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Variation in genetic traits of the lugworm *Arenicola marina*: temperature related expression of mitochondrial allozymes?

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ABSTRACT: Genetic traits of the lugworm *Arenicola marina* were determined for 4 Atlantic populations from France to Norway and compared with a population from the sub-arctic White Sea in Russia. Seven loci were analysed using horizontal starch gel electrophoresis. A low heterozygosity (0.09 to 0.17) and a non-significant heterozygote deficiency were found in all populations. The genetic identity between lugworms of European Atlantic populations was high, whereas similarity of the Atlantic populations with the population from the White Sea was low. The gene flow between the Atlantic and the White Sea populations must be considered negligible, as deduced from the average high and significant gene differentiation F_{ST}. In particular, differences in allele frequencies of glucose phosphate isomerase (*Gpi*) and phosphoglucomutase (*Pgm*) showed that the White Sea population differed significantly from the others. A very strong correlation existed between the frequency of the alleles of isocitrate dehydrogenases 2-A and -B (*Idh*2-A and *Idh*2-B) and the average water temperature. It is concluded that temperature had a selective influence on isocitrate dehydrogenase 2, which, in contrast to isocitrate dehydrogenase 1, was identified as a mitochondrial enzyme. These findings support the hypothesis that mitochondria play a key role in temperature adaptation and the adjustment of critical temperatures.

KEY WORDS: *Arenicola marina* · Genetics · Geographic cline · Isozyme · Lugworm · Polychaeta · Temperature · Mitochondria

INTRODUCTION

Differences in the metabolic reaction to temperature changes have been observed between 2 populations of the lugworm *Arenicola marina* from the German North Sea and from the Russian White Sea (Sommer et al. 1997). The tolerance of both populations to temperature fluctuations is limited, as indicated by the existence of low and high critical temperatures (Tc_I and Tc_{II}), both of which are characterised by the onset of anaerobic metabolism. In White Sea lugworms these critical temperatures were shifted to lower values when compared with North Sea specimens. Additionally, the ability of North Sea lugworms to adapt to temperatures beyond the critical temperatures was poor compared to that of White Sea lugworms (Sommer et al. 1996, 1997). The larger range of temperature fluctuations at the White Sea was seen as a reason for the higher adaptational capacity of the sub-polar lugworms.

The exact mechanisms enabling survival under extreme temperature conditions and causing an adaptive shift of the Tc are unknown. A hypothesis was developed that, among other mechanisms, Tc values are set by an adjustment of mitochondrial density and thus, aerobic capacity (Pörtner et al. 1997, Sommer et al. 1997). The differences in the ability to acclimate to temperature changes may, therefore, be linked to differences in the capacity to adjust mitochondrial density. This may either be explained as (phenotypic) acclimation of congenial populations to environmental temperatures or as (genetic) adaptation of remote pop-

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ulations (Nevo 1978). Statistical, biochemical and physiological correlates provide circumstantial evidence supporting the hypothesis that environmental heterogeneity can be a major factor in maintaining and structuring genetic variation in natural populations. However, direct experimental evidence establishing cause-effect relationships between ecophysiological and genetic structures is still sparse (e.g. Hilbish et al. 1982, Hoffmann & Parsons 1994).

As a further step toward unravelling the differences in temperature acclimation in lugworms, whether it is phenotypic acclimation or genetic adaptation, the degree of relationship between the different populations of lugworms, from the North Sea and White Sea, was assessed by determination of the genetic constitution through electrophoretic isoenzyme analysis. For an identification of eventual geographic patterns in the genetic variability of the lugworm, some additional populations at more southern (warmer) and northern (sub-arctic) locations were sampled in the Oosterschelde (Netherlands), Gironde (France) and near Tromsø (Norway).

MATERIAL AND METHODS

At each sampling station (Fig. 1) lugworms were collected from intertidal flats between mean tidal level and low water level. The stations were located at: (1) France, Bay of Arcachon, 44° 40.5' N, 1° 11.0' W, (2) The Netherlands, Oosterschelde sea arm, Yerseke, 51° 29.5' N, 4° 03.5' E, (3) Germany, Wadden Sea, 53° 42' N, 8° 35' E, (4) Norway, Tromsø, 69° 38.8' N, 18° 54.3' E and (5) Russia, White Sea, Kandalaksha Bay, Chupa Inlet, 66° 20.8' N, 33° 35.8' E.

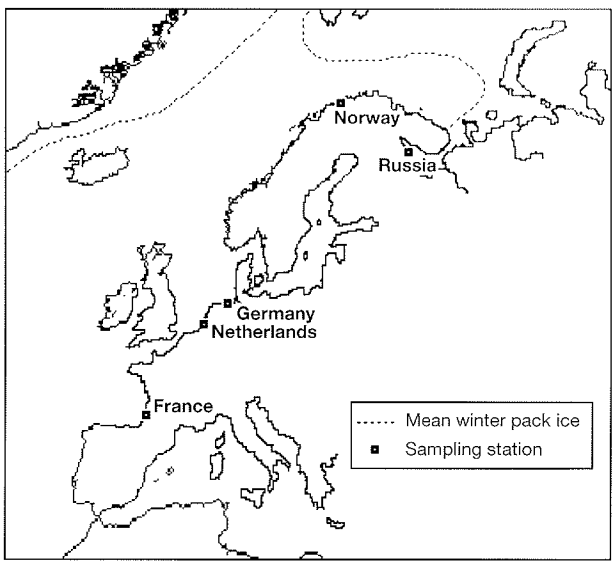

Fig. 1. Sampling stations in the Atlantic (France, Bay of Arcachon; The Netherlands, Oosterschelde sea arm; Germany, Wadden Sea near Bremerhaven; Norway, near Tromsø) and in the White Sea (Russia near Kartesh)

Heads and tails of the worms were dissected, gonads and intestines were removed and only the body wall musculature was frozen in liquid nitrogen. The genetic constitution of the worms was examined using electrophoretic isoenzyme analysis of 7 loci according to Menken (1982) and Hummel et al. (1995): glucose phosphate isomerase (*Gpi*, E.C. 5.3.1.9), NADP-dependent isocitrate dehydrogenase 1 and 2 (*Idh*, E.C. 1.1.1.42), malate dehydrogenase (*Mdh*, E.C. 1.1.1.37), malic enzyme (*Me*, E.C. 1.1.1.40), phosphogluconate dehydrogenase (*Pgd*, E.C. 1.1.1.44), and phosphoglucomutase (*Pgm*, E.C. 5.4.2.2). Fractions of the body walls (about 0.5 g) of 40 to 80 lugworms were homogenized individually for a few seconds in about 0.2 ml of gel buffer using a hand-made mortar and pestle. Electrophoresis was carried out in horizontal 12% starch gels (50% Sigma, 50% Connaught) at a temperature of 0°C. The buffer systems used were Tris-citric acid gel buffer (8 and 3 mM resp.; pH 6.7) and Tris-citric acid electrode buffer (0.223 and 0.086 M resp.; pH 6.3). The electrophoresis was performed for 5 h with a constant current of 100 mA. Staining procedures used Bush B Tris-hydrochloric acid (0.102 M; pH 8.4) according to Menken (1982).

The fastest allele is called A, the slower B, C, and so on. The data were analysed and statistically tested for allele frequencies, heterozygosity, conformance to Hardy-Weinberg equilibrium [fixation index F_{IS}; approximating the deviation of the observed heterozygosity from the expected one (Ho – He)/He], coefficient of gene differentiation (fixation index F_{ST}; measure of differences in allele frequencies at each locus between populations) and genetic identities of genes between populations (standard genetic identity according to Nei 1975) by the Biosys computer programme (Swofford & Selander 1981). Differences in allele frequencies and heterozygote frequencies of different groups were tested with the χ^2 analysis (Sokal & Rohlf 1995). All statistics were performed with Bonferroni correction. The *F*-statistics F_{IS} and F_{ST} are defined according to Nei (1977), and have properties similar to that of Wright's (1965) definition. F_{IS} measures the deviation of genotype frequencies from Hardy-Weinberg proportions and the null hypothesis $F_{IS} = 0$ was tested for significance with $\chi^2 = NF_{IS}^2(b-1)$ and $b(b-1)/2$ degrees of freedom (N is the number of specimens analysed in the sub-populations, and b is number of alleles) (Li 1955). F_{IS} measures the degree of genetic differentiation of sub-populations and was tested for significance with $\chi^2 = 2NF_{ST}(b-1)$ and df = $(b-1)(n-1)$ (n is the number of sub-populations) (Workman & Niswander

1970). Computation of F_{IS} is not appropriate for almost-monomorphic loci (i.e. the dominant allele has a frequency >0.8) because of the predominant influence of rare alleles. These statistics were performed with Bonferroni correction, at a critical probability level of $a' = 0.05/x$ (x is number of repetitions of the same test, i.e. 4 for F_{IS} and 8 for F_{ST}; Sokal & Rohlf 1995).

For preparation of mitochondria, about 1.5 g of body wall tissue was homogenized in 40 ml buffer containing 40 mM Tris (pH 7.5) and 0.55 M glycine as well as 0.25 M saccharose, 4 mM EDTA and 0.2% bovine serum albumin. The homogenate was centrifuged at 4000 × g for 15 min at 0°C. Mitochondria were found in the pellet after a second centrifugation for 60 min at 16000 × g. The pellets were further used for electrophoretic isoenzyme analysis and treated as the body wall sections described above.

RESULTS AND DISCUSSION

The loci *Mdh*, *Pgd*, *Me* and *Idh*1 were monomorphic according to the 5% criterion (Table 1). The other loci had a higher allelic variability, but the 2 most common alleles together had a frequency of 0.9 or more (Table 1). The average heterozygosity across the 7 loci was around 0.13 (Table 1). A trend toward heterozygote deficiency (F_{IS} = 0.09) occurred, yet the deviations from Hardy-Weinberg equilibrium were, in all but 1 example (*Pgm* in the Russian population), non-significant (Table 1). Such a heterozygote deficiency trend is a common, but not yet understood, phenomenon in marine invertebrates (Berger 1983, Singh & Green 1984, Zouros 1987, Zouros & Mallet 1989, Gaffney 1994).

The genetic diversity, as measured by heterozygosity, of the lugworm is low when compared to bivalves analysed in the same geographic territory with the same set of isoenzymes. In bivalves with a strong gene flow, *Cerastoderma edule*, *Macoma*

Table 1. *Arenicola marina*. Allele (A–F) frequencies and measures of genetic variability in lugworm populations from the Atlantic (Nether.: The Netherlands) and the White Sea (see Fig. 1). N = number of specimens; for abbreviation of isoenzymes see 'Materials and methods'; He = expected heterozygosity; Ho = observed heterozygosity; F_{IS} = conformance to Hardy-Weinberg equilibrium, F_{ST} = gene differentiation; n alleles = average number of alleles. Bonferroni correction for tests on significance of F_{IS}: $a' = a/4$; Bonferroni correction for tests on significance of F_{ST}: $a' = a/8$; *p < 0.05, **p < 0.01, na: not applicable

		Population					F_{ST}	
		France	Nether.	Germany	Norway	Russia	All	All – Russia
	N:	80	80	80	40	80	[avg F_{IS}]	
Mdh	A	0.006	0.063	0.063	0.025	0.000	0.024	0.016
	B	0.988	0.938	0.931	0.975	1.000	0.023	0.014
	C	0.006	0.000	0.006	0.000	0.000	0.004	0.003
Avg F_{ST}							0.023	0.015
Ho		0.025	0.125	0.138	0.050	0.000		
He		0.025	0.118	0.130	0.049	0.000		
Pgd	A	0.050	0.019	0.056	0.013	0.000	0.018	0.011
	B	0.950	0.975	0.938	0.988	1.000	0.018	0.011
	D	0.000	0.006	0.006	0.000	0.000	0.004	0.003
F_{ST}							0.018	0.011
Ho		0.100	0.050	0.125	0.025	0.000		
He		0.096	0.049	0.119	0.025	0.000		
Me	A	0.006	0.038	0.025	0.063	0.000	0.020	0.013
	B	0.988	0.925	0.962	0.938	1.000	0.023	0.013
	C	0.006	0.013	0.013	0.000	0.000	0.005	0.003
	D	0.000	0.025	0.000	0.000	0.000	0.020	0.019
F_{ST}							0.020	0.013
Ho		0.025	0.125	0.075	0.125	0.000		
He		0.025	0.143	0.073	0.119	0.000		
Pgm	A	0.013	0.038	0.025	0.112	0.006	0.040	0.034
	B	0.988	0.944	0.925	0.837	0.287	0.414**	0.042
	C	0.000	0.019	0.038	0.013	0.706	0.581**	0.011
	D	0.000	0.000	0.013	0.038	0.000	0.021	0.019
F_{ST}							0.426**	0.034
Ho		0.025	0.063	0.100	0.275	0.287		
He		0.025	0.108	0.143	0.288	0.421		
F_{IS} [avg]		na	na	na	na	0.313**	[0.233]	na
Gpi	A	0.031	0.106	0.081	0.000	0.025	0.033	0.033
	B	0.956	0.875	0.900	1.000	0.700	0.105*	0.038
	C	0.013	0.013	0.019	0.000	0.000	0.006	0.004
	D	0.000	0.000	0.000	0.000	0.269	0.227**	0.000
	E	0.000	0.006	0.000	0.000	0.000	0.005	0.005
	F	0.000	0.000	0.000	0.000	0.006	0.005	0.000
F_{ST}							0.113*	0.033
Ho		0.087	0.225	0.138	0.000	0.412		
He		0.085	0.224	0.184	0.000	0.440		
F_{IS} [avg]		na	na	na	na	0.056	[0.070]	[0.082]
*Idh*1	A	0.006	0.013	0.006	0.000	0.013	0.003	0.003
	B	0.994	0.962	0.981	1.000	0.988	0.011	0.013
	C	0.000	0.025	0.013	0.000	0.000	0.013	0.012
F_{ST}							0.010	0.011
Ho		0.013	0.050	0.025	0.000	0.025		
He		0.013	0.073	0.037	0.000	0.025		
*Idh*2	A	0.669	0.531	0.469	0.237	0.100	0.175**	0.097*
	B	0.325	0.463	0.525	0.762	0.900	0.180**	0.100*
	C	0.000	0.006	0.006	0.000	0.000	0.004	0.003
	D	0.006	0.000	0.000	0.000	0.000	0.005	0.005
F_{ST}							0.176**	0.098**
Ho		0.375	0.575	0.438	0.275	0.175		
He		0.450	0.507	0.508	0.367	0.181		
F_{IS} [avg]		0.161	−0.141	0.133	0.241	n.a	[0.080]	[0.085]
Averages								
F_{ST}							0.205**	0.059
Ho		0.093	0.173	0.148	0.107	0.129		
He		0.103	0.175	0.171	0.121	0.152		
F_{IS} [avg]		0.097	0.011	0.135	0.116	0.151	[0.092]	[0.076]
n alleles		2.6	3.1	3.1	2.0	2.0		

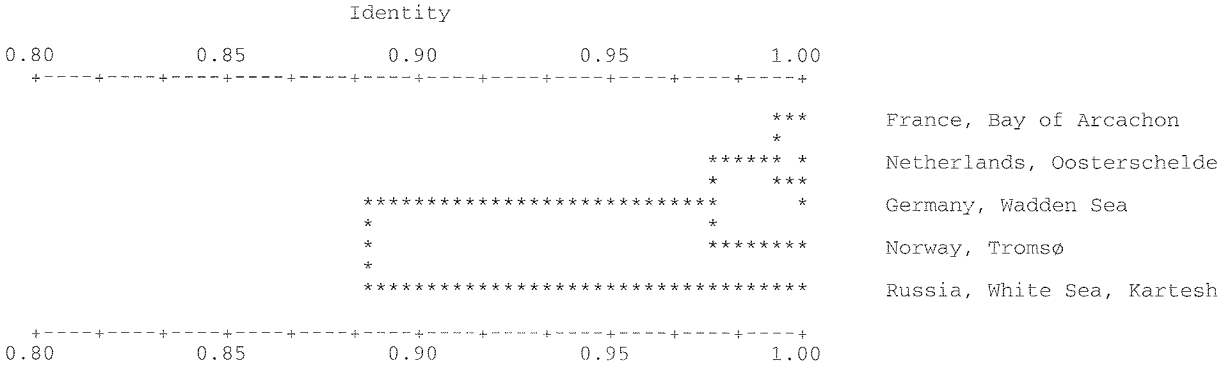

Fig. 2. *Arenicola marina*. Cluster analysis on the genetic identity between lugworm populations using the unweighted pair group method (coefficient used: Nei's genetic identity)

balthica and *Mytilus edulis*, the heterozygosity ranged from 0.21 to 0.39 (vs 0.09 to 0.17 in lugworms) (Hummel et al. 1989, 1994, 1995). However, in the cockle *Cerastoderma glaucum*, a species with restricted gene flow inhabiting semi-isolated shallow non-tidal biotopes and estuaries, the heterozygosity was as low as in the lugworm, 0.09 to 0.17 (Hummel et al. 1994). The picture for other polychaetes is not as uniform as in the abovementioned bivalves; but, similar to the lugworms, the genetic diversity of other polychaetes seems to be lower in general. In *Hediste limnicola*, *H. diversicolor* and *H. japonica* heterozygosity ranged from 0.01 to 0.02 (Fong & Garthwaite 1994), in *Neanthes succinea* from 0.02 to 0.04 (Abbiati & Maltagliati 1992), and in 3 alvinellide polychaetes (*Alvinella pompejana*, *A. caudata* and *Paralvinella grasslei*) from 0.10 to 0.24 (Jollivet et al. 1995). Seven *Nephtys* species from different European locations proved even to be monomorphic in all 6 isoenzyme systems investigated (Schmidt & Westheide 1994).

The genetic identities (Nei 1975) between (the Atlantic) lugworms from France, through The Netherlands and Germany, to Norway were high (Fig. 2). The genetic identity of the Atlantic populations with the Russian White Sea population was low (0.89). A genetic identity below 0.9 might indicate that the populations belong to different subspecies (Avise 1974, Thorpe 1983). Confusion with the recently recognized black lugworm *Arenicola defodiens* (Cadman & Nelson-Smith 1990, 1993) is thought to be of no importance. The allelic patterns distinctive for *A. defodiens* at the diagnostic loci *Pgd* and *Gpi* (Cadman & Nelson-Smith 1990) did not occur at all in our populations.

The differentiation between the Atlantic stations and the Russian station is also indicated by the average high and significant gene differentiation F_{ST} (on average 0.21) when including the Russian station, but low and non-significant (F_{ST} = 0.06) when excluding the Russian station (Table 1). For marine bivalves with high gene flow, the average gene differentiation amounts to 0.01 to 0.03 between populations at geographic distances of hundreds of kilometres, and 0.04 to 0.06 at distances of thousands of kilometres (Skibinski et al. 1983, Dillon & Manzi 1992, Grant et al. 1992, Sarver et al. 1992, Saavedra et al. 1993, Hummel et al. 1994, 1995). Similarly, the gene differentiation among the Atlantic populations, at mutual distances of several thousands of kilometres, amounted to 0.06. In populations with limited gene flow, a much higher F_{ST} can be found, e.g. 0.19 as found for *Cerastoderma glaucum* (Hummel et al. 1994). Therefore, gene flow between *Arenicola marina* populations from the Atlantic coasts can be considered to be strong, whereas gene flow between the White Sea and Atlantic populations must be considered negligible. Although *A. marina* has no pelagic larval stage (eggs are spawned at the sediment surface, hatching larvae immediately penetrate into the sediment), transport of eggs by currents and migration during post-larval stages has been observed frequently (Wolff 1973). Thus, transport by currents and migration are strong enough in the Atlantic to cause a considerable gene flow. The low or absent gene flow between the Atlantic and the White Sea is then probably due to the geographic and hydrographic isolation of the White Sea (Zenkevitch 1963). Very strong currents at the outer parts of the White Sea (in Gorlo and Voronca sounds) cause very violent turbulences of the whole water column. As a consequence the sea bed is covered with an extremely hard sediment which is poor in fauna and flora. Moreover, these tidal oscillations do not bring water of the Barents Sea into the White Sea. They only shift the masses of water within Gorlo sound, causing a separation of water masses and building a barrier for larvae or gametes. This seperation is probably so strong that even for the bivalve *Macoma balthica*, which has a pelagic larval stage of several weeks, a similar hampered gene flow was observed between the Atlantic and White Sea populations (Hummel et al. 1997).

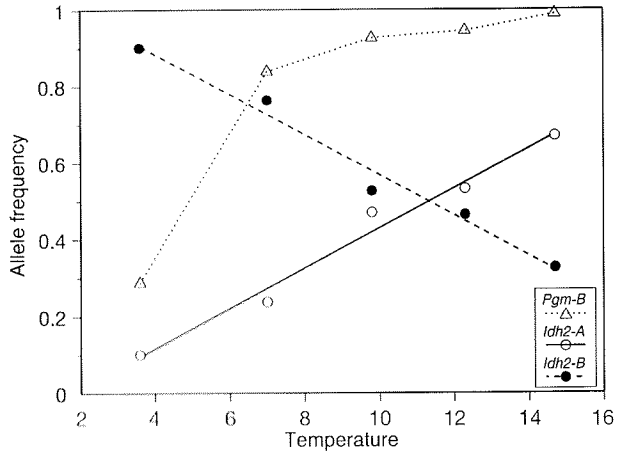

Fig. 3. *Arenicola marina*. Relation between allele frequencies of various enzymes in lugworms and the annual average temperature at the sampling locations [temperatures for France and The Netherlands from Hummel et al. 1995; for Germany from Becker 1981; for Norway from Treshnikov 1985; for Russia from T. Bek and O. Kozlova (based on daily measurements in 1987–1991, WSBS Poyakonda, Moscow State University, pers. comm.)]

The strong gene differentiation between the Atlantic and White Sea populations coincided with abrupt changes in the allele frequencies of *Gpi* and *Pgm* (Table 1, Fig. 3). A χ^2 analysis of differences in allele frequencies of *Gpi* and *Pgm* showed that indeed only the White Sea population differed significantly from all others (Table 2). In contrast, the changes in allele frequencies of *Idh*2 were more gradual when going from France to Russia (Table 2), as can be deduced from the gradually increasing χ^2 with increasing distance between stations, marking, e.g., a significant difference between France and Norway but no significant difference between Norway and the White Sea. A significant gene differentiation for *Idh*2 between the Atlantic populations was still found when the White Sea station was excluded (Table 1). The gradual geographic cline is most remarkable for the allele frequencies of *Idh*2-A and *Idh*2-B: a very strong correlation between the allele frequency and annual average water temperature existed, even when the Russian station was excluded (Fig. 3; $r = 0.99$, $p < 0.01$ for both comparisons).

Table 2. Statistical analyses (χ^2) of differences in allele frequencies between *Arenicola marina* populations for the loci *Gpi*, *Pgm* and *Idh*2. $^*p < 0.05$, $^{**}p < 0.01$

	The Netherl.	Germany	Norway	Russia
Gpi				
France	4.05	2.01	1.81	26.26**
The Netherlands		0.87	5.45	29.08**
Germany			4.29	27.89**
Norway				15.00*
Pgm				
France	2.58	4.55	10.41	87.52**
The Netherlands		1.78	5.76	81.99**
Germany			5.31	76.82**
Norway				54.40**
Idh2				
France	3.20	6.58	20.54**	55.75**
The Netherlands		0.62	9.80	35.24**
Germany			6.38	27.52**
Norway				4.03

Geographic clines in genotypes within a species, especially in a north-south direction, are a common phenomenon, and are mostly related to temperature and salinity (Koehn et al. 1976, 1980b, Endler 1977, Theisen 1978, Buroker 1983, Burton 1983, Rose 1984, Hoffman 1985, Dillon & Manzi 1992, Hummel et al. 1995). The direct cause of such clines is not clearly known, although for leucine aminopeptidase (*Lap*) in *Mytilus edulis* and *Gpi* in *Metridium senile* it has been shown that differential activities of allozymes are coupled to temperature or salinity (Koehn et al. 1976, 1980a, Koehn & Siebenaller 1981, Hoffmann 1985). This may lead to genotype dependent differential selection, most probably during juvenile stages (Levinton & Lassen 1978, Hilbish 1985). Some clines might also be caused by introgression of races or subspecies (Levinton & Lassen 1978, Theisen 1978, Beaumont 1982, Koehn et al. 1984, Väinölä & Varvio 1989). Yet, the genetic similarity of the Atlantic populations in this study clearly showed no difference at subspecies levels. So, the geographic cline found in this study is most probably an adaptive variation connected to temperature. Moreover, when a substantial migration between different populations exists, then the selective pressure of an environmental factor must be very strong, so that a specific allele can be fixed in the genome of a population (Pogson 1987). Since gene flow is strong between the Atlantic populations ($F_{ST} = 0.06$), we can conclude that the selective pressure of temperature on the *Idh* loci must be considerable.

The electrophoretic isoenzyme analysis of the mitochondrial fraction identified *Idh*2 to be a mitochondrial enzyme, whereas *Idh*1 seems to be found in the cytosol. Further study revealed that latitudinal cold adaptation in *Arenicola marina* is linked to mitochondrial proliferation and an increase in the activity of cytochrome oxidase (A. Sommer & H. Pörtner unpubl.) suggesting that mitochondria are an important site of temperature adaptation. This finding agrees with the picture arising from studies in cold ocean fish (Guderley 1997) and suggests that cold adaptation in general appears to be achieved by an increase in mitochondrial density as well as in oxidative capacity of individual mitochondria. This phenomenon led to the general hypothesis that changes in the density and functional properties of

mitochondria are involved in setting the critical temperatures as the upper and lower limits of temperature tolerance (Sommer et al. 1997, Pörtner et al. 1997).

Expression of different *Idh*2 isoenzymes with changing temperature may play an important role in this context. *Idh* has a central role in regulating the flow through the citric acid cycle (Stryer 1990), and therewith in metabolic regulation. Nothing is known about the differences in kinetic properties between the 2 isoforms *Idh*2-A and -B. However, these differences should optimize function at the respective temperatures and may involve a maintenance of K_m values and maximum velocity at low temperatures. Together with mitochondrial proliferation and the rise in aerobic capacity this could explain the observed shift in critical temperatures at the whole-animal level (Sommer et al. 1996, 1997, Sommer & Pörtner unpubl.).

In conclusion, the genetic constitution of the specimens studied showed that the genetic identity for the lugworm in European Atlantic populations is high, whereas the similarity with a population from the White Sea is low. Both geographic and hydrographic isolation of the White Sea as well as average water temperatures seem to influence the genetic constitution of the populations. Whereas the expression of *Gpi* and *Pgm* seems to be influenced by geographic and hydrographic isolation, the expression of *Idh*2 isoforms is predominantly influenced by temperature.

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