



Effect of moderate hypoxia at three acclimation temperatures on stress responses in Atlantic cod with different haemoglobin types

Caroline Methling^{a,*}, Neelakanteswar Aluru^b, Mathilakath M. Vijayan^b, John F. Steffensen^a

^a Marine Biological Laboratory, University of Copenhagen, DK-3000 Helsingør, Denmark

^b Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

ARTICLE INFO

Article history:

Received 1 December 2009

Received in revised form 4 April 2010

Accepted 6 April 2010

Available online 11 April 2010

Keywords:

Fish

Gadus morhua

Cortisol

hsp70

Lactate

Heat shock response

Thermal acclimation

Stressor

ABSTRACT

This study examines stress responses in Atlantic cod (*Gadus morhua*) when exposed to a moderate and transient reduction (35% O₂ sat.) in dissolved oxygen at a range of temperatures (5 °C, 10 °C and 15 °C), conditions occurring in some areas they inhabit. Given their geographical distribution pattern, and differences in preferred temperature of cod with different haemoglobin types, the study was extended to include haemoglobin polymorphism. We hypothesised that the differences in temperature preference between Hbl-1 and Hbl-2 type cod might also be reflected in a difference in stress response to hypoxia exposure. Two hsp70-isoforms (labelled a and b) were detected and they differed in expression in the gills but not in the liver of Atlantic cod. Acclimation temperature significantly affected the expression of hsp70 in the liver, and in an isoform-specific manner in the gills. Hypoxia exposure increased the expression of hsp70 in the liver, but not the gills, of cod and this response was not influenced by the acclimation temperature. The expression of hsp70 in both tissues did not differ between fish with different haemoglobin types. Acclimation temperature significantly impacted plasma cortisol but not lactate levels. Also, acute oxygen limitation or Hbl-type significantly elevated plasma cortisol and lactate levels but these responses were not modulated by acclimation temperature. Taken together, our results suggest that both temperature acclimation and acute hypoxic exposure influence the organismal and cellular stress responses in Atlantic cod. We hypothesise that Hbl-2 fish are more tolerant to short-term hypoxic episodes than Hbl-1 fish, and this adaptation may be independent of tissue hsp70 expression.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Hypoxia is a frequently occurring phenomenon in Danish coastal areas, especially during late summer months where oxygen saturation can decrease to 20–40% (Nielsen and Gargas, 1984) due to the thermally stratified water columns. Cod inhabiting these areas are thus faced with sometimes daily fluctuations in oxygen availability at a range of temperatures. Although Atlantic cod has been shown to be moderately tolerant to low levels (40–20% sat.) of oxygen (Plante et al., 1998; Herbert and Steffensen, 2005), few studies have examined the physiological stress response under these conditions. Hypoxia in teleosts elicits an organismal adaptive stress response (Wendelaar Bonga, 1997; Iwama et al., 1998, 2006), consisting of a rapid release of hormones, including catecholamines and cortisol followed by a suite of secondary responses including changes in metabolism and haematological features (Randall and Perry, 1992; Barton, 2002; Iwama et al., 2006), and a cellular stress response is characterised by the increased expression of a group of proteins collectively referred to

as heat shock proteins (hsps). Hsps function as molecular chaperones, assisting assembly and refolding of denatured proteins (Feder and Hofmann, 1999). The most widely studied member belongs to the 70kDa family (hsp70). In fish, increased synthesis of hsp70 has been reported in response to a wide array of stressors, including hypoxia, heat shock, pathogens and contaminants exposure (Iwama et al., 1998; Currie et al., 2000; Basu et al., 2002; Vijayan et al., 2005; Cara et al., 2005). The hsp70 expression in response to hypoxia has not previously been studied in Atlantic cod.

A haemoglobin polymorphism, including three different genotypes controlled by two allelic genes 'Hbl¹' and 'Hbl²', two homozygous types referred to as 'Hbl-1' and 'Hbl-2' and one heterozygous type referred to as 'Hbl-1/2', was described for Atlantic cod more than forty years ago (Sick, 1961). The Hbl² allele is vastly more frequent in the northern (cold) regions of the distribution area, while the Hbl¹ allele is more frequent in the southern (warm) regions (Sick, 1965a,b). Petersen and Steffensen (2003) found that cod with the Hbl-1 type prefers a higher temperature (15.4 °C) than cod with the Hbl-2 type, (8.2 °C). Petersen and Steffensen (2003) also showed that moderate hypoxia (35% oxygen sat.) introduced at their preferred temperature caused fish with the Hbl-1 type to lower their preferred temperature to 9.8 °C, whereas fish with the Hbl-2 type did not. Seeking a lower temperature

* Corresponding author. Tel.: +45 35321971; fax: +45 35321951.

E-mail addresses: cmethling@bio.ku.dk (C. Methling), mvijayan@sciborg.uwaterloo.ca (M.M. Vijayan).

when oxygen is limited is a sound strategy for cod, given the increased solubility of oxygen in cold water, a depressed metabolism at lower temperatures, and a temperature-dependent critical oxygen saturation being 30.3%, 23.2% and 16.5% at 15 °C, 10 °C and 5 °C, respectively (Schurmann and Steffensen, 1997). Based on these observations, cod with the Hbl-2 seemingly gains no advantage by seeking lower temperatures when faced with moderate hypoxic conditions at their preferred temperature. However, there is no known physiological basis for this difference in responses between the two homozygous types, and it is not known if cod with the Hbl-2 type is truly more tolerant to hypoxia.

The aim of the present study was to examine the stress responses in Atlantic cod, homozygous for either the Hbl-1 or the Hbl-2 haemoglobin type, upon exposure to hypoxic conditions at different temperatures. The level of hypoxia (35% oxygen saturation), and the acclimation temperatures (5 °C, 10 °C and 15 °C) were chosen to mimic conditions that are typical for cod in Danish waters (Nielsen and Gargas, 1984). Liver and gill hsp70 protein expressions were measured as indicator of cellular stress response, while plasma cortisol and lactate levels were measured as indicators of primary hormonal and secondary metabolic responses, respectively.

2. Materials and methods

2.1. Animals and experimental conditions

The experimental period was from late September 2005 to February 2006. Juvenile Atlantic cod (*Gadus morhua*, Gadidae) were caught by trawl and traps in the vicinity of the Marine Biological Laboratory in the northern part of Oresund, Denmark. In this area, the distribution of Hbl-types was approximately 50%, 30%, and 20% for Hbl-1/2, Hbl-1 and Hbl-2, respectively. Post capture, fish were transferred to the laboratory where they were kept in 10 °C recirculated, aerated seawater (35 ppt) with a 16 h light:8 h dark photoperiod. Fish were fed *ad libitum* twice a week with chopped herring (*Clupea harengus*). After a minimum of two weeks acclimation to laboratory conditions, the fish were individually tagged with a PIT tag and a blood sample was collected for determination of haemoglobin type as described by Petersen and Steffensen (2003). Fish were allowed a one week recovery after tagging and there were approximately equal numbers of males and females in each tank.

2.2. Experimental procedure

After determination of Hbl-type, fish were evenly distributed between experimental tanks (500 L) and acclimated (1 °C day⁻¹) to 5 °C, 10 °C, or 15 °C (2 tanks per temperature (control+hypoxia) for a minimum of 3 weeks. After this, water oxygen content was lowered to 35% saturation in the hypoxic groups, while control groups received no further treatment, by circulating water from the tank through a deoxygenation tower, mixing it with compressed nitrogen. The 35% saturation level was achieved in approximately 40 min and it was maintained at this level for 4 h. O₂-saturation was monitored by a LoliOxy Oxygen Analyzer and Regulator system (Loligo system, Denmark) connected to a solenoid valve controlling the flow of nitrogen gas.

2.3. Sampling and plasma analysis

Fish were killed by a sharp blow to the head and approximately 0.5 mL of blood was immediately taken from the caudal vein using a heparinised ice cold syringe. After collecting the blood samples, liver tissue samples and gill tissue samples were removed, immediately frozen in liquid nitrogen and stored at -80 °C. Blood samples were centrifuged (5 min at 8000 g at 4 °C) and the plasma was stored frozen at -20 °C for analysis later. Plasma lactate was measured

spectrophotometrically at 340 nm (Pharmacia Ultrospec 2000) using a commercially available kit (Sigma 826-UV), while plasma cortisol was assayed with an enzyme-linked immunoassay test kit (ADALTISS EIAgen Cortisol L14003K).

2.4. Hsp70 analysis

2.4.1. Homogenising and preparation for western analysis

Tissue homogenization for protein immunodetection was carried out as described by Boone and Vijayan (2002). Total protein concentration was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard (Sigma-Aldrich, St. Louis, MO, USA). Briefly, liver and gill tissues were homogenized in Tris buffer (50 mM, pH 7.5) supplemented with a 1:100 protease inhibitor cocktail (Sigma P2714). The homogenate was mixed with equal volume of 2× Laemmli's buffer (0.35 M SDS, 0.5 M sucrose, 0.289 mM bromophenol blue, 125 mM Tris and 0.05% β-mercaptoethanol) and the samples were boiled for 5 min at 95 °C, cooled and stored at -20 °C.

2.5. SDS-PAGE and Western blotting

Equal protein (50 µg lane⁻¹) was loaded on to an 8% one-dimensional SDS polyacrylamide gel. A pre-stained marker (Biorad, Hercules, CA, USA) was loaded on to every gel, to confirm the molecular mass. One randomly chosen cod liver sample served as the reference and this sample was loaded on to every gel to facilitate inter-gel comparisons. Samples were electrophoresed for 45 min at 200 V using a 1X Tris Glycine SDS (TGS) solution [250 mM Tris, 1.92 M glycine, 1% SDS] as running buffer. After electrophoresis, proteins were transferred to nitrocellulose membranes using a Semi-Dry transfer unit (Biorad) set at 20 V for 20 min in a transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Subsequently, membranes were stained with 0.1% PonceauS stain to ensure equal loading. Membranes were washed in TTBS until the stain was completely removed, soaked in blocking solution [5% skimmed milk powder, 0.05% sodium azide in TTBS (20 mM Tris (pH 7.5), 300 mM NaCl, 0.01% Tween 20)] for 1 h, followed by addition of the primary antibody (clone BRM-22, Sigma-Aldrich; Zakhartsev et al., 2005) in blocking solution (1:3000). After one hour in primary antibody, membranes were washed (2×5 min in TTBS) and the secondary antibody (1:3000) added in the blocking solution without sodium azide. The secondary antibody was an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich). After one hour in the secondary antibody, membranes were washed 3 times for 5 min in TTBS and 10 min in TBS (TTBS without Tween 20). Protein bands were detected by the addition of Nitro Blue Tetrazolium (NBT; 50 µg/mL in 70% dimethylformamide) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; 50 µg/mL in 100% dimethylformamide). The scanned protein bands were quantified using the AlphaEase software (AlphaEase Innovatech, CA, USA). The density of the protein bands was expressed as a percentage of a reference sample (liver tissue), which was used on all gels to normalize for gel to gel variability, and shown as arbitrary units. The hsp70 antibody (clone BRM-22, Sigma-Aldrich) detected two hsp70 proteins, a 72 kDa (hsp70 a) and a 70 kDa (hsp70 b), in the gill and liver (Fig. 1) and this was also shown before in Atlantic cod (Zakhartsev et al., 2005). Although the two bands have been identified in mammals as the constitutive (hsp73) and the inducible isoform (hsp72), this identity has not been confirmed in cod.

2.6. Statistical analysis

All data was log₁₀ transformed prior to any statistical analysis to normalize the data sets. The transformed data was tested for normality and homogeneity of variance. The level of significance chosen for all tests was α = 0.05. The effects of temperature, oxygen saturation and haemoglobin type on hsp70 expressions, plasma

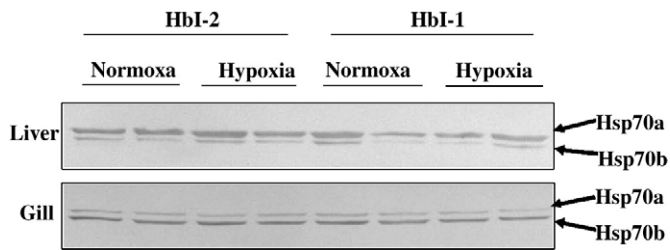


Fig. 1. Tissue-specific hsp70 expression in Atlantic cod after three weeks acclimation to 5 °C, 10 °C, or 15 °C. Representative images of Western blots showing two isoforms, hsp70a and hsp70b expressed in liver and gill tissues. Refer to text for details. Duplicate samples are loaded in adjacent lanes for each treatment. Hbl-2 and Hbl-1 designates two homozygous haemoglobin types. Hsp70s were immunodetected using a monoclonal mouse anti-Hsp70 antibody (Sigma-Aldrich).

cortisol, and lactate levels were analysed with a 3-way ANOVA, followed by Holm–Sidak post hoc tests. All tests were conducted with the SigmaPlot V. 11.0 software (Systat Software, Inc., USA).

3. Results

A descriptive summary of the experimental animals is given in Table 1. There were no significant differences in either body length or body mass between the experimental groups.

3.1. Hsp70 response (3-way ANOVA output: Table 2)

The two hsp70-isoforms, herein labelled hsp70a and hsp70b, were detected in both liver and gill tissues (Fig. 1). The expression of hsp70-isoforms did not differ between Hbl-types, and so these two haemoglobin groups were pooled for hsp70 data analysis (Table 2). There was a significant difference in the expression of hsp70 isoforms in the gills, but not the liver (Fig. 2); hsp70b expression was greater than hsp70a expression in the gills (Fig. 2B and D). Temperature acclimation affected hsp70 expression both in the liver and gills (Table 2). In the liver, hsp70 expression was significantly lower in the 10 °C-acclimated fish compared to the 5 °C and 15 °C-acclimated groups ($p = 0.016$ and $p < 0.001$ for 5 °C and 15 °C, respectively), (Fig. 2A and C). In the gill, there was a significant interaction between acclimation temperature and hsp70-isoforms (Table 2). Hsp70a expression was significantly higher in 15 °C-acclimated ($p < 0.001$) fish compared to the other two temperatures (Fig. 2B), whereas hsp70b expression was significantly lower in 10 °C-acclimated fish compared to 5 °C and 15 °C-acclimated fish ($p < 0.001$; Fig. 2D). Hypoxia exposure had a significant effect on hsp70 protein expression in the liver but not gill. A significantly higher hepatic hsp70 expression was observed in response to hypoxia compared to the normoxic group (Fig. 2A and C). There was no interaction between hypoxia and temperature on hsp70 expression in either the liver or gills (Table 2).

3.2. Plasma cortisol and lactate levels (3-way ANOVA output: Table 3)

The temperature acclimation, hypoxia exposure and haemoglobin types all had a significant effect on plasma cortisol concentration (Table 3; Fig. 3A–C). Plasma cortisol concentration was significantly

Table 2

3-way ANOVA summary table. Main factors are: temperature, O₂-saturation (hypoxia) and hsp70-isoforms (hsp70). Abbreviations are: DF: degrees of freedom; SS: sum of squares; MS: mean square; F: F-statistic, and P: p-value; Statistical significance is shown in bold ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P	Treatment effects
<i>Liver</i>						
Temperature	2	1.551	0.776	7.063	0.001	5 °C and 15 °C > 10 °C
Hypoxia	1	0.671	0.671	6.106	0.015	Hypoxia > normoxia
Hsp70	1	0.020	0.020	0.186	0.667	
Temp × hypoxia	2	0.534	0.267	2.430	0.091	
Temp × hsp70	2	0.404	0.202	1.840	0.162	
Hypoxia × hsp70	1	0.085	0.085	0.773	0.381	
Temp × hyp × hsp	2	0.332	0.166	1.513	0.224	
Residual	156	17.132	0.110			
Total	167	20.833	0.125			
<i>Gill</i>						
Temperature	2	1.816	0.908	30.371	<0.001	5 °C and 10 °C < 15 °C
Hypoxia	1	0.003	0.003	0.089	0.766	
Hsp70	1	15.719	15.719	525.792	<0.001	Hsp70b > hsp70a
Temp × hypoxia	2	0.031	0.015	0.513	0.599	
Temp × hsp70	2	1.247	0.623	21.335	<0.001	See Fig. 2B and D
Hypoxia × hsp70	1	0.005	0.005	0.170	0.681	
Temp × hyp × hsp	2	0.019	0.001	0.322	0.725	
Residual	156	4.664	0.030			
Total	167	22.688	0.136			

lower in the 15 °C-acclimated fish compared to the other two temperatures. The hypoxic treatment resulted in significantly elevated plasma cortisol concentrations regardless of temperature acclimation compared to the normoxic group (Table 3). There was no significant interaction between acclimation temperature and hypoxia exposure on plasma cortisol levels in the present study. A significant difference between Hbl-types was found in plasma cortisol levels with the Hbl-1 type having significantly higher plasma cortisol concentrations compared to the Hbl-2 type (Table 3). There were no significant interactions between temperature, O₂ saturation and haemoglobin type (Table 3).

Temperature acclimation did not significantly affect plasma lactate levels in the present study (Table 3). Hypoxia exposure significantly elevated plasma lactate levels compared to the normoxic group (Table 3; Fig. 3D–F). A difference between Hbl-types was found in plasma lactate levels with the Hbl-1 type having significantly higher lactate concentration compared to the Hbl-2 type. There were no significant interactions between temperature, O₂ saturation and haemoglobin type on plasma lactate concentration (Table 3).

4. Discussion

We demonstrate that temperature acclimation and acute moderate hypoxic exposure elicit organismal and cellular stress responses in Atlantic cod. While the haemoglobin polymorphism did not influence tissue hsp70 expression, plasma cortisol and lactate levels, two indicators of organismal stress response, were significantly different between the two types (Hbl-1 and Hbl-2 fish). Based on these results,

Table 1

A descriptive summary of the experimental groups. Values represent mean ± standard deviations ($n = 7–12$).

	5 °C		10 °C		15 °C	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
Mass (g)	324.6 ± 120.2	352.5 ± 105.4	298.9 ± 95.8	275.8 ± 77.4	355.9 ± 103.2	291.2 ± 111.0
Length (cm)	32.8 ± 4.2	33.9 ± 3.5	32.6 ± 3.4	32.2 ± 2.4	32. ± 2.9	33.5 ± 3.6
Hbl-type (1/2)	10/7	10/8	12/10	10/10	10/7	8/9

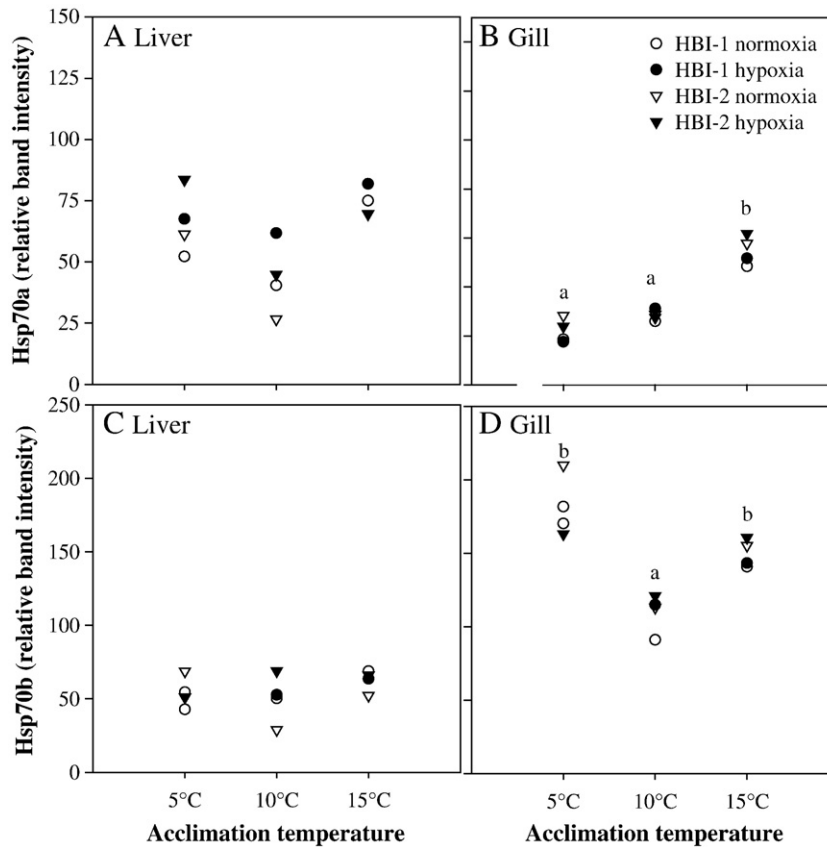


Fig. 2. Hsp70-expression in Atlantic cod exposed to 4 h hypoxia (35% oxygen saturation) after 3 weeks acclimation to 5 °C, 10 °C or 15 °C. Controls were kept normoxia (>80% oxygen saturation). (A, B) Liver and gill hsp70a expression. (C, D) Liver and gill hsp70b expressions. Hbl-1 (circle) and Hbl-2 (triangle) represents the two homozygous haemoglobin types; open symbols are normoxic group (controls), while closed symbols are hypoxic groups. Values represent mean ($n = 4-8$ fish). Different letters denote significant difference between acclimation temperatures. Refer to text for details.

we hypothesise that fish with the Hbl-2 type are more tolerant to stressor exposure and this response may be independent of hsp70 expression.

Table 3

3-way ANOVA summary table. Main factors are: temperature, O_2 -saturation (hypoxia) and Hbl-type. Abbreviations as in Table 2. Statistical significance is shown in bold ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P	Treatment effects
<i>Cortisol</i>						
Temperature	2	17.765	8.883	15.488	<0.001	5 °C and 10 °C > 15 °C
Hypoxia	1	2.355	2.355	4.107	0.046	Hypoxia > normoxia
Hbl-type	1	2.623	2.623	4.573	0.035	Hbl-1 > Hbl-2
Temp × hypoxia	2	0.217	0.109	0.190	0.828	
Temp × Hbl-type	2	0.357	0.179	0.312	0.733	
Hypoxia × Hbl-type	1	0.001	0.001	0.002	0.967	
Temp × hyp × Hbl-type	2	1.836	0.918	1.601	0.208	
Residual	85	48.749	0.574			
Total	96	74.115	0.772			
<i>Lactate</i>						
Temperature	2	0.358	0.179	2.825	0.065	
Hypoxia	1	3.027	3.027	47.765	<0.001	Hypoxia > normoxia
Hbl-type	1	0.368	0.368	5.810	0.018	Hbl-1 > Hbl-2
Temp × hypoxia	2	0.208	0.104	1.641	0.200	
Temp × Hbl-type	2	0.0360	0.018	0.284	0.753	
Hypoxia × Hbl-type	1	0.009	0.009	0.137	0.712	
Temp × hyp × Hbl-type	2	0.081	0.041	0.639	0.530	
Residual	90	5.703	0.063			
Total	101	9.839	0.097			

Conditions of limited oxygen availability leading to hypoxemia, increase the significance of haemoglobins O_2 -carrying-capacity. The oxygen affinity of these different haemoglobins i.e. Hbl₁₁, Hbl₁₂ and Hbl₂₂ has been studied under different conditions (Karpov and Novikov, 1981; Brix et al., 1998). Most recently, Brix et al. (2004) found that at 4 °C, the Hbl₂₂ molecule had a higher affinity than the Hbl₁₁ molecule ensuring O_2 -loading at the gills, while at 12 °C, the Hbl₂₂ molecule had a lower affinity than the Hbl₁₁ molecule thereby ensuring the unloading of O_2 at the tissues. While the Hbl-type may be playing a critical role in oxygen delivery at different temperatures, our results suggest that the activation of the organismal stress response and the associated metabolic adjustments may be Hbl-type-dependent. The differences in plasma cortisol and lactate levels between haemoglobin types, together with the haemoglobins O_2 transport features may reflect a lower capacity for fish with the Hbl-1 type to cope metabolically with a hypoxia stressor relative to the Hbl-2 type fish. The concept that oxygen limitation determines thermal tolerance in ectothermic animals (reviewed by Pörtner et al., 2001) has been studied in Atlantic cod (Satoris et al., 2003; Lanning et al., 2004). These studies found a decrease in venous blood O_2 content with increasing and decreasing temperatures pointing to circulatory performance as a limiting factor in setting the thermal tolerance window. However, these studies did not differentiate between haemoglobin types. Hypothetically, if a difference in the temperature-dependent hypoxemia exists between haemoglobin types, then this would be reflected in differences in the thermal tolerances between haemoglobin types, either by a directional shift or a narrowing of the thermal tolerance window. In turn, this could be a possible explanation for the difference in temperature preferences between haemoglobin types (Petersen and Steffensen, 2003). The connection between temperature preferences,

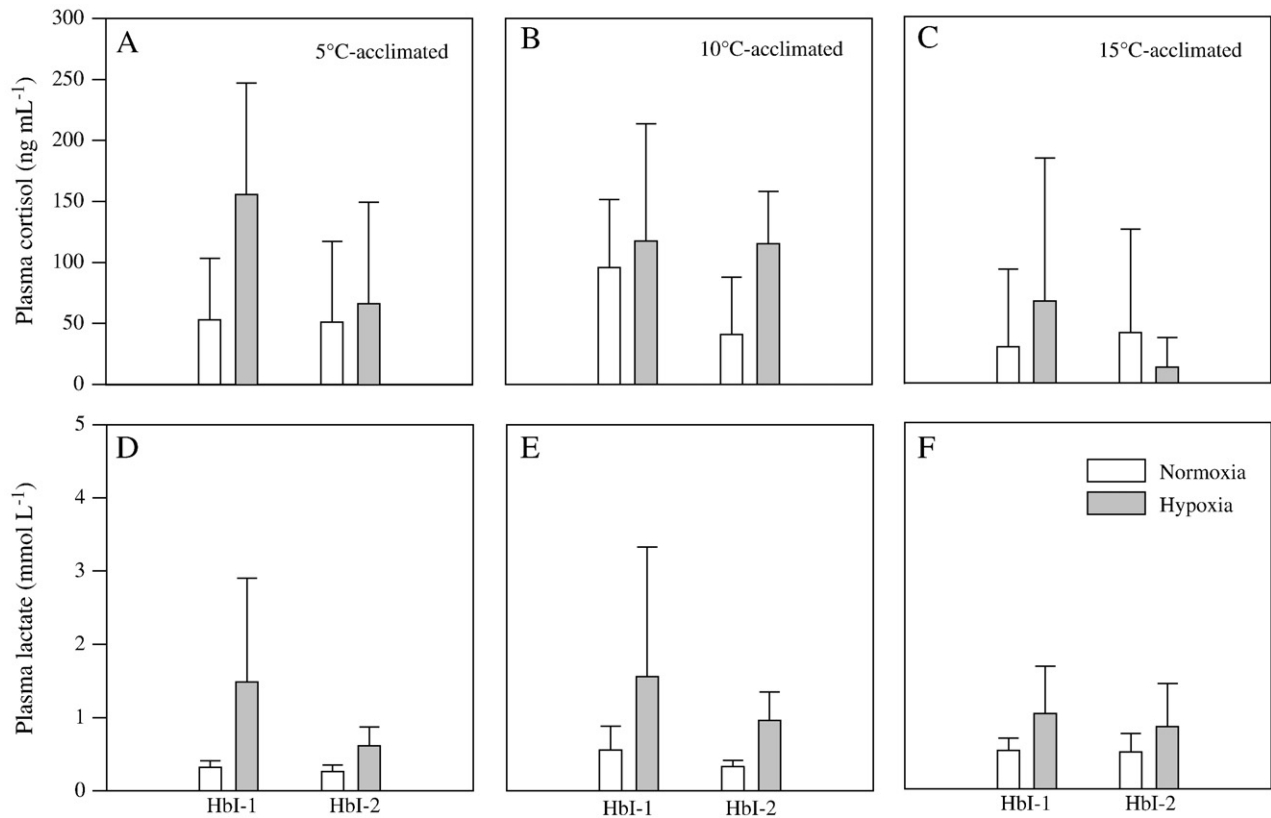


Fig. 3. Plasma cortisol (ng mL⁻¹) and plasma lactate (mmol L⁻¹) levels in Atlantic cod after three weeks temperature acclimation; 5 °C, 10 °C or 15 °C, and 4 h of moderate hypoxia (35% oxygen saturation; shaded bars). Controls were kept at normoxia (>80% oxygen saturation; open bars). Values represent mean ± standard deviations ($n = 6-10$ fish).

thermal tolerance limits, circulatory capacities and metabolic adjustments in these HbI-types is an interesting subject for further studies.

Hsp70 protein expression was influenced by acclimation temperature in a tissue- and isoform-specific manner in Atlantic cod. Tissue-specific responses of heat shock proteins have previously been shown in Atlantic cod (Zakhartsev et al., 2005) and in several other species of fish (Koban et al., 1991; Dietz and Somero, 1993; Airaksinen et al., 1998; Currie et al., 2000). Temperature-dependent changes in hsp70 expression have been reported for a number of other fish species in both natural and experimental conditions (Buckley and Hofmann, 2002; Fader et al., 1994; Lund et al., 2002; Basu et al., 2002). However, Zakhartsev et al. (2005) reported a lack of hsp70 response in wild Atlantic cod to both short and long term temperature changes (4 °C to 15 °C). Zakhartsev et al. (2005) argued that hsp70s play a secondary role in defining thermal tolerance limits in Atlantic cod, in accordance with the hypothesis that thermal tolerance follows a systemic to molecular hierarchy (Pörtner et al., 2001), and suggested that constitutive hsp70 levels are sufficiently high to overcome temperature related stress. However, we demonstrate a temperature-dependent expression of hsp70s in Atlantic cod within a physiological range of temperatures. Although we and the other study used the same antibody for hsp70 detection, the difference between the two studies may be related to the measurement of total hsp70 by ELISA (Zakhartsev et al., 2005), whereas we separated the two isoforms of hsp70. Our study shows that there are isoform-specific differences and this may be masked when examining the total hsp70 content. The higher expression of gill hsp70b-isoform (and hsp70 in liver tissue) at 5 °C and 15 °C compared to 10 °C-acclimated animals may point to cellular adaptation to protect protein function as the animals move further away from their optimal temperature.

The organismal stress response seen in the present study in response to moderate hypoxic exposure suggests a metabolic disturbance regardless of temperature acclimation in this species.

Indeed, high plasma lactate levels were reached during severe hypoxia (Claireaux and Dutil, 1992) or chasing fish to exhaustion (Herbert and Steffensen, 2005), implying that the fish in the current study may be suffering from mild metabolic disturbances. This is in accordance with the known critical oxygen saturation (Scrit) for Atlantic cod, defined as the point where the standard metabolism can no longer be maintained. Scrit is temperature dependent and is 30.3%, 23.2% and 16.5% at 15 °C, 10 °C and 5 °C, respectively (Schurmann and Steffensen, 1997). Thus, 35% saturation is above the Scrit for all experimental temperatures and, therefore, represents a minor threat to the fish in the present study. Our plasma cortisol levels in the unstressed fish are comparable to those reported for Atlantic cod of similar size (Caipang et al., 2008). The lower plasma cortisol levels at 15 °C compared to 5 °C and 10 °C suggest that the highest temperature may inhibit the cortisol response in this species. The lower cortisol levels at high temperatures may involve either a direct inhibitory effect of higher temperature on cortisol production and/or alteration in this steroid turnover. The other possibility is the exhaustion of the pituitary corticotrops and/or interrenal tissue due to constant temperature stimulation of cortisol production during the three week acclimation period. However, the recent observation that gradual temperature acclimation did not affect plasma cortisol levels over a 20 day period in cod (Perez-Casanova et al., 2008) supports the notion that higher temperature may be directly inhibiting cortisol production/clearance in this species, but this remains to be verified. Our results are at odds with a recent study showing a higher plasma cortisol levels in cod acclimated to a higher temperature compared to 4 °C (King et al., 2006). However, these fish were much smaller (~19 g) relative to our study pointing to a size/developmental effect on the stress response in this species. For instance in Danish coastal areas, during summer months, small cod inhabit shallow high temperature high oxygen saturated waters, while larger cod are found in deeper, colder and sometimes hypoxic areas.

In summary, we demonstrate that temperature acclimation influences plasma cortisol concentration and tissue hsp70 expression in Atlantic cod. A moderate hypoxic exposure (stressor) of short duration elevated plasma cortisol and lactate levels and increased hsp70 expression in the liver. While hsp70 expression was not affected by Hbl-type, plasma cortisol and lactate levels were higher in the Hbl-1 fish compared to the Hbl-2 fish. Based on the stress responses evident from this study, we hypothesise that fish with the Hbl-2 type may be metabolically more tolerant to hypoxia than cod with the Hbl-1 type, and this response may be independent of hsp70 expression.

Acknowledgements

The study was supported by the Danish National Science Research Council (JFS), the Norwegian Research Council, the Natural Sciences and Engineering Research Council of Canada (MMV) and the Elisabeth and Knud Petersen Foundation.

References

- Airaksinen, S., Råbergh, C.M.I., Sistonen, L., Nikinmaa, M., 1998. Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *J. Exp. Biol.* 201, 2543–2551.
- Barton, B., 2002. Stress in fishes: a diversity of responses with particular reference to circulating corticosteroids. *Integr. Comp. Biol.* 42, 517–525.
- Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. *Gene* 295, 173–183.
- Boone, A.N., Vijayan, M.M., 2002. Glucocorticoid-mediated attenuation of the hsp70 response in trout hepatocytes involves the proteasome. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283, 680–687.
- Brix, O., Forås, E., Strand, I., 1998. Genetic variation and functional properties of Atlantic cod hemoglobins: introducing a modified tonometric method for studying fragile hemoglobins. *Comp. Biochem. Physiol.* 119, 575–583.
- Brix, O., Thorkildsen, S., Colosimo, A., 2004. Temperature acclimation modulates the oxygen binding properties of the Atlantic cod (*Gadus morhua* L.) genotypes-Hbl* 1/1, Hbl*1/2, and Hbl*2/2-by changing the concentration of their major haemoglobin components (results from growth studies at different temperatures). *Comp. Biochem. Physiol.* 138, 241–251.
- Buckley, B.A., Hofmann, G.E., 2002. Thermal acclimation changes DNA-binding activity of heat shock factor 1 (HSF1) in the goby *Gillichthys mirabilis*: implications for plasticity in the heat-shock response in natural populations. *J. Exp. Biol.* 205, 3231–3240.
- Caipang, C.M.A., Brinckmann, M.F., Berg, I., Iversen, M., Eliassen, R., Kiron, V., 2008. Changes in selected stress and immune-related genes in Atlantic cod, *Gadus morhua*, following overcrowding. *Aquacult. Res.* 39, 1533–1540.
- Cara, J.B., Aluru, N., Moyano, F.J., Vijayan, M.M., 2005. Food-deprivation induces HSP70 and HSP90 protein expression in larval gilthead sea bream and rainbow trout. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 142, 426–431.
- Claireaux, D., Dutil, J.-D., 1992. Physiological response of the Atlantic cod (*Gadus morhua*) to hypoxia at various environmental salinities. *J. Exp. Biol.* 163, 97–118.
- Currie, S., Moyes, C.D., Tufts, B.L., 2000. The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: in vivo and in vitro comparisons. *J. Fish Biol.* 56, 398–408.
- Dietz, T.J., Somero, G.N., 1993. Species- and tissue-specific synthesis patterns for heat-shock proteins HSP 70 and HSP 90 in several marine teleost fishes. *Physiol. Zool.* 66, 863–880.
- Fader, S.C., Yu, Z., Spotila, J.R., 1994. Seasonal variation in heat shock proteins (hsp70) in stream fish under natural conditions. *J. Therm. Biol.* 19, 335–341.
- Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 61, 243–282.
- Herbert, N.A., Steffensen, J.F., 2005. The response of Atlantic cod, *Gadus morhua* to progressive hypoxia: fish swimming speed and physiological stress. *Mar. Biol.* 147, 1403–1412.
- Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M., 1998. Heat shock protein expression in fish. *Rev. Fish Biol. Fish.* 8, 35–56.
- Iwama, G.K., Alfonso, L.O.B., Vijayan, M.M., 2006. Stress in Fish. In: Evans, D.H., Claiborne, J.B. (Eds.), *The Physiology of Fishes*, Third edition. CRC Press, Boca Raton, Florida, pp. 319–342.
- Karpov, A.K., Novikov, G.G., 1981. Hemoglobin alloforms in cod, *Gadus morhua* (Gadiformes, Gadidae), their functional characteristics and occurrence in populations. *J. Ichthyol.* 6, 45–49.
- King, W.V., Buckley, L.J., Berlinsky, D.L., 2006. Effect of acclimation temperature on the acute stress response in juvenile Atlantic cod *Gadus morhua* L., and haddock, *Melanogrammus aeglefinus* L. *Aquacult. Res.* 37, 1685–1693.
- Koban, M., Yup, A.A., Agellon, L.B., Powers, D.A., 1991. Molecular adaptation to environmental temperature: heat-shock response of the eurythermal teleost *Fundulus heteroclitus*. *Mol. Mar. Biol. Biotechnol.* 1, 1–17.
- Lanning, G., Bock, C., Satoris, F.J., Pörtner, H.O., 2004. Oxygen limitation of thermal tolerance in cod, *Gadus morhua* L., studied by magnetic resonance imaging and on-line venous oxygen monitoring. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 287, 902–910.
- Lund, S.G., Caissie, D., Cunjak, R.A., Vijayan, M.M., Tufts, B.L., 2002. The effects of environmental heat stress on heat shock mRNA and protein expression in Miramichi Atlantic salmon (*Salmo salar*) parr. *Can. J. Fish. Aquat. Sci.* 59, 1553–1562.
- Nielsen, G., Gargas, E., 1984. Oxygen, nutrients, and primary production in the open Danish waters. *Limnologia (Berlin)* 15, 303–310.
- Perez-Casanova, J.C., Rise, M.L., Dixon, B., Afonso, L.O.B., Hall, J.R., Johnson, S.C., Gamperl, A.K., 2008. The immune and stress responses of Atlantic cod to long-term increases in water temperature. *Fish Shellfish Immunol.* 24, 600–609.
- Petersen, M.F., Steffensen, J.F., 2003. Preferred temperature of juvenile cod (*Gadus morhua*) with different haemoglobin genotypes at normoxia and moderate hypoxia. *J. Exp. Biol.* 206, 359–364.
- Plante, S., Chabot, D., Dutil, J.-D., 1998. Hypoxia tolerance in Atlantic cod. *J. Fish Biol.* 53, 1342–1356.
- Pörtner, H.O., Berdal, B., Blust, R., Brix, O., Colosimo, A., de Wachter, B., Giuliani, A., Johansen, T., Fischer, T., Knust, R., Lannig, G., Naevdal, G., Nedenes, A., Nyhammer, G., Sartoris, F.J., Serendero, I., Sirabella, P., Thorkildsen, S., Zakhartsev, M., 2001. Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*). *Cont. Shelf Res.* 21, 1975–1997.
- Randall, D.J., Perry, S.F., 1992. Corticosteroids. *Fish Physiol.* 12, 255–300.
- Satoris, F.J., Bock, C., Serendero, I., Lanning, G., Pörtner, H.O., 2003. Temperature-dependent changes in energy metabolism, intracellular pH and blood oxygen tension in the Atlantic cod. *J. Fish Biol.* 62, 1239–1253.
- Schurmann, H., Steffensen, J.F., 1997. Effects of temperature, hypoxia and activity on the metabolism of juvenile cod. *J. Fish Biol.* 50, 1160–1180.
- Sick, K., 1961. Haemoglobin polymorphism in fishes. *Nature* 192, 894–896.
- Sick, K., 1965a. Haemoglobin polymorphism of cod in the Baltic and the Danish belt sea. *Hereditas* 54, 19–48.
- Sick, K., 1965b. Haemoglobin polymorphism of cod in the North Sea and in the North Atlantic Ocean. *Hereditas* 54, 49–69.
- Vijayan, M.M., Prunet, P., Boone, A.N., 2005. Xenobiotic impact on corticosteroid signaling. In: Moon, T.W., Mommsen, T.P. (Eds.), *Biochemical and Molecular Biology of Fishes. : Environmental Toxicology*, Vol. 6. Elsevier, Amsterdam, pp. 365–394.
- Wendelaar Bonga, S.E., 1997. The stress response in fish. *Physiol. Rev.* 77, 591–625.
- Zakhartsev, M., De Wachter, B., Johansen, T., Pörtner, H.O., Blust, R., 2005. Hsp70 is not a sensitive indicator of thermal limitation in *Gadus morhua*. *J. Fish Biol.* 67, 767–778.