelectric points (*pI*)

Table 1 Normalized protein spot intensities (correspond to relative amounts of protein expression) of constitutively expressed proteins (no significant differences between freshwater and brackish water animals) in foot muscle of *Theodoxus fluviatilis* ($n = 6$ each)

Spot no.	Freshwater animals		Brackish water animals	
	Mean	SD	Mean	SD
1	12.1	1.6	16.1	5.7
2	12.6	1.6	15.8	6.0
3	13.9	0.9	11.2	3.0
7	11.5	1.4	12.5	4.1
8	8.5	2.3	7.5	2.2
9	12.7	1.4	15.0	5.7
10	14.8	0.8	15.4	5.7
11	14.0	1.4	15.3	6.2
12	12.3	1.2	12.8	4.9
13	11.7	1.6	13.4	5.1
14	12.2	1.1	12.5	5.1
16	7.6	2.8	12.0	4.2
17	12.4	1.5	15.4	6.0
19	11.7	1.7	11.2	4.2
20	13.1	1.6	14.7	5.9
21	9.8	2.9	12.1	3.8
24	10.7	1.5	9.2	2.2
27	9.9	1.3	12.1	5.0
28	8.8	1.2	8.1	2.9
29	5.7	1.3	7.2	1.9
31	14.5	1.4	13.7	5.0
33	4.2	2.0	5.8	2.8
34	14.8	1.0	15.5	5.9
37	5.8	3.0	4.2	3.3
38	9.8	1.6	11.2	4.3
39	6.1	3.7	7.4	2.9
40	5.6	3.9	9.3	3.8

abundant in brackish water animals than in freshwater animals (Fig. 6, panel c). However, the amounts of three proteins (spots 5, 22 and 30) increased in freshwater animals exposed to brackish water ("fw to bw") but did not decrease upon transfer of brackish water animals to APW ("bw to fw"). Finally, in contrast to the substantial changes in protein amounts in spots 5, 22 and 30, the protein represented by spot 15 had low expression levels in animals which had been transferred to different salinities, irrespective of the animal's origins. These results indicate that the regulation of protein abundance in proteins represented by the spots 5, 15, 22 and 30 may be rather complex.

Discussion

Our results confirm previous reports (Neumann 1960; Kangas and Skoog 1978) that *T. fluviatilis* is euryhaline and osmotolerant, at least in the range of external salinities of 0.5 and 16‰. However, osmotolerance is more pronounced in animals of the brackish water population than in animals of the freshwater population (Fig. 2). Animals of the freshwater populations that were exposed to sea water (32‰, 769 mOsmol/kg H_2O) remained inactive, did not open their operculi, and did not reach a new steady state after 24 h in sea water (Fig. 2). Approximately, 10% of these animals died within 24 h. However, animals collected at brackish water locations and transferred into sea water (32‰ salinity) in the laboratorium, opened their operculi within 2 h after the transfer and started to move around. These animals survived such a treatment for much more than 2 days and were still alive after 3 months.

The relative loss of soft tissue weight (approximately 30%) within the first 2 h upon transfer of freshwater animals to medium with a salinity of 16‰ (Fig. 1b) was

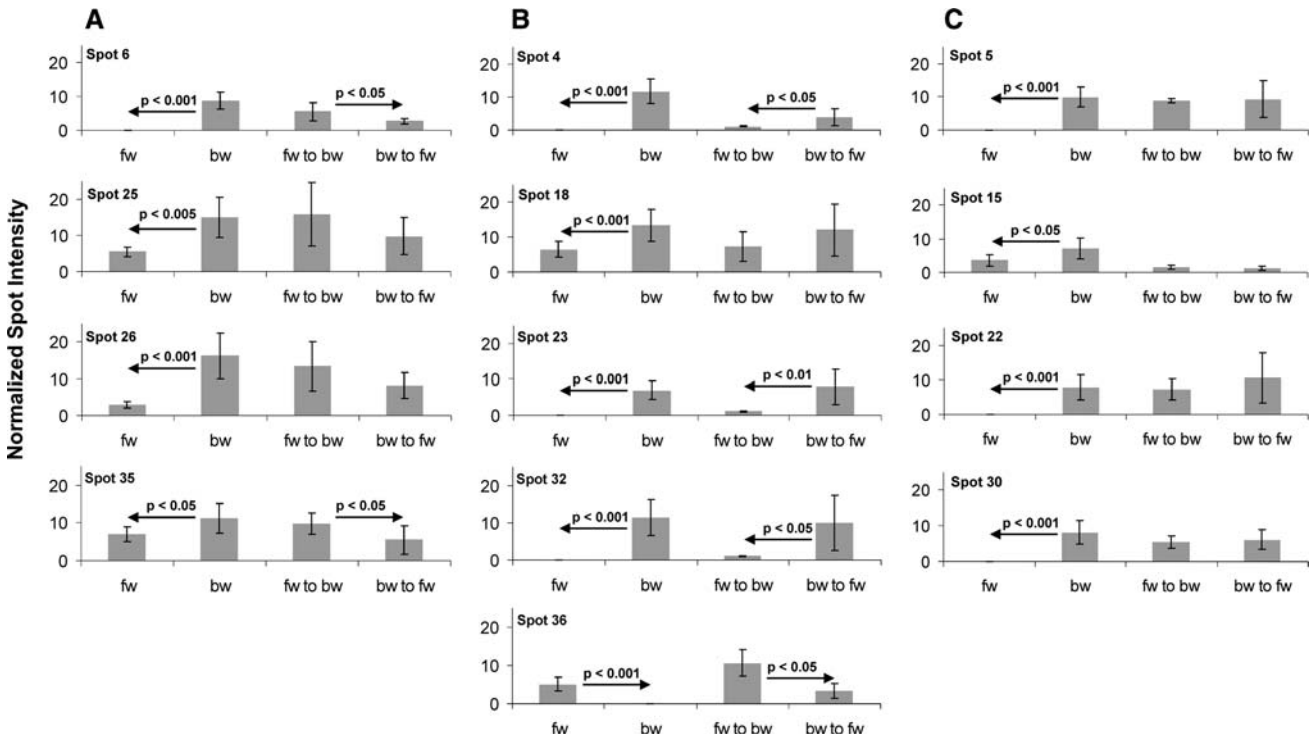

Fig. 6 Relative amounts of protein expression in foot muscle of *Theodoxus fluviatilis* (*fw* freshwater population, *bw* brackish water population, *fw to bw* freshwater animals acclimated to 16‰ salinity (382 mOsmol/kg H_2O) for 14 days, *bw to fw* brackish water animals acclimated to APW (25 mOsmol/kg H_2O) for 14 days). **a** Proteins regulated according to the physiological condition of the animals. **b** Proteins expressed according to the origin of the animals (freshwater or brackish water) and not affected by the physiological condition of the animals. **c** Proteins with complex expression patterns, $n = 6$, each. Significant differences of mean (ANOVA) are indicated

smaller than the relative increase in hemolymph osmolality (Fig. 1a), which indicates that animals of the freshwater populations are able to limit osmotic volume loss. Such a response to hyperosmotic stress may depend on the ability to accumulate low molecular weight osmolytes in the body fluids (Gilles and Jeuniaux 1979; Pierce 1982).

We tested the hypothesis that the difference in osmotolerance in the two groups results from different capabilities of accumulating organic osmolytes in the body fluids. While both, animals from freshwater and brackish water populations, do accumulate organic osmolytes (Fig. 4), this ability is far more pronounced in the brackish water animals. The NPS concentration in foot muscle tissue in freshwater animals was only half of that found in brackish water animals where it reached 70 µmol/g fresh weight upon transfer of the animals to sea water (32‰). Similar levels of NPS accumulation were generally found in estuarine molluscs transferred to hyperosmotic media, e.g., in *Rangia cuneata* transferred to medium with a salinity of 20‰ (Henry et al. 1980) or in *Villorita cyprinoides* exposed to 10‰ salinity (Vinu Chandran and Damodaran 2000).

If it is true that the brackish water population of *T. fluviatilis* of the Baltic Sea is indeed evolutionarily derived from limnic ancestors in northern Germany as proposed by Bunje (2005), these animals must have regained or retained the ability of quickly generating organic osmolytes under hyperosmotic stress. This conclusion is justified, since animals of the freshwater populations which were acclimated to medium with a salinity of 16‰ for 10 days were still not able to accumulate NPS beyond a level of approximately 50 µmol/g fresh weight upon transfer to sea water with a salinity of 32‰ (data not shown).

These data indicate that the difference in the ability to generate osmolytes may not solely be due to physiological adaptation, but may have a genetic basis and represent local adaptations (greater fitness of individuals in their local habitats due to natural selection). Earlier work has failed to show genetic differences between individuals of the freshwater population and those of the brackish water population comparing 16S RNA- and cytochrome C sequences (Bunje 2005; Bunje and Lindberg 2007). We reasoned that coding DNAs for 16S RNA or cytochrome C may have been much more conserved in evolution than regulatory or housekeeping proteins involved in salt and water balance and that such difference may show up when comparing protein expression patterns in animals of the freshwater and brackish water populations under osmotic stress as, for instance, observed in different locally adapted populations of the European flounder (Larsen et al. 2008).

Most of the soluble proteins in *T. fluviatilis* foot muscle (27 of 40) were equally present in freshwater and brackish water animals (Table 1); such a high degree of coincidence indicates that both groups of snails are indeed closely related. In 13 protein spots, however, protein expression between the two *Theodoxus* populations differed (Table 2). Four proteins had higher expression levels in individuals exposed to brackish water and lower in animals maintained in APW independently of the origin of the animals (Fig. 6, panel a) indicating that the expression levels of these proteins are physiologically regulated by the osmotic status of the animal and not genetically fixed. It would be interesting to know the identity of these proteins to study their role in osmotic acclimation. Owing to limitations in database information, we did not attempt to identify the relevant proteins in the 2D gels. *Theodoxus* is considered to belong to a basal group of gastropods (Neritidae) and is evolutionarily very distant to other gastropods studied more intensely (Colgan et al. 2003; Ponder and Lindberg 1997).

The expression pattern of five proteins, however, was clearly associated with the origin of the animals (freshwater or brackish water) and not with any of the physiological conditions the animals were subjected to (Fig. 6, panel b) pointing to population-specific genetic determinants. We only inspected protein expression, and we cannot generally exclude the possibility that we have missed post-translationally or proteolytically modified forms of individual proteins; such proteins would also run at different positions in the 2D gels, but would not be related to genetic differences in the *Theodoxus* populations in freshwater or brackish water. However, it seems highly unlikely to us that such an explanation applies to all five proteins. Even if some of the observed changes in the expression of these proteins are not due to different genetic backgrounds in the respective genes, but instead, result from differences in splicing or protein processing, these mechanisms must per se be differently organized in animals from freshwater and brackish water populations. Again, this may point toward subtle genetic differences in animals from freshwater and brackish water populations.

The expression patterns of four other proteins were more complex (Fig. 6, panel c). In all cases, protein expression was higher in brackish than in freshwater animals. The abundance of three proteins was similar in brackish water and in freshwater animals exposed to ABW (16‰ salinity). At first glance, this expression pattern indicates a physiological mode of regulation. This conclusion, however, does not fully hold because the abundances of these proteins were exactly the same in brackish water animals acclimated to freshwater. Similarly, the protein represented by spot 15 was less abundant in animals acclimated to different salinities, regardless of their original habitat; such a mode of regulation of proteins points toward a combination of physiological and genetic determinants of protein expression or towards a combination of two or more relevant physiological determinants (e.g., osmotic condition and general stress).

Although genetic structuring in animal species has been described before, the evolutionary significance of these often minute genetic differences are still poorly understood. Only recently, systematic studies have been attempted to elucidate the adaptive relevance of low genetic differentiation among fish populations from different salinities (Larsen et al. 2008). Our results provide initial support for the hypothesis that freshwater and brackish water populations of *T. fluviatilis* may represent locally adapted subgroups of the same species. Whether there are any reproductive limitations associated with the observed subtle changes in osmotolerance, as indicated by snail-free zones between freshwater and brackish water habitats (Zettler et al. 2004), may be evaluated by performing breeding experiments over several generations.

Table 2 Estimated molecular mass (kDa) and pI (isoelectric point) of differentially expressed soluble foot muscle proteins of freshwater or brackish water *Theodoxus fluviatilis*

Spot no.	Molecular mass (kDa)	pI	Level of significance, *P*
4	24	5.5	<0.001
5	26.6	5.5	<0.001
6	39	7.3	<0.001
15	21	7.4	<0.05
18	38	5.6	<0.001
22	37	7	<0.001
23	38	7.1	<0.001
25	21.5	5.6	<0.005
26	22	5.7	<0.001
30	22.5	6.4	<0.001
32	18	5.2	<0.001
35	24	5.8	<0.005
36	21.5	5.8	<0.001

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