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Hsp70 is not a sensitive indicator of thermal limitation in *Gadus morhua*

M. Zakhartsev*†, B. De Wachter‡, T. Johansen§, H. O. Pörtner¶ and R. Blust‡

**Biochemical Engineering, International University of Bremen, Campus Ring 1, 28759 Bremen, Germany, ‡Department of Biology, University of Antwerp, Antwerp, Belgium, §Department of Fisheries and Marine Biology, University of Bergen, P. O. Box 7800, N-5020 Bergen, Norway and ¶Marine Biology/Ecological Physiology, Alfred Wegener Institute for Polar and Marine Research, D-27568 Bremerhaven, Germany*

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The levels of heat-shock proteins of the 70 kDa family (Hsp70s) were measured in different soft tissues of Atlantic cod *Gadus morhua* from different locations and after exposure to various thermal conditions: acute temperature increments (1° C day^{-1}), mid-term (73 days at 4–15° C) and long-term thermal acclimation (278 days at 8–15° C), and seasonal and latitudinal temperature variations (field samples). Tissue specific distribution patterns of Hsp70s were observed: liver > gills > red blood cells > brain > white muscle. Thus, different tissues may have required different levels of protection by Hsp70s, and possibly this was related to the rate of protein synthesis. There were no differences in tissue Hsp70s between Arctic cod populations (Arctic, *i.e.* Barents and White Seas, Norwegian coast, and North or Baltic Seas). No changes in Hsp70s levels were observed in response to temperature variation of any intensity (acute fluctuation or seasonal and latitudinal) within the range of physiological temperatures (4–15° C) in wild and laboratory Atlantic cod. This confirms previous observations that changes in Hsp70 caused by such temperature variation are often small in fishes. Probably, the constitutive level of Hsp70s in Atlantic cod was high enough to overcome potentially harmful effects of temperature variations within the physiological range. A suppressing effect of high temperature (15° C) has already been observed at a systematic level (as reduced rate of somatic growth), whereas it is not reflected in modified Hsp70s. Therefore, Hsp70s apparently played a secondary role in defining thermal tolerance limits in Atlantic cod. These conclusions are in line with a recent concept of thermal tolerance which indicated that the first line of thermal limitation in the cold and warm is a loss in aerobic scope.

Key words: acute temperature; *Gadus morhua*; heat-shock protein; Hsp70; temperature variation.

INTRODUCTION

Heat-shock proteins (Hsps) are a family of molecular chaperones found in both prokaryotes and eukaryotes (Ellis, 1996). These proteins are synthesized both constitutively (to assist the folding and maturation of housekeeping proteins)

†Author to whom correspondence should be addressed. Tel.: +49 421 200 3121; fax: +49 421 200 3229; email: m.zakhartsev@iu-bremen.de

and in response to a wide range of biotic and abiotic stressors (stress-induced). Therefore, Hsps are also referred to as stress proteins. These chaperones are commonly named according to their molecular mass in kilodaltons (kDa). The Hsps have been grouped into several classes (families) on the basis of their size and sequence homology (Hsp100, Hsp90, Hsp70, Hsp60, small Hsp and ubiquitin) and every family comprises multiple members. All Hsps are seen to be involved in the regulation of protein homeostasis (Feder & Hofmann, 1999) or the protection of molecules from denaturation (Zietara & Skorkowski, 1995). Accordingly, a positive correlation exists between the levels of induced Hsps and scope for growth (SFG) (Sanders *et al.*, 1991).

Induction of one or more classes of stress-induced Hsps in response to heat stress has been observed in a large number of species belonging to all major phylogenetic lineages (Feder & Hofmann, 1999) and fishes are no exception to this rule (Yu *et al.*, 1994; diIorio *et al.*, 1996; Iwama *et al.*, 1998; Currie *et al.*, 2000). Therefore, elevated levels of Hsps became one of the most popular indicators of stress conditions induced by temperature (Dietz & Somero, 1993; Hofmann & Somero, 1995).

As a consequence of the protection of proteins by Hsps, they increase tolerance to extreme temperatures in intertidal fishes (Nakano & Iwama, 2002). Increased levels of Hsps, after an initial temperature stress, are interpreted to protect tissues from subsequent lethal stress which would normally result in structural damage (Brown *et al.*, 1992). Increased levels of Hsps found during extended periods of environmental stress suggest an important role of Hsps during long-term adaptation (Sanders & Martin, 1993). A few studies have shown that differences in the levels of constitutive and induced Hsps and the number of isoforms of Hsps were related to the natural environment of the species (Sharp *et al.*, 1994). Animals living in an unstable (*e.g.* intertidal) environment showed a higher concentration of constitutive Hsps than specimens of more closely related species living in a stable environment (Hofmann & Somero, 1995; Carpenter & Hofmann, 2000).

Some species of marine fishes have a wide latitudinal distribution, thus these species are exposed to significantly different environmental regimes in various parts of their distribution range. Atlantic cod *Gadus morhua* L. displays distinct northern and southern distribution limits in the North Atlantic with separate populations which clearly have adapted to significantly different temperature regimes (Table I). This species was therefore chosen for the present study in order to test the hypothesis that Hsp70s correlate with the thermal environment and may be used as a diagnostic marker of resistance to thermal stress in fishes.

The Hsp70s are wide spread stress biomarkers, which can be found in almost every living organism (Lewis *et al.*, 1999). Moreover, previous research on heat shock proteins from carp *Cyprinus caprio* L. has shown that mouse monoclonal anti-Hsp70 (from Sigma) are most suitable commercial antibodies to detect this group of Hsps in fishes (De Wachter *et al.*, 1998). Other classes of mouse antibodies (anti-Hsp90 and Hsp60) from different vendors showed lower cross-reactivity with fish Hsps. These circumstances determined the particular choice to monitor Hsp70s in Atlantic cod.

The following question was investigated in this study: Do different levels in Hsp70s exist between populations and are HPS70s suitable indicators of heat

TABLE I. Average water temperature and levels of salinity across sampling area (averaged for the water column between 0 and 10 m depth)

	Water temperature (° C)		Salinity		
Area	Season	Range	Season		Source
Wadden Sea (North Sea)	Spring	6–7	Spring	27	Brodte (2001)
	Summer	17–18	Summer	31	
Kiel Bight (Baltic Sea)	Spring*	2–4		14	Böhnecke & Dietrich (1951); Fischer *et al.* (1992)
	Summer	13			
Hafrsfjord (Norwegian Sea)	Spring*	3–5		34	Böhnecke & Dietrich (1951); Brodte (2001)
	Summer*	14–15			
Cape Kartesh (White Sea)	Winter	−1		19–24**	Babkov (1982); D. Lajus (pers. comm.)
	Spring	0			
	Summer	10			
	Autumn	5			
	Annual	4			

stress within the natural range of temperatures? The data are interpreted in the light of a recent hypothesis that a systemic to molecular hierarchy exists in thermal tolerance and that Hsps only respond to thermal stress once long-term thermal limits set by an oxygen limitation of thermal tolerance, have already been surpassed (Pörtner, 2002*a*, *b*).

MATERIALS AND METHODS

ANIMALS

Atlantic cod from different populations (North Sea, NSC; Norwegian coastal, NCC; North-east Atlantic, NEAC) were used for this research (Fig. 1). Fish were collected by offshore bottom trawling or were caught by hook and line. Laboratory experiment 2 was carried out on cultured 1 year-old Atlantic cod originating from offspring of coastal Atlantic cod from western Norway (NCC) and of Atlantic cod from the Barents Sea (NEAC). All fish were treated according to 'Guidelines for the treatment of animals in behavioural research and teaching' (Anon, 1998).

FIELD SAMPLING

Atlantic cod were collected by hook and line in the Russian White Sea (Cape Kartesh) in July to September 1998, and by offshore bottom trawling in the North Sea (Tiefe Rinne, Weisse Bank and Bruine Bank) in February 1999 and in the Baltic Sea (Kiel Bay) in April 1999 (Table II).

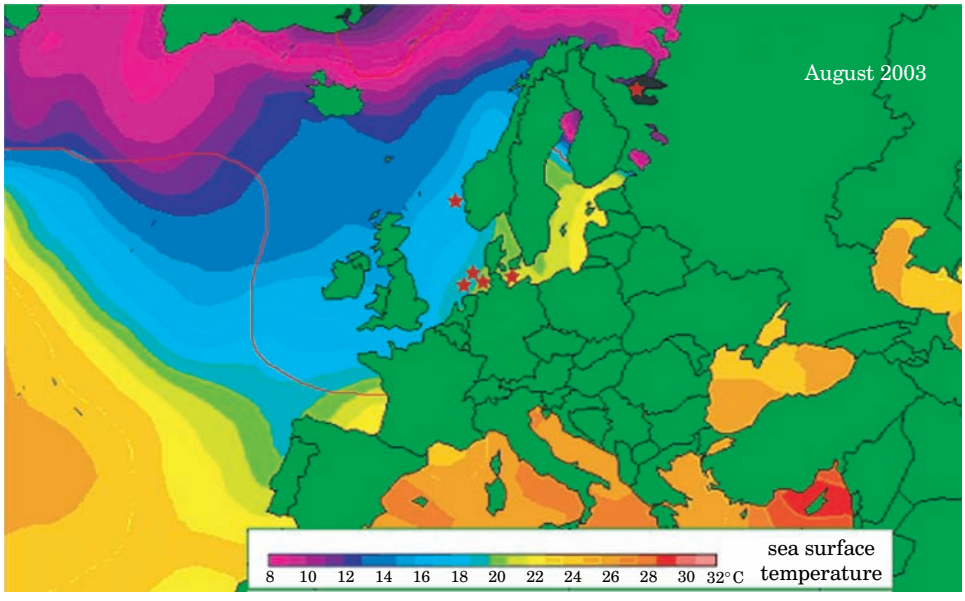

FIG. 1. Approximate distribution area (—) and sampling sites (★, for exact locations see Table II) of *Gadus morhua* were matched with surface water temperature in the North Atlantic. The surface water temperature stratification was obtained from http://www.wunderground.com/MAR/eum.html on 8 August 2003. On the southern edge of the distribution area for Atlantic cod (Wadden Sea) the temperature was *c.* 18–20° C, while on the northern edge (White Sea) it was *c.* 4–8° C. (Table I for more environmental conditions across the sampling area.)

ACUTE TEMPERATURE EXPOSURE

Experiment 1

The NSC were caught near Helgoland Island (Germany) at an environment temperature of 10·5° C. They were delivered to the Alfred Wegener Institute for Marine and Polar Research, Bremerhaven, Germany (AWI; http://www.awi-bremerhaven.de) and pre-acclimated at 10·5° C for 2 weeks. Fish were exposed to acute temperature increments (1° C day^{-1}) from 11 to 16·5° C ($n = 4$ for each temperature).

TABLE II. Details of field sampling for Atlantic cod

	Southern North Sea					
Area	Tiefe Rinne	Weisse Bank	Bruine Bank	Baltic Sea Kiel Bay	White Sea Cape Kartesh	
Position	54°10′ N; 07°54′ E	55°27′ N; 06°31′ E	53°45′ N; 04°16′ E	54°32′ N; 10°48′ E	66°20′ N; 33°40′ E	
Time	Feb. 1999	Feb. 1999	Feb. 1999	Apr. 1999	Jul. to Aug. 1998	Sep. 1998
Temperature (° C)*	5·5	5·5	5·5		13·6–14	10·5
Number of fish	32	81	65	102	71	11

*, sea surface temperature during the sampling.

LONG TERM TEMPERATURE ACCLIMATION

Experiment 2

A growth experiment with 270 cultured 1 year-old NCC and NEAC was carried out at the University of Bergen (The Department of Fisheries and Marine Biology at the High Technology Center in University of Bergen, Norway; http://www.ifm.uib.no). Fish of each strain were individually tagged with passive integrated transponder (PIT) tags and randomly divided into three groups. The growth experiment was carried out between 27 May 1998 and 5 March 1999 (278 days) in circular flow-through tanks (diameter 2 m) at 8, 12 and 15° C. Fresh sea water (salinity 34, oxygen content >90%) was supplied from a remote deep water inlet (*c.* 100 m depth) in a neighbouring fjord outside Bergen. Commercial dry-feed (Nor Aqua Innovation Ltd, Dirdal, Norway) was given in surplus and natural light regimes were simulated corresponding to latitude 60° N (Bergen, Norway).

Experiment 3

A growth experiment with NSC was carried out at the AWI between 27 April 1999 and 10 September 1999 (73 days). Most fish (total length, L_T, 20–33 cm, 84–378 g) were caught with a bottom trawl of RV 'Heincke' in April 1999 in the North Sea at the 'Tiefe Rinne' (Table II). Under slight narcosis with MS-222 (0·05 gl^{-1}) the fish were individually tagged with floy tags which were fixed under the second dorsal fin. The animals were kept in flow-through tanks at 4, 8, 12 and 15° C. Fresh, sea water (salinity 31, oxygen content >90%) was continuously supplied from a remote deep water inlet. Fish were fed daily with an excess of sprats *Sprattus sprattus* (L.) because these wild Atlantic cod did not feed on commercial food pellets.

HEAT SHOCK PROTEIN ANALYSIS

Sampling

At capture from the wild or at the end of the acclimation period fish were killed by 500 mg l^{-1} of 3-aminobenzoic acid (MS-222) in sea water (Ross & Ross, 1999), measured (wet body mass, wet liver mass and L_T) and then tissue samples (liver, intestine, gills, brain, heart, spleen, muscle and blood) were taken. Sampling time was reduced as much as possible; in general, it took <3 min from the killing of the fish until sample fixation. The samples were freeze clamped in liquid nitrogen (−195° C), transported on dry ice (−60–70° C), and stored in a deep freeze (−80° C).

Thawed tissue pieces were homogenized by Ultra-Turrax (IKA Labortechnik, Stanfen, Germany) in a homogenization buffer. Samples were centrifuged at $16 \times 10^3 g$ for 20 min at +4° C. Supernatants were collected and used for further analysis by Western blot and indirect non-competitive ELISA according to De Wachter *et al.* (1998).

Western blot

Western blots were used to detect the presence of the 70 kDa family of heat shock proteins (70 and 72 kDa) (Fig. 2). Samples were diluted (1/1, v/v) with loading buffer to a total protein concentration of 35 μg ml^{-1}, boiled for 5 min, and then 10–20 μl of the sample (*c.* 0·35–0·7 μg of total protein) were separated by vertical PAGE electrophoresis (Hoefer Mighty, Amersham Bioscience, Uppsala, Sweden) at 4° C. Two identical gels were run in parallel, one gel was stained for molecular mass determinations with Commassie R250 (Amersham Biosciences, 1998).

For the detection of Hsp70s the second gel was blotted on PolyScreen PVDF transfer membrane NEF1000 (NEN Life Science Product, Boston, MA, U.S.A.) using Hoefer TE62 Transphor II Cooling Unit (Amersham Bioscience). The Western blot was performed according to general practice described by Amersham Biosciences (1999). Monoclonal anti-Hsp70 mouse IgG_1 (clone BRM-22, Sigma St Louis, MO, U.S.A.) and rabbit anti-mouse IgG_1 horseradish peroxidase conjugate (Sigma) were used to

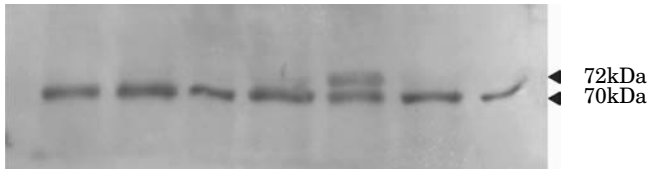

FIG. 2. Western blot of heat-shock proteins of the 70 KDa family (Hsp70 and Hsp 72) from different tissues of *Gadus morhua*. The total Hsp70s content was quantified by indirect ELISA. G, gills; S, spleen; Go, gonads; I, intestine; L, liver; B, brain; St, Hsp70 standard (from bovine brain, Sigma).

detect the fish Hsp70s. The images of both gels were matched to determine the molecular masses of the Hsp70 bands.

ELISA

An indirect non-competitive ELISA was used to quantify the concentrations of Hsp70s in different fish tissues. Homogenized samples were diluted with carbonate buffer to a total protein concentration of 35 μg ml^{-1}; boiled for 5 min and coated on a microplate (COSTAR, Cambridge, MA, U.S.A.) overnight at 4° C during gentle shaking in the dark.

The indirect non-competitive ELISA of fish Hsp70 has been performed according to general practice (Crowther, 1995) using monoclonal anti-Hsp70 mouse IgG_1 (clone BRM–22, Sigma) and rabbit anti-mouse IgG_1 alkaline phosphatase conjugate (Sigma) to detect fish Hsp70. Serial dilution of standard Hsp70 (from bovine brain, Sigma) was used to calculate the Hsp70 concentrations in ng of Hsp70 equivalents per μg of total proteins (ng Hsp70s μg^{-1} total protein).

Protein quantification

Total protein concentrations in the samples were analysed by the Bradford method using a commercial kit (BioRad, Hercules, CA, U.S.A.).

STATISTICS

Prior to any statistical test the normal distribution of the data was checked by the Kolmogorov-Smirnov test. Then ANOVA/MANOVA tests or correlation analyses [StatSoft, STATISTICA (data analysis software system), version 6; www.statsoft.com] were used to check for differences between groups, effects of independent factors and relationships between variables (Sokal & Rohlf, 1995). Linear, non-linear regression analyses and *F*-tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, U.S.A.; www.graphpad.com).

RESULTS

Heat-shock proteins of the 70 kDa family were found in tissues of Atlantic cod in two major distinctive isoforms, which were distinguished by Western blot, as 70 and 72 kDa proteins (Fig. 2). Since Western blot cannot be used for the 'absolute' quantification of Hsps indirect non-competitive ELISA was therefore used. ELISA with mouse monoclonal anti-Hsp70, however, has another limitation: it displays cross-reactivity with both isoforms of the 70 kDa heat-shock protein family. Therefore, the term 'Hsp70s' was used to emphasis that the

combined content of both isoforms of the 70 kDa heat-shock protein family was quantified by indirect immunoassay.

FIELD SAMPLES

MANOVA of Hsp70 in different tissues of field samples ($n = 35$) from the White and North Seas (Fig. 3) showed no significant effect of latitude and seasonal temperature variations (between 5·5 and 14° C) ($P = 0{\cdot}61$ and $P = 0{\cdot}26$, respectively). There was, however, significant effect of tissue type on Hsp70 ($P < 0{\cdot}001$; Fig. 3).

Hsp70s were observed in all examined soft tissues of Atlantic cod, at tissue specific levels in the sequence: liver > gills > red blood cells > brain > white muscle (Fig. 3). This sequence was not different between populations.

EXPERIMENT 1

The relationship between tissue Hsp70 content and temperature was a linear and non-significant ($P > 0{\cdot}05$) (Fig. 4). Further F-tests for slopes comparison did not reveal a significant difference among them and their difference from zero ($F_{4,109}$, $P = 0{\cdot}07$). Therefore the acute temperature increment (1° C day^{-1}) between 11 and 16·5° C had no effect on Hsp70 content in all tissues studied (Fig. 4). Additionally, the F-test revealed significant differences among intercepts ($F_{4,113}$, $P < 0{\cdot}001$), which indicates once again the tissue specific Hsp70 content.

EXPERIMENTS 2 AND 3

No differences in Hsp70s levels of liver (two-way ANOVA, $P > 0{\cdot}05$) were observed between three populations of Atlantic cod (NCC, NEAC and NSC) after mid-term and long-term acclimation (for 73 or 278 days; experiments 2 and 3) at 4, 8, 12 and 15° C (Fig. 5). Levels of liver Hsp70s indicated no correlation with acclimation temperature ($r = -0{\cdot}00274$, $P > 0{\cdot}05$, $n = 237$).

DISCUSSION

Atlantic Cod possess one or two isoforms of the heat-shock proteins 70 and 72kDa in all studied soft tissues (Fig. 2). This fact emphasizes the ubiquitous distribution and relevance of the Hsp70s in fish tissues. Since indirect ELISA with mouse monoclonal anti-Hsp70 does not distinguish between these isoforms, the present study gives the total content of both isoforms in the tissues.

Hsp70s showed tissue specific differences. The sequence of tissues with respect to Hsp70s was almost identical among populations regardless of latitudinal, seasonal and experimental temperature variations (Figs 3 and 4). This observation indicates hierarchy in tissue protection against environmental disturbance including heat shock. It is also very likely, however, that this differentiation depends on tissue specific features such as rate of protein synthesis and rate of protein exchange. It should be emphasized that liver and gills are the most active tissues in fishes in terms of protein synthesis (Lyndon & Houlihan, 1998).

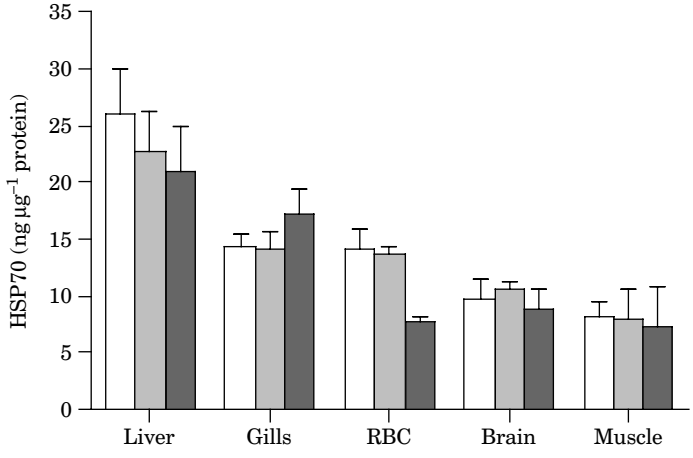

FIG. 3. Mean ± S.D. concentrations of Hsp70 in field samples of *Gadus morhua* from the White Sea in July 1998 at 14° C (□), the White Sea in September 1998 at 10·5° C (□) and the North Sea in February 1999 at 5·5° C (■) is different tissues (RBC, red blood cells). The Hsp70s were quantified by indirect ELISA. There were no significant differences between areas and temperatures ($P > 0{\cdot}05$) but there were between tissues ($P < 0{\cdot}001$).

The measurements of Hsp70s in Atlantic cod from acute heat exposure experiments, acclimation studies and field showed no significant difference in levels of Hsp70s between populations of this species, despite quite significant differences in environmental conditions (Fig. 1 and Table I). Thus, populations of the same species adapted to living in different thermal regimes display equal levels of Hsp70s. This indicates that the levels of Hsp70s are conserved within a species within their physiological range of temperatures (4–15° C).

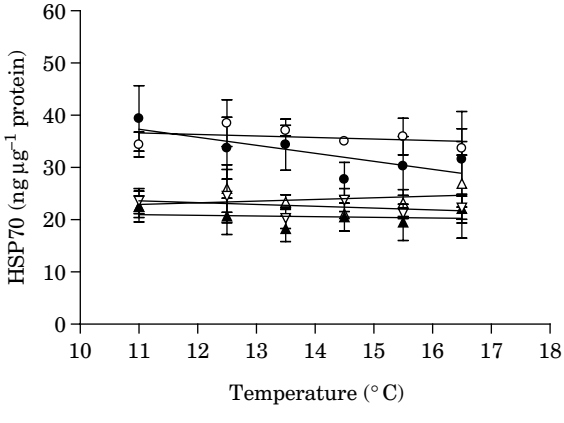

FIG. 4. Mean ± S.D. concentrations of Hsp70s during laboratory exposure of North Sea *Gadus morhua* (NSC) to acute temperature increments (1° C day^{-1}) from 11 to 16·5° C (experiment 1) in red blood cells (▽), muscle (▲), brain (○), gills (△) and liver (●). There was no significant difference in the effect of acute temperature change in the tissues ($P > 0{\cdot}05$) but there was a significant effect of tissue type ($P < 0{\cdot}001$). The Hsp70s were quantified by indirect ELISA.

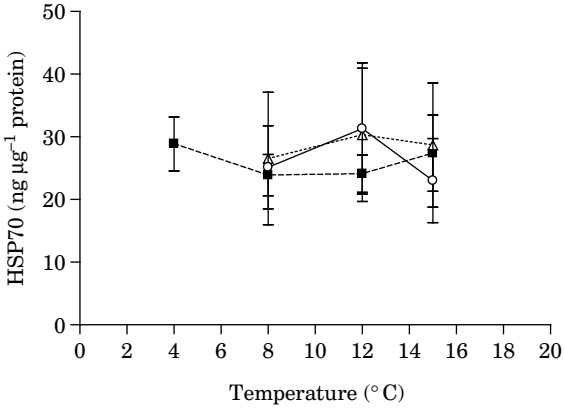

FIG. 5. Mean ± S.D. liver Hsp70 in Norwegian coastal (NCC), north-eastern cod (NEAC), and North Sea (NSC) *Gadus morhua* after acclimation during 73 or 278 days to different temperatures (experiment 2 and 3). The Hsp 70s were quantified by indirect ELISA. There is no effect of population or acclimation temperature on Hsp70 in liver (MANOVA, $P > 0{\cdot}05$; $n = 180$).

No significant variations of Hsp70s were observed in tissues of Atlantic cod exposed to different thermal conditions: acute temperature rise (Fig. 4), mid-term temperature acclimation (Fig. 5), long-term temperature acclimation (Fig. 5), seasonal and latitudinal temperature variation (Fig. 3). This might mean that temperature variation of any intensity (acute fluctuations and long-term seasonal changes) within the physiological range of temperatures (4–15° C) cannot be considered as stressful at the molecular level for Atlantic cod and the observed levels of Hsp70s are constitutive and determined by internal factors.

The data suggest that Hsp70s are poor indicators of environmental temperature stress in Atlantic cod within the range of physiological temperatures. Consequently, Hsp70s may be insensitive indicators of adverse environmental conditions for Atlantic cod. One of the reasons might be that the constitutive levels of Hsp70s in Atlantic cod may be high enough to cover potentially harmful temperature variations within the physiological range. These results are also supported by a limited number of literature reports on high constitutive levels but low levels of stress-induced changes in Hsp70s after heat shock in other fish species (Koban *et al.*, 1991; Yu *et al.*, 1994).

It is well known that acclimation temperature has a significant effect on growth rate of *G. morhua* which follows a bell shaped curve with a distinct thermal optimum. An average optimal temperature for the somatic growth of Atlantic cod is between 10 and 13° C (Jobling, 1988; Otterlei *et al.*, 1999; Pörtner *et al.*, 2001). At 4 and 15° C acclimation temperatures growth rate becomes limited. This effect, however, was not reflected by differences in the levels of Hsp70s between acclimation experiments. At 15° C the Hsp70s do not indicate stressful temperature conditions, although a certain systemic limitation already takes place as reflected by the growth rate decrements (Pörtner *et al.*, 2001). Apparently, these growth limitations are not linked to disturbances of protein homeostasis. These considerations would indicate that the disruption of molecular functions is not of primary importance in setting of physiological thermal

limits. This conclusion is supported by data obtained by Dietz & Somero (1993) for the marine teleosts buffalo sculpin *Enophrys bison* (Girard), speckled sanddab *Citharichthys stigmaeus* Jordan & Gilbert, English sole *Parophrys vetulus* Girard and staghorn sculpin *Leptocottus armatus* Girard collected in Yaquina Bay (Oregon, U.S.A.). These demersal species are usually present in shallow waters (0–500 m) of the North Pacific from California to Alaska (60° N − 30° N; http://www.fishbase.org). Fishes were acclimatized to 10° C and then were exposed to acutely elevated temperatures between 15 and 28° C (2 h exposure and a subsequent 1 h of recovery at 10° C) and then tissue content of Hsp70 and Hsp90 was measured. Common habitat temperature for these species is 10° C (Holland, 1969), and particularly for English sole the optimal growth temperature is 10–14° C (Gadomski & Caddell, 1991). The average Hsp induction thresholds (for different tissues and for both Hsp70 and Hsp90) for these species, however, were 20·3, 20·8, 24 and 28° C, respectively. This observation indicates that the temperature threshold for Hsp induction is almost 10° C above the common habitat temperature. Obviously these thresholds are much above the temperature threshold for long-term survival of these species. There is another indirect evidence of this reasoning based on the fact that the California halibut *Paralichthys californicus* (Ayres), which is a related species to English sole (in terms of order and ecology), displays successful egg hatching at 12, 16 and 20° C and at 8 and 24° C eggs died prior to embryo formation (Gadomski & Caddell, 1991). Therefore, these Hsp induction thresholds probably indicate that the disruption of molecular functions is secondary in the setting of physiological thermal limits or long-term survival limits.

The data are also in line with a recent hypothesis of a systemic to molecular hierarchy in thermal tolerance limits. Limited oxygen supply and a fall in aerobic scope are the first line of thermal limitation in both the cold and the warm which leads to performance reductions and may affect growth performance. Heat shock proteins are secondary in indicating long-term thermal limitation. Only in animals regularly but transiently subjected to thermal extremes, such as in the intertidal zone, may Hsp expression be finely tuned to respond to such thermal stress events, which regularly occur beyond the oxygen limits of thermal tolerance (Pörtner, 2002*a*, *b*).

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