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The significance of intracellular amino acid regulation for freezing tolerance of the mussel *Mytilus edulis* L.

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Abstract

'Winter'-adapted North Sea mussels (*Mytilus edulis* L.) are able to continue isosmotic regulation of intracellular amino acid concentrations in the adductor muscle during extracellular freezing. At the same time the intracellular water content of the muscle cells remains constant. Among the amino acids, low-molecular glycine constitutes the major component, followed by taurine. These two amino acids amount to about 73 % of the entire amino acid pool in the adductor muscle. In contrast, mussels from the western Baltic Sea exhibit lower intracellular amino acid concentrations. During freezing, these frost-sensitive animals show only a minor potential of amino acid regulation.

Keywords: freezing tolerance, amino acids, *Mytilus edulis*.

Introduction

Various behavioural, morphological, physiological and biochemical features, which are the basis of well-developed freezing tolerance in intertidal marine invertebrates, have been reported (Theede 1973, Aarset 1982, Murphy 1983, Storey & Storey 1989). Freezing injuries after extracellular ice formation are mainly associated with the loss of critical amounts of water below a minimum cell volume (Meryman 1970). Elevated amounts of specific intracellular polyhydroxy antifreeze-compounds, such as glycerol, sugars or alcohols, are lacking in marine mussels (Kanwisher 1966, Williams 1970, Theede *et al.* 1976, Storey & Storey 1989). However, intracellular amino acids contribute essentially to the maintenance of cell osmolarity (Lange 1972, Gilles 1972, Pierce & Greenberg 1973, Deaton *et al.* 1989). By measuring total intracellular amino acid content and changes in concentration of amino acids during freezing, we studied the significance of intracellular amino acid regulation for the freezing tolerance of *Mytilus edulis*.

Material and methods

Mytilus edulis L. from the North Sea (shell length 5.2±0.3 cm) were supplied by the Biologische Anstalt Helgoland, Wattenmeerstation Sylt; Baltic Sea individuals (shell length 5.0±0.6 cm) were collected in Kiel Fjord during January/February 1990. The animals were kept in circulating sea water without food and pre-adapted for at least three weeks to defined environmental conditions: North Sea mussels to 34 ‰S and 3°C in combination with short-day conditions (8 h light, 16 h darkness; referred to as 'winter-adapted') or to 16°C in combination with long-day conditions (16 h light, 8 h darkness; referred to as 'summer-adapted'). Baltic Sea individuals were treated similarly at 17 ‰S.

For freezing experiments, the animals, with their mantle fluid, were placed in empty glass jars (volume: 300 ml) without sea water and immersed in a Lauda cooling-thermostat at a constant temperature of −10°C (± 0.02°C). Decline in body temperature was monitored by a thermistor (Figure 1). Chilling was achieved at a rate of 0.5±0.2°C · min^{-1} (n = 6). After reaching the super-cooling point, the heat of crystallization caused a low temperature increase (Figure 1). The latent heat of freezing delayed cooling rate to 0.06±0.03°C · min^{-1}. Following different exposure times (1-168 h), the animals were thawed by adding sea water (+10°C; 34 or 17 ‰S). Temperature increase during thawing occurred at a rate of 3.4±0.5°C · min^{-1} (Figure 1). One hour after thawing, the animals were tested for ciliary activity of the terminal gills. This was used as an indicator for freezing injuries (Theede 1965).

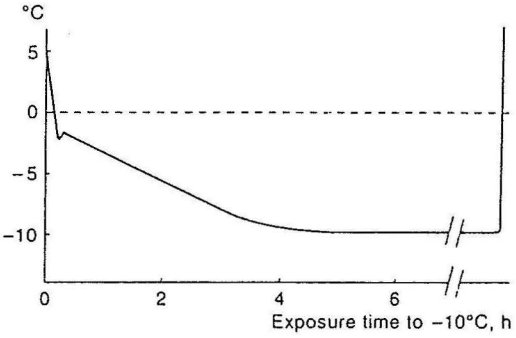


Figure 1.
Mytilus edulis. Representative temperature course inside the mantle cavity of mussels during exposure to −10°C.

Individual adductor muscles were cut out and deep-frozen in liquid nitrogen. The frozen material was ground and homogenized with five volumes of ice-cold 0.6 M perchloric acid. After centrifugation (15 min at 14 500 *G*), the supernatant was neutralized with 5 M KOH and 3 M $KHCO_3$ and centrifuged again (5 min at 14 500 *G*). Perchloric acid precipitated proteins without hydrolysis; this was proved by extraction of a bovine serum albumin standard.

Amino acids were separated by High-Performance Liquid Chromatography (HPLC). For derivatization, a 60 µl sample was mixed with 75 µl OPA-reagent, consisting of 50 mg ortho-phtalaldehyde, 625 µl methanol, 5.75 ml 0.4 M borate buffer (pH 10.4) and 50 µl mercaptoethanol. 15 µl norvaline (100 pmol · µl^{-1}) was added as an internal standard. After derivatization, 100 µl was injected on a Kontron-HPLC equipment (for chromatographic conditions see Table 1) and compared with an amino

Table 1.
Chromatographic conditions for HPLC analysis.
Solvent A: 60 ml acetonitrile, 940 ml acetate buffer (0.4 M; pH 7.7).
Solvent B: 600 ml acetonitrile, 400 ml water.
Flow rate: 1.5 ml · min^{-1}.
Excitation: 340 nm;
Emission: 455 nm.

Time, min	Solvent B, %
0	2
10	5
45	25
55	60
60	2

acid standard. Proline, a secondary amine which is not derivatized by the OPA-reagent, was quantified by the phenylisothiocyanate method (Yaegaki *et al.* 1986).

Intracellular water content of the adductor muscles was measured using the radioactive markers ^{14}C-carboxyl-inulin or ^{14}C-carboxyl-dextran. 20 µl of a marker (370 Bq = 10 nCi) was injected into an adductor muscle. After an equilibration period of one day at room temperature, the mussels were frozen (air temperature −10°C) for different periods (1.5-72 h) as described above. After exposure, the adductor muscles were dried at 60°C and total water content of the tissue was calculated as the difference of wet and dry weight. Dried tissues were homogenized and hydrolysed in 1 ml of 1 M NaOH for two days at room temperature. Immediately after thawing, the extrapallial liquid (haemolymph) of the mussels was collected. Radioactivity in muscle and haemolymph samples was measured in a TRI-CARB 460C Automatic Liquid Scintillation System (Packard Instruments) using 10 ml Hionic-Fluor™ scintillation solvent (Packard). To determine the disintegrations per minute (dpm) counts per minute (cpm) were corrected to 100 % efficiency by external standardization procedures. Quenching corrections were also determined using ^{14}C-n-hexadecane (8720 dpm) as an internal reference standard. Variations of the two methods of quenching correction were less than 5 %. Extracellular tissue water content was calculated according to Freeman & Shuttleworth (1977):

$$E = \frac{A_M}{A_H},$$

where E = extracellular water content, A_M = activity of the muscle sample (dpm · g^{-1} wet wt), A_H = activity of the haemolymph sample (dpm · g^{-1}).

Intracellular water was calculated as the difference of total tissue water and extracellular water. No significant differences in the determination of the intracellular water content between the two markers, ^{14}C-inulin and ^{14}C-dextran, were found.

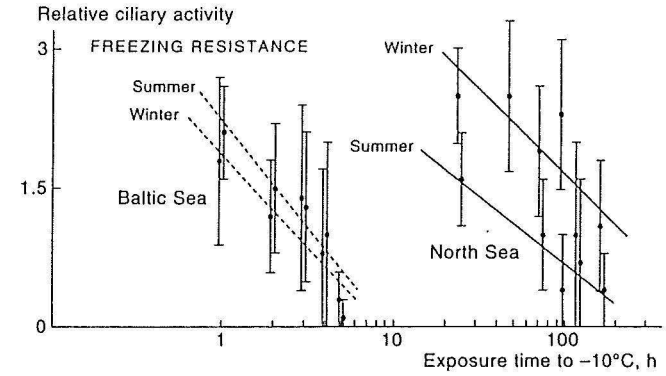
Data were analysed by using a two-tailed Student's *t*-test (significance level: 5 %).

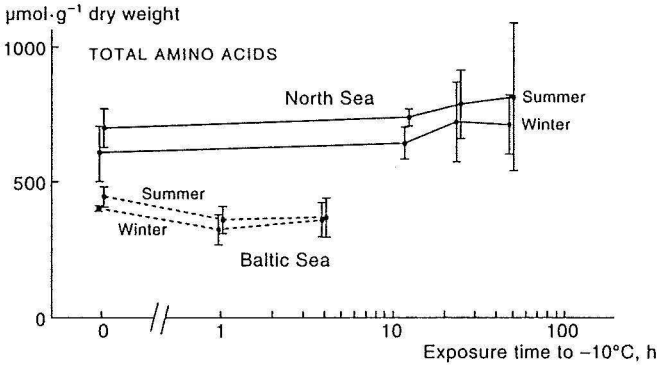
Table 2A.
Mytilus edulis from the North Sea. Concentrations of selected amino acids in adductor muscles during exposure to −10°C after adaptation to winter conditions (W: 3°C; 8/16 h light/dark period) or summer conditions (S: 16°C; 16/8 h light/dark period) for at least three weeks. Values are in µmol · g^{-1} dry wt ± SD. Bold figures: Significant differences ($p<0.05$) to animals without exposure to cold.
*: Significant differences ($p<0.05$) of summer-adapted to winter-adapted animals.

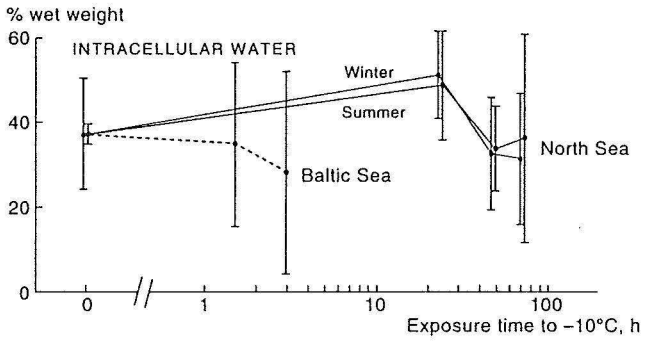
		Exposure time to −10°C (hrs)				
		0	12	24	48	72
	W:	(n=7)	(n=4)	(n=5)	(n=3)	(n=3)
	S:	(n=9)	(n=4)	(n=4)	(n=5)	(n=3)
Glycine	W:	233.9±53.5	220.4±40.0	328.4±130.9	**337.8± 90.3**	**331.8±29.0**
	S:	233.3±76.4	249.0±66.4	271.8± 71.4	238.4±121.3	217.8±24.4*
Taurine	W:	214.7±35.3	**280.1±24.5**	211.2± 18.3	216.4± 26.4	**136.5±48.8**
	S:	259.6±26.9*	287.6±32.4	251.9± 33.7*	331.1±146.1	**220.3±30.4***
Arg	W:	55.3±25.2	41.2± 7.6	35.7± 14.8	23.1± 7.1	29.2± 9.5
	S:	57.6±17.0	59.4±12.5*	54.7± 12.4	**37.1± 17.3**	52.9± 4.6*
Asp	W:	42.6± 7.8	48.2± 8.5	**56.6± 12.8**	52.1± 13.5	30.0±13.8
	S:	30.7± 4.5*	26.5± 9.2*	**41.1± 10.4**	**50.8± 20.1**	**54.4± 8.3***
Ala	W:	22.0±14.0	17.0± 4.4	**38.8± 11.7**	22.8± 12.4	24.8± 8.7
	S:	12.1± 6.7	11.1± 2.6*	**28.2± 6.7**	**29.4± 9.1**	**43.7±17.3**
Glu	W:	17.3± 4.8	14.3± 3.7	18.3± 3.8	16.7± 6.1	14.5± 3.2
	S:	15.0± 3.2	11.8± 3.4	18.8± 3.5	**20.1± 5.0**	**21.4± 3.1***
Ser	W:	5.9± 2.8	7.7± 3.2	**9.8± 2.5**	**18.2± 14.4**	5.8± 1.0
	S:	27.4± 9.6*	23.6± 5.1*	**49.4± 23.1***	27.2± 8.2	**16.4± 2.7***
Σ	W:	612.1±96.7	651.0±59.0	730.2±146.6	718.3±111.2	593.6±94.8
	S:	703.9±74.6*	744.2±34.3*	794.4±125.5	821.6±277.8	706.9±57.1

Table 2B.
Mytilus edulis from the Baltic Sea. Concentrations of selected amino acids in adductor muscles during exposure to −10°C. For other details see Table 2A.

		Exposure time to −10°C (hrs)				
		0	1	2	3	4.2
	W:	(n=4)	(n=4)	(n=4)	(n=5)	(n=5)
	S:	(n=4)	(n=4)	(n=4)	(n=4)	(n=4)
Glycine	W:	186.9±11.3	**109.2±23.9**	155.5±43.2	**135.2±25.2**	**133.1±30.4**
	S:	141.9±12.6*	**101.7±16.1**	**95.6±33.4***	134.3±48.4	**82.0±26.5***
Taurine	W:	77.1±11.7	80.2±17.0	**124.6±15.9**	79.9±14.5	79.9±17.7
	S:	125.2±31.9*	**81.9±11.0**	108.2±10.3	112.6±19.8*	102.1±13.3
Arg	W:	25.4± 5.9	29.1± 5.7	**49.6± 4.2**	**38.6± 6.9**	38.2±12.0
	S:	44.3± 7.6*	**30.8± 7.5**	43.8±14.7	**34.5± 4.8**	**31.2± 7.3**
Asp	W:	53.5± 1.8	**45.5± 6.1**	55.8±10.2	41.0±13.7	**41.5±10.5**
	S:	56.3± 9.3	**67.3± 4.1***	62.5±20.5	59.6±15.6	72.4±12.3*
Ala	W:	14.6± 3.8	12.3± 6.0	20.8± 9.6	17.0± 8.4	19.2± 6.5
	S:	16.3± 3.0	17.3± 7.6	**25.2± 6.7**	16.1± 6.3	16.5± 6.7
Glu	W:	20.3± 3.7	18.2± 3.2	26.8± 8.2	19.6± 3.7	18.0± 3.2
	S:	20.9± 5.1	21.3± 3.6	24.0± 3.9	16.6± 3.4	21.1± 6.4
Ser	W:	7.7± 2.4	9.8± 3.0	**4.7± 0.9**	8.2± 2.0	9.4± 3.1
	S:	10.0± 3.4	7.0± 1.3	7.7± 2.5*	7.6± 2.0	8.6± 1.9
Σ	W:	404.5± 3.6	**326.9±52.8**	**464.2±21.0**	**363.3±32.3**	364.4±61.4
	S:	451.2±35.1*	**364.1±48.4**	423.1±77.9	424.6±87.4	370.8±70.6







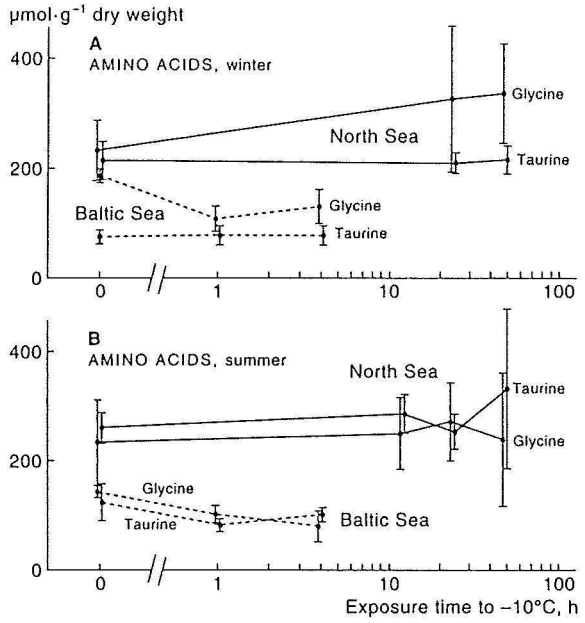


Figure 2. (left, top)
Mytilus edulis. Freezing resistance of North Sea and Baltic Sea individuals (mean ± SD; n = 6-29). Ciliary activity after exposure to −10°C and subsequent thawing was estimated according to Theede (1965): 0 = no ciliary activity, 1.5 = activity half-reduced, 3 = normal activity. Animals were adapted to winter conditions (3°C, 8/16 hrs light/dark period) for at least three weeks.

Figure 3. (left, centre)
Mytilus edulis. Regulation of total amino acid content in adductor muscles of North Sea and Baltic Sea individuals during exposure to −10°C (mean ± SD; n = 3-9). For other details see Table 2A.

Figure 4. (left, bottom)
Mytilus edulis. Regulation of intracellular water content in adductor muscles of North Sea and Baltic Sea individuals during exposure to −10°C (mean ± SD; n = 2-5). Baltic individuals were used for experiments after capture in summer. For other details see Table 2A.

Figure 5. (right)
Mytilus edulis. Regulation of glycine and taurine in adductor muscles of North Sea and Baltic Sea individuals during exposure to −10°C (mean ± SD; n = 3-9). For other details see Table 2A.

Results

Freezing resistance of North Sea individuals was dependent on previous adaptation: the effective dose for decreasing ciliary activity by 50% (ED_{50} time) was four days freezing in winter-adapted mussels whereas the ED_{50} time in summer-adapted animals was one day (Figure 2). *M. edulis* from the Baltic Sea on the other hand had only a minor freezing tolerance. Their ED_{50} values were about two hours, regardless of the pre-treatment (Figure 2).

During freezing, North Sea mussels showed only small changes in the total amount of amino acids in the adductor muscle. After one day exposure to −10°C, amino acid concentrations tended to increase (Figure 3; Table 2A). In contrast, the more sensitive Baltic Sea individuals showed a tendency for decreasing amino acid content after a few hours at −10°C (Figure 3; Table 2B). At the same time, the intracellular water content in these animals also decreased, whereas it remained at a high level for more than 20 hours in North Sea mussels (Figure 4). Summer-adapted mussels had higher amino acid concentrations than winter-adapted animals (Figure 3; Table 2).

In particular, the amount of glycine, comprising nearly half of the amino acid concentration of the adductor muscle of winter-adapted North Sea *Mytilus*, rose significantly during freezing (Figure 5A; Table 2A). Glycine levels were higher in winter-adapted mussels than in summer-adapted individuals. Glycine and taurine constituted about 73% of the total amino acid pool. In summer-adapted North Sea mussels glycine and taurine made up about 70% but the glycine level showed no significant changes during freezing (Figure 5B; Table 2A).

Glycine content of *M. edulis* from the Baltic Sea declined significantly after a few hours at −10°C in both experimental groups (Figure 5A & B; Table 2B). In these frost-sensitive animals glycine and taurine together constituted about 65% of the total amino acid concentration in winter-adapted and 59% in summer-adapted individuals.

Discussion

Several species of marine macrofauna are capable of surviving sub-zero temperatures in the upper littoral zone of temperate and subarctic regions. Freezing tolerance increases during winter months (Theede 1965, 1972, Aarset 1982, Murphy 1983, Storey & Storey 1989). North Sea mussels, which are adapted to cold and short day conditions (winter-adapted), are more tolerant to freezing than summer-adapted individuals. The freezing tolerance of Baltic Sea mussels on the other hand is considerably lower and independent on previous adaptation. According to Theede & Stein (1989) mussels are able to survive formation of extracellular ice crystals, which is initiated by nucleating agents. Extracellular ice formation increases the osmolarity of the unfrozen extracellular fluid and could cause intracellular dehydration. When a critical amount of tissue water is converted to ice, freezing injuries occur e.g. by distortion of cells, disruption of membranes or increase of electrolyte concentration to toxic levels. Finally, intracellular freezing is lethal. Thus, freezing-tolerant animals such as *M. edulis* have to avoid intracellular ice formation.

Effects of extracellular freezing are comparable to those of exposure to hyperosmotic sea water. If euryhaline invertebrates are exposed to increased salinity, the augmented production of osmotically effective organic compounds, mainly amino acids, inhibits strong shrinkage of the cells (i.e. isosmotic cell volume regulation; Lange 1972, Gilles 1972, Pierce & Greenberg 1973, Deaton *et al.* 1989). Our study shows that regulation of intracellular dissolved free amino acids continues during extracellular freezing. The concentration of total amino acids in North Sea mussels increases, and the intracellular water content remains nearly constant. Thus, no water leaves the cells. In contrast, the amino acid concentrations of Baltic Sea individuals tend to decrease. It seems that the amino acid regulation of these frost-sensitive animals is of minor importance.

Glycine and taurine are the most important amino acids for osmotic regulation (Lange 1972, Zandee *et al.* 1980). These compounds constitute about 73 % of the whole amino acid content in the adductor muscle of winter-adapted North Sea individuals and 65 % in winter-adapted Baltic Sea mussels. During exposure to –10°C, the concentration of the low-molecular glycine of winter-adapted North Sea mussels rises, whereas its content in Baltic Sea animals declines.

Zandee *et al.* (1980) found distinct fluctuations of amino acids in *M. edulis* during the course of a year: the concentration of aspartate and glycine in the adductor muscle increased during winter and spring. The results of the present study are consistent with these earlier observations. Most amino acids (which occur in lower concentrations) reach their highest levels in summer-adapted animals, whereas the concentration of glycine increases in winter-adapted mussels. Thus, glycine contributes prominently to the regulation of osmolarity during exposure to cold.

Mytilus edulis is an osmoconforming organism. Our study shows that Baltic Sea individuals exhibit a much lower intracellular amino acid pool in comparison with North Sea animals (cf. Gilles 1972). Changes in freezing tolerance resulting from salinity acclimation are among others related to changes in osmotically active substances in the body fluids. Thus, acclimation to low salinity may decrease the freezing tolerance by raising the melting point of the tissue water on a colligative basis. The amount of tissue water, frozen at any sub-freezing temperature, will then be increased, leading to lethal dehydration to cells of brackish water animals (Aarset 1982). Probably, their cells are damaged before the adaptation to winter conditions can become effective. Thus, no effect of temperature adaptation could be found in Baltic Sea mussels. The reduction of amino acid concentrations and especially of glycine after a few hours of exposure to cold is probably caused by disruption of the membranes. In contrast, higher intracellular amino acid concentrations of North Sea mussels inhibit water extraction and a critical shrinkage of the cells. These animals are able to continue the isosmotic regulation of their intracellular amino acid pool during extracellular freezing.

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References

Aarset, A.V., 1982. Freezing tolerance in intertidal invertebrates (a review). – Comp. Biochem. Physiol. 73A: 571-580.

Deaton, L.E., J.G.S. Derby, N. Subhedar & M.J. Greenberg, 1989. Osmoregulation and salinity tolerance in two species of bivalve molluscs: *Limnoperna fortunei* and *Mytilopsis leucophaeta*. – J. exp. mar. Biol. Ecol. 133: 67-79.

Freeman, R.F.H. & T.J. Shuttleworth, 1977. Distribution of water in *Arenicola marina* (L.) equilibrated to diluted sea water. – J. mar. biol. Ass. U.K. 57: 501-519.

Gilles, R., 1972. Osmoregulation in three molluscs: *Acanthochitona discrepans* (Brown), *Glycymeris glycymeris* (L.) and *Mytilus edulis* (L.). – Biol. Bull. 142: 25-35.