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Effect of salinity on spatial distribution and cell volume regulation in two sibling species of *Marenzelleria* (Polychaeta: Spionidae)

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ABSTRACT: *Marenzelleria* sibling species Types I and II are characteristically distributed within the salinity gradient of the Elbe estuary. For the first time F_1-hybrids between the 2 *Marenzelleria* species were found. Type I inhabits mainly the isoosmotic/hyperosmotic range, while Type II and the F_1-hybrids prevail in the hypoosmotic range. A sympatric occurrence could be inferred in salinity ranges from 3.3 to 12.3 ppt. *Marenzelleria* sibling species Type II has been found for the first time in the North Sea (Elbe estuary). The influence of salinity on the distribution of *Marenzelleria* spp. was examined by laboratory experiments. In a time-course experiment with salinity changes from 10 to 25 ppt and from 25 to 10 ppt, respectively, enzyme activities and free amino acids were determined. The activities of alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase were always higher in Type I than in Type II. A salinity increase from 10 to 25 ppt resulted in a faster increase of the concentrations of D,L-alanine, glycine, serine, glutamate and threonine in Type I. The biochemical results showed that the capacity of cell volume regulation could be involved in the different spatial distributions of the 2 sibling species.

KEY WORDS: *Marenzelleria* spp. · Sibling species · Hybrids · Cell volume regulation · Free amino acids · D-alanine · Enzyme activities · Allozyme electrophoresis

INTRODUCTION

Salinity is an important factor for the spatial distribution of animals living in brackish water (Kolding 1985, Arndt 1989), although factors such as food availability, substrate type, predation and water depth have to be taken into consideration as well (Webb et al. 1997). In this context, biological invasions are of particular interest. Successful colonisation mainly depends on the genotype of the immigrant, as well as on specific characteristics (e.g. salinity) of the new habitat.

If sibling species immigrate at roughly the same time, they should — according to evolutionary adaptation — access separate habitats (niches). However, when individuals of such sibling species occur in overlapping areas, they may possibly even hybridise, although hybridisation may not be inherent in their area of origin.

Abundant populations of *Marenzelleria* spp. were reported for the first time in Europe in the North Sea during the late 1970s (Elliott & Kingston 1987) and then in the Baltic Sea in 1985 (Bick & Burckhardt 1989). The most probable mode of immigration was via the ballast water of ships from the Atlantic coast of North America to Europe. Originally, it was assumed that a single non-indigenous species, *M. viridis* (Verrill, 1873), had invaded European waters (Essink & Kleef 1993, Bastrop et al. 1997). Genetic analysis by means of starch gel electrophoresis of allozymes and sequencing of a segment of mitochondrial 16S rDNA showed later that 2 different, sibling species of *Marenzelleria* were present in Europe. *Marenzelleria* Type I (*M.* cf. *wireni*) is found in the North Sea only, whereas Type II (*M.* cf. *viridis*) exists in both the Baltic Sea and, probably, the North Sea, namely in the Elbe estuary (Bastrop et al.

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1995, 1997, 1998, Röhner et al. 1996a,b, Bick & Zettler 1997). The North Sea worms of Type I correspond to the Type I specimens found in coastal waters between Nova Scotia (Canada) and Cape Henlopen (Delaware, USA), while *Marenzelleria* Type II from the Baltic Sea corresponds to *Marenzelleria* Type II worms from the Arctic (Tuktoyaktuk Harbor, Northwest Territories, Canada), New Hampshire and coastal waters between Chesapeake Bay southward to the Ogechee River in Georgia, USA (Röhner et al. 1996b, Bastrop et al. 1997, 1998).

The genetic studies showed clearly that at about the same time 2 sibling species of the genus *Marenzelleria* were introduced into the North and the Baltic Sea, respectively (Bastrop et al. 1998).

Marenzelleria Type I appears to colonise habitats with a higher or fluctuating salinities. Both sibling species are hyperosmoregulators/osmoconformers, like *Nereis diversicolor* (Hohendorf 1963, Bastrop et al. 1997). The osmolality of the coelomic fluid after acclimation to various salinities between 0.25 and 18 ppt is the same for both sibling species (Bastrop et al. 1997).

The aim of the present research was to unravel the spatial distribution and differences in cell vollume regulation between the sibling species in the Elbe estuary, in order to derive hypotheses related to interaction between genotype and habitat. Occurrences of both *Marenzelleria* species in the Elbe estuary could be regarded as a natural experiment to establish the influence of salinity. By allozyme electrophoresis we analysed for the first time the occurrence of *Marenzelleria* Type II and were interested in the following questions: (1) How are the sibling species distributed within the salinity gradient of the Elbe River estuary? (2) Does hybridisation occur?

The field study has proven that both species of *Marenzelleria* can be found in the Elbe estuary (see 'Results') and led to the hypothesis that cell vollume regulation in the osmoconformal range (>10 ppt) may be responsible for different distribution patterns of the species. To test this hypothesis laboratory experiments have been conducted.

As an indicator for cell vollume regulation, the concentration of free amino acids (FAAs) was determined during salinity acclimation for both sibling species. The adjustment of intracellular FAA concentrations plays an important role in acclimation to salinity changes in polychaetes (Oglesby 1978) and other marine invertebrates (Gilles 1975, 1979, Henry 1995).

When osmoconformers are exposed to changing salinity regimes, they will gain or lose water along the osmotic gradient. High intracellular FAA concentrations counterbalance high inorganic ion concentrations in the extracellular fluid of polychaetes exposed to elevated salinities (Oglesby 1978, Reitze et al. 1989). In *Marenzelleria* spp. only a few non-essential amino acids such as alanine and glycine are major contributors to the response (Schiedek 1999).

Alanine aminotransferase (GPT) catalyses the synthesis of L-alanine from glutamate and pyruvate (Stryer 1996). According to Jürss et al. (1999), this enzyme has a higher level of activity in *Marenzelleria* Type I compared to *Marenzelleria* Type II. Therefore, Type I is expected to react with a faster increase in the alanine level towards hyperosmotic stress. To evaluate this hypothesis, a time-course experiment was carried out using the salinity regime known from studies at Stn 5, which is characterised by a fluctuating salinity and an almost complete disappearance of *Marenzelleria* Type II, in comparison to Stn 4, where both species occur sympatrically. FAA levels, activities of 3 enzymes of amino acid metabolism, i.e. alanine aminotransferase, aspartate aminotransferase (AAT) and glutamate dehydrogenase (GDH), as well as 2 enzymes of the energy metabolism (citrate synthase [CS] and octopine dehydrogenase [ODH]) were measured. The synthesis of citrate from oxaloacetate and acetyl coenzyme A by the key enzyme CS is an important control point of citric acid cycle (Stryer 1996). Therefore, CS was used as an indicator for aerobic metabolism. For pelagic chaetognaths, nemerteans and annelids (Polychaeta), an excellent correlation between the metabolic rate (O_2 consumption) and CS activities has been found (Thuesen & Childress 1993a,b).

For some invertebrates (e.g. polychaetes), it is known that the opine dehydrogenases function along with or replace lactate dehydrogenase (LDH) during anaerobic metabolism (Livingstone 1991, Thuesen & Childress 1993b). For example, in the polychaete genus *Tomopteris* high lactate dehydrogenase as well as very low octopine dehydrogenase activities were found (Thuesen & Childress 1993b). Surprisingly, in *Marenzelleria* spp. lactate, strombine and alanopine dehydrogenase showed only low activities, while high activities of ODH could be detected. Furthermore, octopine is the only measurable end product of cytosolic glycolysis in *Marenzelleria* spp. (Schiedek 1997a,b, Schiedek et al. 1997). Therefore, ODH was taken as an indicator for anaerobic metabolism.

MATERIALS AND METHODS

Starch gel electrophoresis of allozymes. Worms were collected from the Elbe estuary in May 1997 (Fig. 1) and from the Ringkobing Fjord (RkF) and Darss-Zingst Bodden Chain (DZBC) in July 1997. Data concerning the last 2 populations are given in Jürss et al. (1999).

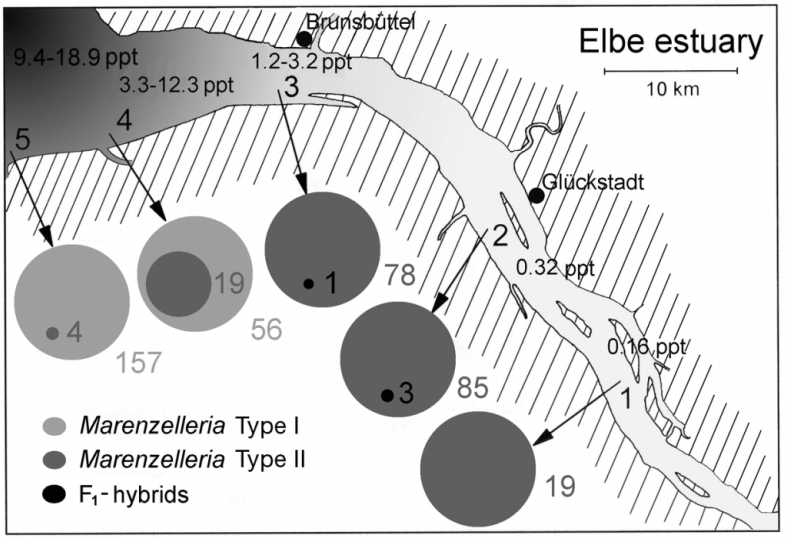

Fig. 1. *Marenzelleria* spp. Salinity-dependent distribution in the Elbe River estuary (Germany). Sampling locations and maximum salinity fluctuations during low and high tide on 20 and 21 May 1997 are indicated (data obtained from: www.cux.wsd-nord.de/htm/start.asp). Stn 1: Twielefleth; Stn 2: Wischhafen; Stn 3: Brunsbüttel; Stn 4: Neufelder Reede; Stn 5: Altenbrucher Bogen (green buoy 41). Circles and numbers next to each circle show the number of species investigated at the sampling location

Sediment samples were taken by a Van Veen grab (Elbe estuary) or by means of a corer at the other 2 sampling sites. Specimens were separated by sieving and immediately frozen in liquid nitrogen. The numbers of worms investigated for each species are shown in Fig. 1.

A total of 8 enzymes representing 10 loci were analysed: isocitrate dehydrogenase (IDH, EC 1.1.1.42), aspartate aminotransferase (AAT, EC 2.6.1.1), octopine dehydrogenase (ODH, EC 1.5.1.11), malate dehydrogenase (MDH, EC 1.1.1.37), glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), arginine kinase (APK, EC 2.7.3.3) and adenylate kinase (AK, EC 2.7.4.3). The methods used for allozyme electrophoresis and statistical evaluation are described in Bastrop et al. (1995), Röhner et al. (1996a,b) and Jürss et al. (1999).

Expt 1. Specimens of *Marenzelleria* spp. were collected in a brackish estuary in the North Sea (Type I; Ringkobing Fjord, Denmark) and the Baltic Sea (Type II; Bodstedter Bodden, Darss-Zingst Bodden Chain) (Table 1). Sediment samples were taken by the means of a corer and subsequent sieving. Individuals were transported alive to the laboratory.

In each case, 20 specimens were kept in plastic aquaria containing aerated artificial seawater (Red Sea Salt, Coral Reef Fishpharm Ltd, Israel). The bases of the aquaria had been covered with 2 cm of washed sand. Incidences of mortality observed during the first days after sampling were attributed to stress during sampling and transport. No mortality was observed during the course of the experiment.

Prior to the experiments, the worms were acclimated for 51 d to 10°C and 10 ppt salinity and a photo period of 12 h light/12 h dark. The acclimation salinity corresponds to the salinity at the beginning of isoosmia for both species (Bastrop et al. 1997). The acclimation temperature was chosen because sexual maturing in both types is less rapid at 10°C (Bochert 1996). The worms were fed regularly with cultured *Chlorella* sp. until 1 wk before the experiments commenced.

After the period of acclimation, the animals were transferred to 25 ppt salinity. In order to reduce the risk of mortality caused by stress, water was sucked off with a flexible tube to leave the sediment undisturbed. Aerated artificial seawater of 25 ppt was then carefully filled in. For each time point, 3 aquaria were set up per species. Additionally, 3 further aquaria per species were prepared as control aquaria with 10 ppt. To ensure the same conditions in both test and control aquaria, the water of the latter was changed as well.

After 3, 6, 12, 24, 72, 144, 288, 672 h, respectively, the worms of all 3 aquaria for each species were taken from the aquaria and frozen in liquid nitrogen until further analysis.

It was assumed that the polychaetes had reached a new steady state after 672 h (28 d). After 672 h, the salinity within the aquaria was changed from 25 ppt back to 10 ppt (672 h was considered a new reference value). At 12, 24, 48 and 96 h after changing the water, worms were taken from the aquaria and frozen.

The average weight of specimens of both types was almost equal (Type I: 0.0359 g [0.0113 g to 0.1301 g]; Type II: 0.0436 g [0.0123 g to 0.0926 g]).

Table 1. *Marenzelleria* spp. Salinity and temperature regimes at the sampling locations (RkF: Ringkobing Fjord; DZBC: Darss-Zingst Bodden Chain)

	Sampling locations	Date	Salinity (ppt)	Temperature (°C)
Expt 1	RkF	Jul 2001	11.9	22.0
	DZBC	Jul 2001	4.6	27.0
Expt 2	RkF	Sep 2002	11.4	24.5
	DZBC	Aug 2002	3.3	19.0

Free amino acids: Frozen worms were cut into 4 equal pieces. Piece 1 (head end) and Piece 3 were homogenised in ice-cold, 1 M perchloric acid (2 × 30 s) using an ultrasonic homogeniser (Bandelin HD 60). After centrifugation at 24 000 × *g* for 10 min at 0°C, the supernatant was removed and neutralised with 5 M K_2CO_3. After incubation on ice for 30 min, the precipitated $KClO_4$ was removed by means of centrifugation (20 min, 24 000 × *g*, 0°C). The supernatant was frozen at –20°C until analysis.

In order to determine the dry weight, Pieces 2 and 4 (tail end) were dried at 60°C for 24 h in a heating oven (Heraeus).

For analysis of the FAAs, a Shimadzu-HPLC system with a Grom column (Germany) was used. Spectrophotometric detection of amino acids was performed at 570 nm, or at 440 nm in the case of proline.

Using an external standard, identification was performed with the Shimadzu-HPLC software. For each sample, 3, 5 and 10 µl of supernatant were analysed and averaged.

The content of the amino acids D,L-alanine (D,L-ala), glycine (gly), proline (pro), histidine (his), arginine (arg), aspartate (asp), serine (ser), threonine (thr) and glutamate (glu) is expressed as µmol g^{-1} dry wt.

Enzyme activities: The preparation of the worm homogenates and the measurement of the activities of the enzymes AAT, EC 2.6.1.1, GPT, EC 2.6.1.2, ODH, EC 1.5.1.11 and CS, EC 4.1.3.7 were carried out according to Jürss et al. (1999). Glutamate dehydrogenase (GDH, EC 1.4.1.4) was determined according to Peng et al. (1994). Total protein concentrations were determined with the bicinchoninic acid kit (Sigma), with bovine serum albumin as standard. The chemicals used were purchased from Sigma, AppliChem or Roche Diagnostics.

Expt 2 (D,L-ala). The specimens for the second experiment were collected from Ringkobing Fjord (Denmark) and from Bodstedter Bodden (DZBC, Germany; Table 1) and were treated as described for Expt 1.

After 36 h at 25 ppt, 5 worms of each species were taken out and frozen in liquid nitrogen.

Homogenates were analysed using optical tests for D-ala and L-ala (Graßl & Supp 1985, Williamson 1985) or HPLC (Agilent series 1100; column RP C18, Knauer). D-ala and L-ala were separated according to Nimura & Kinoshita (1986) and Görs (2002). As prelabelling reagents, N-acetyl-L-cysteine and *o*-phthalaldehyde were used.

Statistics. All data are presented as means ± standard error (SE). Statistical significance was tested only within each species. Differences between means were tested for significance by Student's *t*-test in the case of equal variances, or by Welsh's *t*-test otherwise. Statistical significance was accepted at $p < 0.05$.

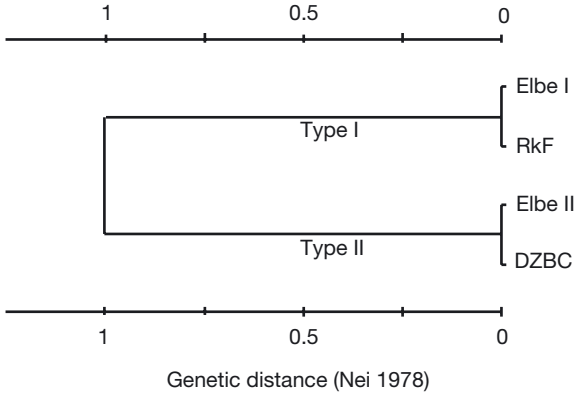

Fig. 2. *Marenzelleria* spp. UPGMA tree of Nei's (1972) genetic distance

RESULTS

Distribution of species and detection of hybrids

The genetic analysis has established for the first time incidences of both *Marenzelleria* species (Types I and II) in the North Sea, namely in the Elbe estuary. Both species are characteristically distributed along the salinity gradient of the Elbe estuary (Fig. 1). *Marenzelleria* Type I inhabits the osmoconformal range, while *Marenzelleria* Type II prefers the oligohaline area (hyperosmotic regulation). At Stns 4 and 5, both species appear sympatrically. The populations sampled in the Elbe estuary were compared with those from the RkF and the DZBC. The *Marenzelleria* Type I population from the Elbe and the population from RkF showed a genetic distance of $D = 0.001$, while a genetic distance of $D = 0.000$ was established between the *Marenzelleria* Type II population from the Elbe and that from the DZBC. This proves that they are virtually identical populations of Type I or Type II (Fig. 2). Based on these similarities and due to sampling difficulties, we alternatively used *Marenzelleria* samples from RkF and DZBC for laboratory experiments.

Apart from establishing the occurrence of both *Marenzelleria* sibling species in the Elbe estuary, incidences of hybridisation have been found for the first time. Fig. 3a shows the electrozymogram of *IDH* from worms of Stn 1 (Twielenfleet) and Stn 4 (Neufelder Reede). Like all other loci examined in this paper, i.e. *MDH-II*, *GAPDH*, *AK* and *APK*, *IDH* represents a diagnostic locus (Ayala & Powell 1972), which allows 100% classification of the specimens to either *Marenzelleria* Type I or Type II (Bastrop et al. 1995, 1997, Röhner et al. 1996a,b). Fig. 3b shows the electrozymogram of *IDH* for Type I and II, as well as for a hybrid.

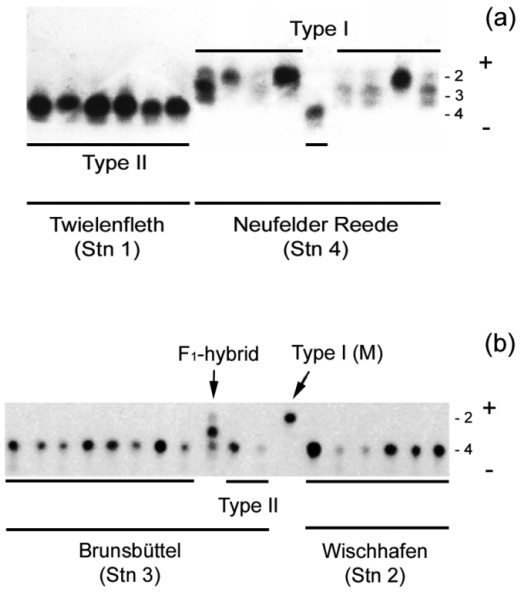

Fig. 3. *Marenzelleria* spp. (a) Zymogram of *IDH**; sampling locations and genetic Types I and II are indicated. Alleles denoted by *2*, *3* and *4*. (b) Zymogram of *IDH**; sampling locations, genetic Types I, II and an F_1-hybrid are indicated. Alleles denoted by *2* and *4*. M: individual used as marker

All data for the diagnostic loci indicated the presence of 3 F_1-hybrids at Stn 2 and 1 F_1-hybrid at Stn 3 (Fig. 1).

The genotype frequencies of the investigated samples, parameters characterising genetic variation and the coefficients of genetic identity and genetic distances of the populations are presented in Table 3 & Appendix 1.

Enzyme activities

Fig. 4 shows the results of the enzyme activity comparisons of the sibling species (Table 2) after long-term acclimation (51 d at 10 ppt = 10 ppt/0 h; 28 d at 25 ppt = 25 ppt/672 h). After acclimation to an isoosmotic and hyperosmotic environment *Marenzelleria* Type I shows noticeably higher activity levels of enzymes involved in amino acid metabolism than *Marenzelleria* Type II. With regard to CS and ODH, such quantitative differences do not exist. Apart from the CS of *Marenzelleria* Type I, no salinity acclimation can be observed on an enzyme level. Thus, the enzymes involved in amino acid metabolism exhibit a constitutively higher level in Type I than in Type II.

Table 2. *Marenzelleria* spp. Expt 1. Ratio of enzyme activities after acclimation (10 ppt/10°C; control) and at new steady state (25 ppt/672 h). V_{max} ratio: maximal rate of an enzyme under saturating conditions

Enzyme	Days	Salinity (ppt)	V_{max} ratio RkF Type I/DZBC Type II
CS	51	10	1.06
	28	25	1.33
ODH	51	10	1.08
	28	25	1.16
GDH	51	10	1.49
	28	25	1.81
AAT	51	10	1.37
	28	25	1.45
GPT	51	10	1.74
	28	25	1.96

Free amino acids

After long-term acclimation (51 d at 10 ppt; 28 d at 25 ppt), the 2 sibling species showed an almost identical pool of total amino acids (Fig. 5). Under these conditions, the concentration of gly, ser and his was species specific. The amino acid gly was 1.53- to 1.78-fold higher in *Marenzelleria* Type I, while in Type II ser and

Table 3. *Marenzelleria* spp. Genetic variability at 10 loci in all populations (±SE). Expected heterozygosity according to Nei's (1978) unbiased estimate

Population	Type	Average sample size per locus	Average no. of alleles per locus	Percent polymorphic loci *P*(0.95)	Average heterozygosity Observed	Expected
Elbe, North Sea	I	199.2 (9.0)	1.6 (0.2)	30.0 (0.060)	0.121 (0.059)	0.124
Ringkobing Fjord, North Sea	I	69.8 (0.1)	1.7 (0.2)	30.0 (0.066)	0.135 (0.067)	0.142
Elbe, North Sea	II (6.9)	186.4 (0.5)	2.6 (0.042)	10.0 (0.039)	0.064	0.060
Darss-Zingst Bodden Chain, Baltic Sea	II (0.0)	70.0 (0.4)	1.9 (0.035)	20.0 (0.041)	0.059	0.066

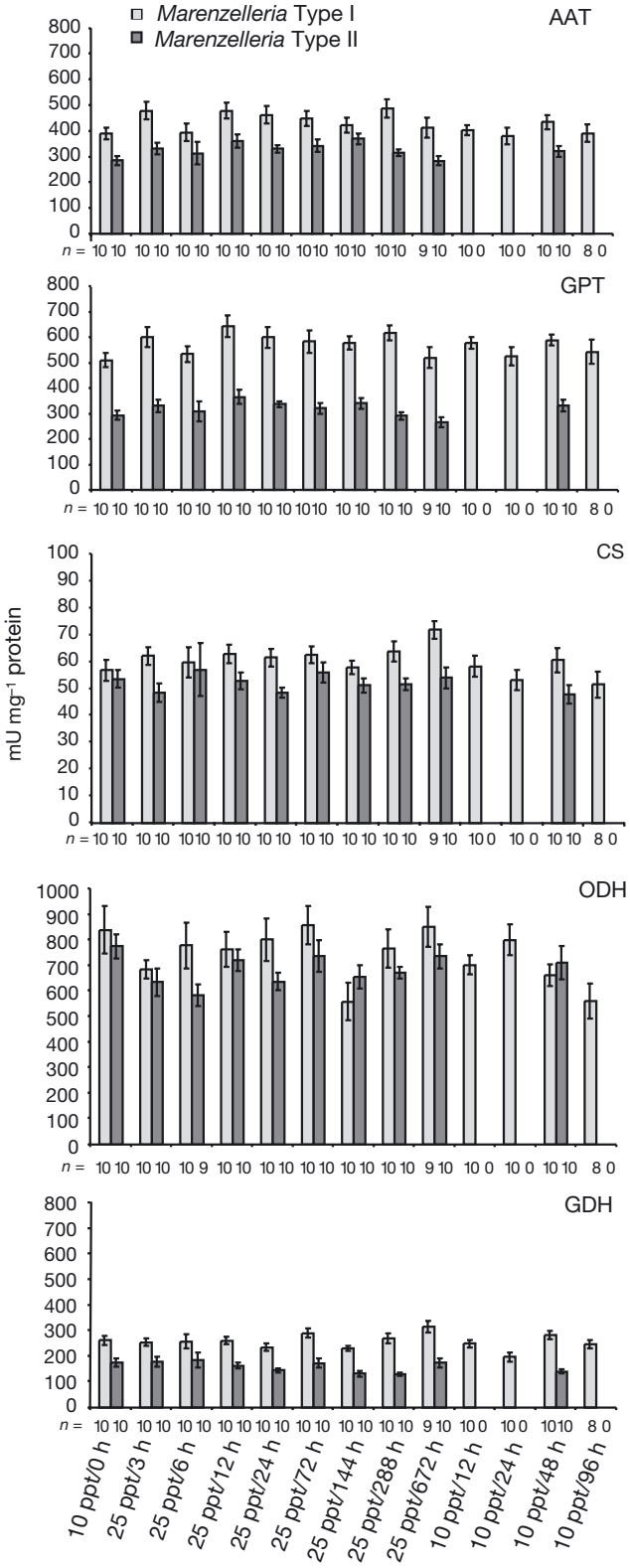

Fig. 4. *Marenzelleria* spp. Expt 1. enzyme activities (mU mg^{-1} protein ± SE) at different points in time during the time-course experiment. Number of specimens analysed is indicated under each column

his showed 2.49- to 1.71-fold and 3.51- to 2.07-fold higher concentrations, respectively (Fig. 6). Arg was the only amino acid examined that is not influenced by external salinity. For both *Marenzelleria* species, concentrations of all other FAAs were significantly altered during the time after salinity change. In both species, concentrations of D,L-ala and pro rose fastest with an increase in salinity from 10 to 25 ppt. D,L-ala, gly and pro showed the highest increase in concentration during salinity acclimation. In the initial phase of hyperosmotic stress, D,L-ala was most important for both *Marenzelleria* species to adjust the intracellular osmotic concentration. The most striking difference between the sibling species was found in the time course of D,L-ala, 3 and 6 h after changing salinity, the D,L-ala level of Type I in 25 ppt rose significantly by 1.33- and 1.95-fold, respectively. For Type II, the first significant rise in D,L-ala occurred only after 6 h (1.61-fold). The level of other FAAs also increased faster and to a higher extent in *Marenzelleria* Type I compared to control specimens (Fig. 6).

Within the first 24 h in 25 ppt, D,L-ala, gly, ser, glu and thr concentrations increased much faster in *Marenzelleria* Type I than in Type II (Fig. 6). Therefore, the total amino acid pool of Type I in 25 ppt was significantly enhanced already after 6 h in worms compared to control specimens in 10 ppt. For *Marenzelleria* Type II, the same effect was not observed until after 24 to 72 h. When specimens were kept at 25 ppt for 24 h, the total FAA pool both Type I and Type II increased 1.97- and 1.28-fold, respectively.

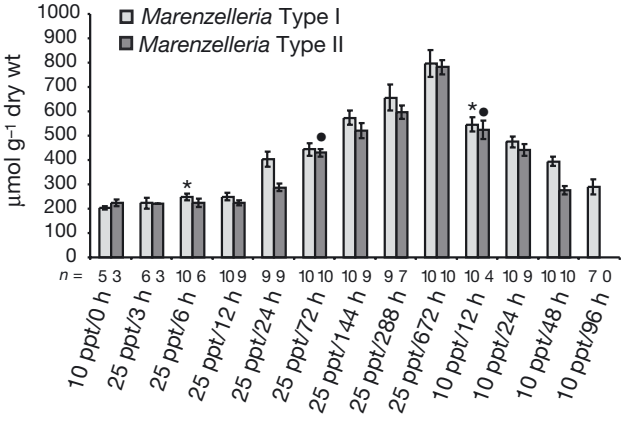

Fig. 5. *Marenzelleria* spp. Expt 1. Salinity-dependent change of the total free amino acid (FAA) pool (D,L-ala, gly, pro, his, arg, asp, ser, thr, glu) during the time-course experiment. Data are mean (µmol g^{-1} dry wt) ± SE values. Asterisks indicate first significant increase and decrease compared to the control (10 ppt/0 h) and steady state (25 ppt/672 h) in Type I. Dots indicate first significant increase and decrease in Type II. Number of specimens analysed is indicated under each column

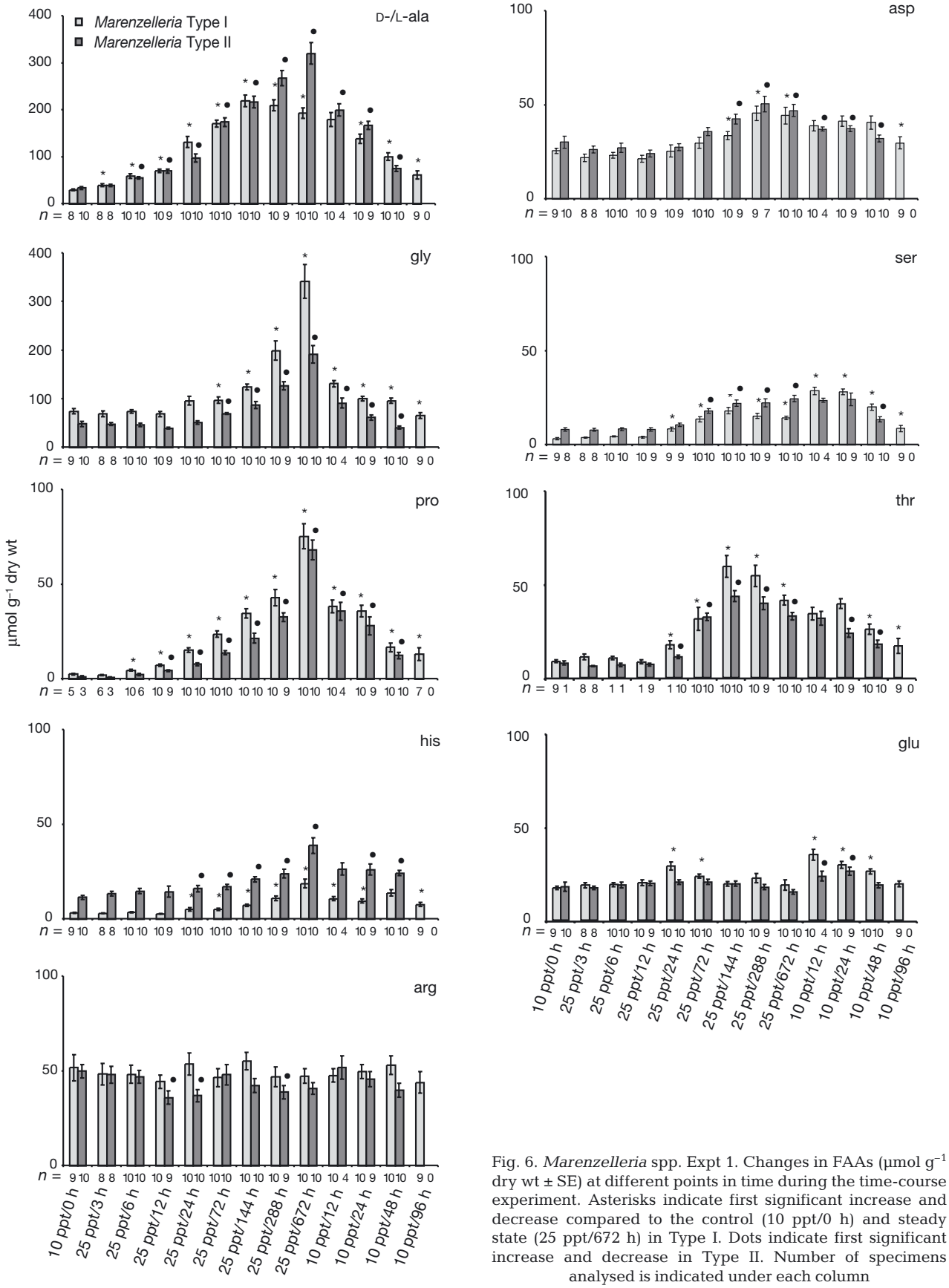

Fig. 6. *Marenzelleria* spp. Expt 1. Changes in FAAs (µmol g^{-1} dry wt ± SE) at different points in time during the time-course experiment. Asterisks indicate first significant increase and decrease compared to the control (10 ppt/0 h) and steady state (25 ppt/672 h) in Type I. Dots indicate first significant increase and decrease in Type II. Number of specimens analysed is indicated under each column

When a salinity change was made from the new steady state (672 h/25 ppt) to 10 ppt, the level of gly and pro declined very quickly in both sibling species. Especially for *Marenzelleria* Type II, the concentration of D,L-ala declined rather fast. After 12 h in 10 ppt, the concentration of D,L-ala had decreased to 93% of its previous level in *Marenzelleria* Type I and to 62% in Type II (Fig. 6). Glu and ser are the only 2 amino acids that rise in concentration after dilution of the environmental water. For a period of 24 h, the concentration of glu was much higher in both sibling species, while ser increased only in Type I compared to the original salinity (25 ppt). Then, 48 h after the salinity change from 25 to 10 ppt, the total pool of FAAs was 50% of the control value (672 h/25 ppt) in Type I and 35% in Type II. Altogether, the sibling species seemed to react more similarly towards a salinity decrease from 25 to 10 ppt than towards an increase from 10 to 25 ppt.

Expt 1 has shown a specific function of ala in connection with hyperosmotic stress. L-ala and D-ala were recorded together in the time-course experiment. A separate Expt 2 was carried out to establish whether D-ala is found in the amino acid pool of *Marenzelleria* spp. By 2 independent methods, D-ala was found in both *Marenzelleria* Type I and Type II. Both methods delivered almost identical results for D-ala. HPLC values are slightly higher for L-ala than results obtained by the optical test (Fig. 7). However, this does not seem to be a particularly important aspect in the context of this paper. The amount of D-ala in this experiment was 35 or 32% of the total alanine (D + L) amount in *Marenzelleria* Type I, and 26 or 19%, in Type II. The total amount of ala (D + L) determined in Expt 2 corresponds to the value from Expt 1 for the time spans of 24 h (at 25 ppt) to 72 h (at 25 ppt).

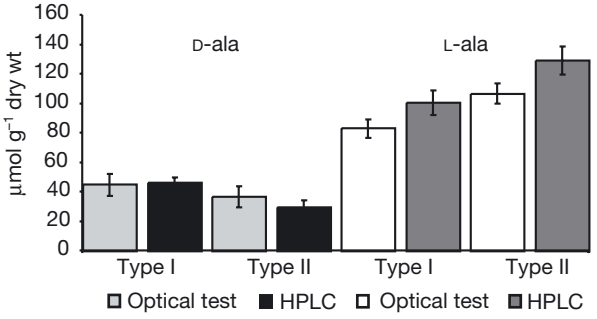

Fig. 7. *Marenzelleria* spp. Expt 2. Determination of D-alanine (black and light grey columns) and L-alanine (white and dark grey columns) with both the optical test and HPLC after 36 h in 25 ppt; mean (µmol g^{-1} dry wt) ± SE; n = 5

DISCUSSION AND CONCLUSION

Genetic and ecological diversity

Estuaries characterised by low and fluctuating salinities are also subjected to variations in other factors such as pH, temperature, dissolved oxygen and particulate loadings. However, salinity seems to represent the environmental factor that best explains the distribution of *Marenzelleria* spp. in the Elbe estuary, although the influence of other abiotic factors cannot be ruled out.

Both *Marenzelleria* species are hyperosmoregulators at below ~10 ppt and osmoconformers at above ~10 ppt salinity (Bastrop et al. 1997). *Marenzelleria* Type I prevails in the Elbe estuary at salinity fluctuations within the osmoconformal range (Stn 5; Fig. 1), while Type II, together with 4 F_1-hybrids, was found mainly in the hypoosmotic range. Between those 2 ranges, there is an area with salinity fluctuations in the hypo-isoosmotic range in which both sibling species occur sympatrically (Stn 4). On the Atlantic coastline of the US, the area of origin of the *Marenzelleria*, the 2 sibling species have never been found to occur sympatrically (Röhner et al. 1996b, Bastrop et al. 1997, 1998).

The population of *Marenzelleria* Type II in the salinity gradient of the Ogeechee River estuary corresponds largely to the *Marenzelleria* Type II found in the Elbe estuary (Dörjes & Howard 1975). In all of our studies *Marenzelleria* Type I was mainly found at higher salinities, whereas Type II prefers oligo- to mesohaline biotopes (Table 1 in Bastrop et al. 1997).

The F_1-hybrids are only found in the oligohaline environment (hyperosmotic osmoregulation) of the Elbe estuary. In the detailed work on hybridisation of marine animals, Gardner (1997) did not mention any example of hybridisation among polychaetes. Therefore the existence of hybrids of the *Marenzelleria* sibling species in the Elbe River is very remarkable.

In order to interpret the distribution of the *Marenzelleria* sibling species in the Elbe estuary, it is important to look into reproduction as well. During the reproduction phase of *Marenzelleria* spp., the gametes of numerous spawning individuals are released simultaneously into water. The larvae of *Marenzelleria* spp. have a planktonic phase lasting several weeks. Sometimes several million larvae per cubic metre were found (Bochert 1997). Juvenile worms also have been found in the plankton (George 1966, Dauer et al. 1980, 1982, Bochert & Bick 1995). Accordingly, place of reproduction and place of benthic life of the adults examined in the present paper do not necessarily have to be identical. The influence of salinity on the gametogenesis and larval development has been examined in detail for *Marenzelleria* Type II (Bochert 1997). A salinity of 10 ppt proved favourable for gametal growth, whereas

≥25 und ≤5 ppt affected it negatively. One-setiger larvae were unable to complete their development to metamorphosis at salinities <5 ppt. Metamorphosis to benthic life was possible at salinities between 10 and 20 ppt. Due to this reproduction strategy one could expect that both *Marenzelleria* species are, in principle, able to colonise the entire Elbe estuary.

Clarke's (Chow & Clarke 2000) model of the distribution of a species in relation to an abiotic environmental variable describes 3 ranges: range predicted from physiological tolerance > actual range > reproduction range. In line with this model, both sibling species of *Marenzelleria* do not fully exhaust their physiological tolerance. The results shown here regarding the distribution of *Marenzelleria* spp. in the Elbe estuary are merely a snap-shot of the 'actual range', since due to the mobility of the larvae and juveniles no statement can be made regarding the 'reproductive range'.

Nevertheless, temperature must also be taken into consideration, since it influences salinity tolerance (Kinne 1971). This leads to the conclusion that the distribution of the *Marenzelleria* species in the Elbe estuary are certainly also influenced by changing seasons. Seasonal effects play a role in spawning behaviour as well. As yet, it was assumed that *Marenzelleria* Type I (spring) and Type II (autumn) reproduce at different times (Bochert 1997). However, the existence of hybrids in the Elbe estuary indicates at least partially an overlap of reproduction times.

In total, salinity as an environmental factor plays an important part in the distribution of the *Marenzelleria* species. In principle, the distribution of sibling species in the Elbe estuary indicates that Type I tolerates salinity fluctuations in the osmoconformic range (≥10 ppt) better than *Marenzelleria* Type II. For the oligohaline range, however, the opposite seems to hold true.

The salinity-related distribution of the mussels *Mytilus trossulus* and *Mytilus edulis* in the areas where Baltic Sea and North Sea meet (Varvio et al. 1988, Väinölä & Hvilsum 1991) parallels the distribution of *Marenzelleria* sibling species in the Baltic Sea and North Sea as well as in the Elbe estuary.

After a comprehensive comparison of the 2 mussel species and their hybrids on an organism level, Gardner & Thompson (2001) suggest differences between the taxa. Their distributional differences are likely to occur at cellular or biochemical levels, rather than reflecting a difference in energy balance between the taxa.

Free amino acids and enzyme activities

Jürss et al. (1999) have suggested that the values of enzyme activities under saturating conditions may be as a suitable starting point for a comprehensive analysis of evolutionary adaptation in *Marenzelleria* spp. For pelagic chaetognatha, nemerteans and annelids (Polychaeta), significant correlations between the metabolic rate (O_2 consumption) and CS activities have been found (Thuesen & Childress 1993a,b). The activities of ODH, as an indicator of the anaerobic potential, and CS lead to the conclusion that the physiological processes in *Marenzelleria* Types I and II function within the same range of ATP synthesis capacity.

The findings for GDH, GPT and AAT show that *Marenzelleria* Type I has generally a higher level of activity than Type II. For GPT, this has already been established in 2 previous experiments (Jürss et al. 1999). All 3 enzymes can be involved in transdeamination. Transdeamination is the primary mechanism for catabolism of amino acids (AAs), e.g. in fish the amino group of a variety of AAs is transferred to α-ketoglutarate to form glu, which is then deaminated by glutamate dehydrogenase (Jürss & Bastrop 1995). Regarding invertebrates, the importance of this metabolic process is not as universal as for vertebrates (Bishop 1976, Bishop et al. 1994). However, for the mussel species *Mya arenaria* indications of functional transdeamination have been reported (Moyes et al. 1985). Findings for the polychaete *Arenicola marina* (Reitze et al. 1989) suggest that transdeamination plays an important role — at least in these animals. This transdeamination can support ammoniogenesis, but also the synthesis of L-ala and asp. Experiments with the transaminase inhibitor aminooxyacetate (AOA) have shown that aminotransferases, especially GPT, play a vital role in the qualitative composition of the total AA pool in connection with the salinity-related rise of FAAs in *Geukensia demissa* (Bishop et al. 1981, 1994) and *A. marina* (Reitze et al. 1989).

In general, from the high constitutive activity level of the enzymes involved in metabolism it could be concluded that, within salinity fluctuations in the osmoconformal range, *Marenzelleria* Type I can carry out cell vollume regulation faster than Type II. On the other hand, Type I has a disadvantage in comparison to Type II at the oligohaline range. This is in line with the argumentation of the Long Island Sound model of selection at the *Lap* (leucine amino peptidase) locus for blue mussels (*Mytilus edulis*; Hilbish et al. 1982, Hilbish & Koehn 1985, Gardner & Kathiravetpillai 1997). This mussel experiences *Lap*-genotype-dependent mortality related to local salinity variation. The differences in mortality arise from higher activities of the *Lap*94 allozyme compared with the other *Lap* allozyme. Mussels carrying copies of the *Lap*94 allele can accumulate cellular FAAs faster than mussels with other alleles, but the same mussels also lose cellular nitrogen at faster rates (Hilbish et al. 1982). Depending on salinity, *Lap*94 means either a selective advantage or disadvantage.

When interpreting the FAA changes in *Marenzelleria*, it must be taken into consideration that only values for whole individuals (cells + blood + coelomic fluid) are available. The fact that the AA concentration is reduced when salinity is decreased from 25 to 10 ppt could be related to metabolism, protein synthesis, or the release of AAs into the environment. In ecophysiological terms, it is important to note that the time course of the FAA changes is relevant, since at the beginning and end of the acclimation period both *Marenzelleria* sibling species show roughly the same total concentration of FAAs. It must also be noted that arg is a non-compatible osmolyte (Clarke 1975, Yancey 1994) that has no primary (direct) function during salinity acclimation. Phosphoarginine plays an important role in the regeneration of ATP. The arginine kinase catalyses the adjustment in both directions of the equilibrium between arg + ATP and phosphoarginine + ADP + H^+. Arg is present at a high and stable concentration. Out of 10 enzymes measured, arginine kinase shows the highest activity in both *Marenzelleria* sibling species (Jürss et al. 1999).

As in a number of other invertebrates (Henry 1995), ala, gly and pro are the most important, well-known organic osmolytes for cell vollume regulation during hyperosmotic stress. In the initial phase after salinity change from 10 to 25 ppt, D,L-ala has the most important function in both *Marenzelleria* species. As early as 3 h after transfer, *Marenzelleria* Type I shows a significant rise in D,L-ala, followed by a significant increase in pro after 6 h. The first significant increases of D,L-ala and of pro in *Marenzelleria* Type II emerge only after 6 and 12 h, respectively. Gly levels accumulate slowly in both species compared to the former 2 AAs. Significantly increased amounts do not occur until 72 h after the change.

Because AA accumulated at very different rates, it has to be noted that the time course of a single AA is not necessarily correlated with the time course of the whole FAA pool. The amount of total FAAs is of clear ecophysiological relevance in acclimation to a new salinity.

It has been established in one field study and 3 laboratory experiments that *Marenzelleria* Type I shows a higher level of GPT activity than Type II (1.88- vs 1.41-fold, Jürss et al. 1999; 1.74- vs 1.96-fold, present study). This means a higher ala synthesis capacity of Type I and could be the reason behind the ecologically relevant, quick rise in ala during the initial phase of hyperosmotic stress.

GPT catalyses the synthesis of L-ala, which could be transferred to D-ala in one step by alanine racemase (Reitze et al. 1989, Low et al. 1996). GPT is essential for the steady-state concentrations of D- and L-ala. In the ribbed mussel *Geukensia demissa*, it has been shown by inhibition with AOA that the ala production was transaminase dependent (Bishop et al. 1994). After hypoosmotic shock, the quantitative reduction of L-ala was almost completely inhibited by AOA in the polychaete *Arenicola marina* (Reitze et al. 1989).

Two diagnostic GPT loci exist in both *Marenzelleria* species (Jürss et al. 1999). Future analysis should establish how far the different GPT activities have their origin in differences in enzyme kinetics and/or in expression of the genes. Oysters (*Crassostrea virginica*) from the Atlantic Ocean accumulate more glycine betaine than oysters from Chesapeake Bay after hyperosmotic stress (Dragolovich 1994). Differences in betaine dehydrogenase and choline dehydrogenase kinetics between the 2 oyster populations seem to be responsible for the observed differences in glycine betaine recovery patterns (Perrino & Pierce 2000a,b).

In terms of cell vollume regulation, euryhaline marine invertebrates generally tend to tolerate declining salinities more easily than elevating salinities (Gilles 1979). Furthermore, the reduction of the FAA pool is almost identical in both *Marenzelleria* sibling species. Due to its higher level in GPT activity, Type I was expected to display a faster decline of ala, while the opposite was measured. It is also remarkable that gly, with its relatively low turnover (Bishop et al. 1994), declines fastest and to the highest extent. In this context, it has to be taken into consideration that the rising level of the FAAs during rising salinity is due to metabolic reactions (proteolysis, transdeamination, de novo synthesis), while during reduction of salinity, the change in FAAs is mainly due to catabolism and excretion into the environment (Bayne 1975, Livingstone et al. 1979, Gilles 1979, Reitze et al. 1989, Ballantyne & Chamberlin 1994).

For euryhaline invertebrates, the excretion of NH_3/NH_4^+ and AAs increases in parallel to the reduction of salinity (Bayne 1975, Gilles 1979, Reitze et al. 1989, Ballantyne & Chamberlin 1994). In contrast to the general tendency of other non-essential AAs, glu temporarily rises after a change of salinity from 25 to 10 ppt in both *Marenzelleria* species, while ser levels increased temporarily in Type I only. Glu could be produced by degradation of pro, while ser could derive from gly (Bishop et al. 1994).

To summarise, it can be concluded that with regard to the time course of the cell vollume regulation by means of FAAs, *Marenzelleria* Type I adapts better to salinity fluctuations in the osmoconformal range (>10 ppt) than *Marenzelleria* Type II. Together with the species-specific distribution of the *Marenzelleria* sibling species in the salinity gradient of the Elbe River, the physiological findings can, at least in part, explain the spatial distribution of sibling species Types I and II in the area of origin (North America) and in the colonised habitats of the North and Baltic Seas.

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Appendix 1. *Marenzelleria* spp. Number of individuals sampled (*N*) and genotype frequencies in 4 populations in the North Sea and Baltic Sea

Locus Genotype	Type I Elbe	Type I Ringkobing Fjord	Type II Elbe	Type II Darss–Zingst Bodden Chain
IDH*				
(N)	212	70	200	70
2–2	0.566	0.571	–	–
2–3	0.406	0.300	–	–
3–3	0.028	0.129	–	–
4–4	–	–	1	1
AAT–I*				
(*N*)	213	70	200	70
1–2	–	0.014	0.010	–
2–2	1	0.986	0.990	1
AAT–II*				
(N)	211	69	182	70
1–3	–	–	0.011	0.014
3–3	–	–	0.967	0.957
3–4	0.081	0.029	0.022	0.029
4–4	0.919	0.971	–	–
AK*				
(N)	202	70	201	70
1–4	–	–	0.005	–
1–7	–	–	0.005	–
3–3	0.990	0.971	–	–
3–6	0.010	0.029	–	–
4–4	–	–	0.527	0.557
4–7	–	–	0.378	0.286
4–8	–	–	0.015	0.014
4–9	–	–	0.015	0.029
4–10	–	–	0.005	0.014
7–7	–	–	0.040	0.086
7–9	–	–	0.005	0.014
9–10	–	–	0.005	–

Locus Genotype	Type I Elbe	Type I Ringkobing Fjord	Type II Elbe	Type II Darss–Zingst Bodden Chain
G3PDH*				
(N)	123	70	136	70
1–3	–	–	0.007	–
3–3	0.317	0.314	0.919	0.857
3–4	–	–	0.066	0.129
4–4	–	–	–	0.014
3–5	0.504	0.514	0.007	–
5–5	0.179	0.171	–	–
GAPDH*				
(N)	213	70	201	70
1–1	1	1	–	–
2–2	–	–	1	1
ODH–1*				
(N)	181	69	173	70
2–3	–	–	0.040	–
3–3	0.724	0.493	0.931	0.971
3–4	0.155	0.304	0.017	0.029
3–5	0.039	0.130	0.012	–
4–4	0.055	0.043	–	–
4–5	0.017	0.029	–	–
5–5	0.011	–	–	–
MDH–I*				
(N)	213	70	201	70
1–1	1	1	0.995	0.971
1–2	–	–	0.005	0.029
MDH–II*				
(N)	211	70	201	70
2–2	–	–	0.995	1
2–5	1	1	0.005	–
APK*				
(N)	211	70	201	70
1–1	–	–	1	1
2–2	1	1	–	–

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