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Preferred temperature of juvenile Atlantic cod *Gadus morhua* with different haemoglobin genotypes at normoxia and moderate hypoxia

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Summary

Atlantic cod *Gadus morhua* has polymorphic haemoglobin, which can be separated into two homozygous types, HbI-1 and HbI-2, and one heterozygous type HbI-1/2. The geographical distribution of Atlantic cod with the different haemoglobin types varies, with the HbI^2 allele occurring at high frequency in northern regions, and the HbI^1 allele dominant in warmer areas. To determine if temperature is a selective parameter in the distribution of the haemoglobin types, the preferred temperature of the homozygous genotypes HbI-1 and HbI-2 was measured. We found that HbI-2 cod preferred a temperature of 8.2±1.5°C while HbI-1 cod preferred 15.4±1.1°C, and this preference was significant. The effect of hypoxia (35% oxygen saturation) on the preferred temperature was also measured. Previous studies showed that the preferred temperature of fish decreases during hypoxia, and this was the case for HbI-1 cod, which preferred 9.8±1.8°C during hypoxia, whereas HbI-2 cod did not show this effect. The results indicate that environmental temperature changes will lead to a distributional change in the different haemoglobin types of Atlantic cod, global warming providing an advantage for HbI-1 cod. However, since HbI-1 cod prefer a low temperature under hypoxic conditions, a combination of increased water temperature and hypoxia could be unfavourable for Atlantic cod stocks.

Key words: haemoglobin, Atlantic cod, *Gadus morhua*, preferred temperature, hypoxia, swimming speed.

Introduction

The different types of Atlantic cod haemoglobin have been known since 1961 (Sick, 1961). Experiments with agar gel electrophoresis revealed that Atlantic cod haemoglobin consists of two homozygous genotypes (HbI-1 and HbI-2) and one heterozygous genotype (HbI-1/2). These genotypes are controlled by two alleles, referred to as HbI^1 and HbI^2. Such polymorphism of fish haemoglobin is prevalent (Kirpichnikov, 1981) and is often essential if fish change habitat during their development (ontogenetic effect), or if they shift between different environments where changes in respiration media, salinity etc. can occur (Jensen et al., 1998). In some cases, however, the reason why some fish, such as Atlantic cod, have more than one haemoglobin type is unknown.

When searching for the reason why Atlantic cod have polymorphic haemoglobin we examined the frequency distribution of the two alleles coding for the different haemoglobin types. The distribution is extremely heterogeneous across the North Atlantic. The HbI^2 allele reaches up to 99% frequency in Greenland waters and is also dominant in northern Norway, Iceland, the Faeroe Islands, Canada and in the northern part of the Baltic, whereas the HbI^1 allele is dominant in the warmer areas (Sick, 1965a,b; Frydenberg et al., 1965). The difference between the homozygous genotypes is limited to an extra histidine-containing peptide in the HbI-1 type, and therefore the structural differences are only minimal (Rattazzi and Pik, 1965). However, differences in biochemical properties have been described for the genotypes. The oxygen affinity of haemoglobin is higher for HbI-2 cod at low temperatures (<10°C) (Karpov and Novikov, 1981; Brix et al., 1998; McFarland, 1998), and for HbI-1 cod, at some blood pH values, at high temperatures (>14°C) (Karpov and Novikov, 1981; Brix et al., 1998). The heterozygous haemoglobin type is generally found to have oxygen affinity values that are intermediate to HbI-1 and HbI-2 (Karpov and Novikov, 1981). This information suggests that temperature could be a selective parameter for the distribution of Atlantic cod with different haemoglobin types. The present study examined whether this was the case by measuring the preferred temperature of HbI-1 and HbI-2 cod.

The preferred temperature of a species is not a fixed value because it can be influenced by other parameters. Seasonal fluctuation of the preferred temperature is highest during summertime and lowest during winter (Clark and Green, 1991). The amount of food consumed by the fish also changes their preferred temperature. Fish fed with the lowest food ration prefer the lowest temperature (Despatie et al., 2001). In addition, hypoxia results in a decrease of the preferred

temperature (Schurmann et al., 1991; Schurmann and Steffensen, 1992). Since hypoxia is a common phenomenon in several habitats where the Atlantic cod is represented, especially in coastal regions such as the Gulf of St Lawrence (Chabot and Dutil, 1999) and the Baltic Sea (Nielsen and Gargas, 1984), we analysed the effect of hypoxia on the preferred temperature for the different haemoglobin types.

Materials and methods

Experimental animals

Juvenile Atlantic cod *Gadus morhua* L. were caught by trawl in the northern part of Øresund (Denmark) during the summer months. They were kept under a 16 h:8 h light:dark cycle at 10°C in aerated water tanks (500 litre) that were continuously supplied with recirculating filtered 30‰ seawater (8 l min^{-1}). The cod were fed regularly with chopped herring *Clupea harengus*, and were in good condition. Several months prior to the experiments they were pit-tagged and their haemoglobin type analysed (as described below). Cod with haemoglobin types HbI-1 and HbI-2 were pre-selected for the preferred temperature experiments.

Determination of haemoglobin genotype

The haemoglobin genotypes were determined by agar gel electrophoresis (Fyhn et al., 1994). 0.2 ml of blood was sampled from the caudal vein with a heparinized syringe. The blood sample was centrifuged at 3000 ***g*** in an Eppendorf tube and the plasma discarded. 50 µl of the red blood cells (RBC) were transferred to a new Eppendorf tube and washed twice with 60 µl of 1.17% NaCl solution. 80 µl of cold distilled water was added to haemolyse the RBC. The sample was refrigerated for 10 min, and subsequently centrifuged for 5 min at 3000 ***g***. To ensure proper sinking in the gel, 40 µl of haemolysed RBC (supernatant) was mixed with 35 µl of 40% sucrose solution. A gel was prepared using 1% agar (Difco bactoagar) dissolved by warming in Smithies buffer (pH 8.6) diluted 1:1 with distilled water. Diluted Smithies buffer was also used as electrophoresis buffer. All samples were electrophoresed at a 40 mA current for 35 min at 10°C, using a Pharmacia EPS600 power supply.

Shuttle box technique

To determine the preferred temperature, cod of body mass 90–200 g were allowed to thermoregulate in an electronic shuttle box. The shuttle box consisted of a warm and a cold water chamber connected by a tube. The fish was able to thermoregulate by shuttling between the two chambers. The temperature difference between the two chambers was 2°C and was kept constant by the use of two pumps (Eheim 1046). When the fish occupied the cold chamber the entire system was cooled down (4°C h^{-1}), whereas the system started heating up when the fish entered the warm chamber (4°C h^{-1}). The position of the fish was registered by a CCD camera connected to a PC video frame-grabber (Visionetics VFG-512 BC), digitising at 25 frames s^{-1}, at a resolution of 256×256 pixels. Three 40 W red light bulbs illuminated the shuttle box from below and made the fish look like a black silhouette on a light background. The contrast between the fish and its surroundings was used by a custom-designed software program to detect the geometric centre of the silhouette of the fish. The *x*,*y* coordinates of the position of the fish were transmitted *via* an RS-232 port to a second computer. The second computer was equipped with Labtech Notebook programmed to switching on either cooling or heating, depending on the position of the fish (for further details, see Schurmann et al., 1991; Schurmann and Steffensen, 1992, 1994).

The oxygen saturation was measured by a microprocessor oximeter (WTW oxy 96) in the warm part of the shuttle box. Test measurements showed that the difference in oxygen saturation between the two chambers never exceeded 2%. The oxygen saturation could be regulated *via* a computer system equipped with Labtech Notebook, which controlled solenoid valves. When the oxygen saturation was above the selected set point (35% oxygen saturation) compressed nitrogen was added to the system.

Experimental protocol

The cod was introduced to the shuttle box 24 h prior to the experiment at a water temperature of 10°C. The experiments were carried out during daytime, where the highest activity and the most precise thermoregulation of the cod were previously observed. The data collection period consisted of 4 h of normoxia (>80% oxygen saturation) followed by a 2 h reduction in oxygen saturation, again followed by 3 h of hypoxia (35% oxygen saturation).

Calculations and analysis

To estimate the body temperature of a fish in a shuttle box system Newton's Law of Cooling may be used:

$$T_b = T_a + (T_i - T_a)e^{-kt}, \quad (1)$$

where T_b is body temperature, T_a is ambient temperature, T_i is the initial body temperature, t is time (min) and k is the rate of change of the core temperature (min^{-1}). Stevens and Sutterlin (1976) previously measured the value of k for another temperate saltwater fish, the sea raven (*Hemitripterus americanus*), and found it to be related to the body mass (W), measured in g:

$$k = 3.32 \times W^{-0.536}. \quad (2)$$

We chose this value of k to calculate the body temperature of Atlantic cod, since it is the most relevant literature value. The preferred temperature of each fish was calculated as the median of the estimated body temperature. Swimming speed was calculated as body lengths (BL) s^{-1} and measured as the distance in pixels (corresponding to a known distance in cm) swum s^{-1}.

Analyses of variance (ANOVA) tested the effects of the independent factors (HbI-genotype and oxygen saturation) and their interactions on the preferred temperature or swimming speed. The Student Newman–Keul method was used in cases

Table 1. *Preferred temperature, lower and upper avoidance temperatures and swimming speed of Atlantic cod with different haemoglobin genotypes*

	N	Preferred temperature (°C)	Lower avoidance temperature (°C)	Upper avoidance temperature (°C)	Swimming speed ($BL\,s^{-1}$)
HbI-1 >80% O_2 saturation	8	15.4±1.1	14.0±0.9	16.0±0.8	0.38±0.05
HbI-2 > 80% O_2 saturation	8	8.2±1.5	7.2±1.6	9.3±1.6	0.29±0.09
HbI-1 35% O_2 saturation	8	9.8±1.8	9.3±1.8	11.2±2.2	0.17±0.08
HbI-2 35% O_2 saturation	8	8.0±2.9	7.3±2.8	9.5±2.9	0.16±0.08

Values are means of medians ± S.D. (temperature values only).

The preferred temperature was significantly different (P=0.0001) between HbI-1 and HbI-2 at normoxia and between HbI-1 at normoxia compared with HbI-1 at hypoxia (P=0.0005).

Swimming speed decreased significantly (P=0.0001) for both haemoglobin types when exposed to hypoxia.

where the two-way ANOVA showed a significant difference (P<0.05).

Results

The preferred temperature was calculated as the median preferred temperatures ± S.D. of eight fish during normoxia (>80% oxygen saturation) and eight fish during hypoxia (35% oxygen saturation). The experiment was done for both HbI-1 and HbI-2 cod (Table 1). During normoxic conditions HbI-1 cod preferred a significantly higher (P<0.0001) temperature (15.4±1.1°C) compared with HbI-2 cod (8.2±1.5°C). When exposed to hypoxia, however, no difference was observed between the two genotypes. This was as a result of HbI-1 cod decreasing the preferred temperature significantly (P<0.0005) (9.8±1.8°C), while the preferred temperature of HbI-2 cod was relatively unchanged (8.0±2.9°C). The upper and lower avoidance temperatures indicate that the cod are precise thermoregulators. The avoidance temperatures are calculated as the median of the highest or lowest temperatures experienced by the fish and show that the fish shuttles within a narrow range of temperatures. Despite the difference in preferred temperature between the two haemoglobin types at normoxia, the swimming speed did not differ significantly (0.38±0.05 and 0.29±0.09 $BL\,s^{-1}$). However, both haemoglobin types decreased their swimming speeds significantly (P<0.0001) when facing hypoxia and reached a similar level of 0.16±0.08 and 0.17±0.08 $BL\,s^{-1}$. Changes in preferred temperature and swimming speed for the two haemoglobin types, as described above, are illustrated in Fig. 1, which includes an experimental period where the fish is exposed to normoxia and hypoxia.

Discussion

A highly significant difference was found between the preferred temperatures of HbI-1 and HbI-2 cod. The temperature preferred by HbI-1 cod (15.4±1.1°C) compared to HbI-2 cod (8.2±1.5°C) proves that temperature is a selective parameter in the heterogeneous distribution of the haemoglobin types. This conclusion is illustrated in Fig. 2, which shows the frequency of the HbI^1 allele throughout the Atlantic cod's range, plotted on a sea surface temperature map. Schurmann and Steffensen (1992) previously examined the preferred temperature of juvenile cod captured near Helsingør in Denmark. They found a preference for 13.9±2.7°C without taking different haemoglobin types into consideration. Sick (1965a) found the distribution of HbI-1, HbI-1/2 and HbI-2 in this area to be 31%, 51% and 18%, respectively. While the preferred temperature of HbI-1/2 is not known, the result of Schurmann and Steffensen (1992) indicates that it may be situated somewhere between those of HbI-1 and HbI-2. Clark and Green (1991) and Despatie et al. (2001) also measured the preferred temperature of Atlantic cod in Canada, where the HbI^2 allele is dominating, and in both studies the preferred temperature was lower than 10°C.

Periods of hypoxia occur in coastal areas, and in the late summer months the oxygen saturation can be as low as 20–40% in Danish waters (Nielsen and Gargas, 1984). In the present study an oxygen saturation of 35% resulted in a significant decrease in the preferred temperature for cod with HbI-1 genotype from 15.4±1.1 to 9.8±1.8°C. There are several physiological advantages in lowering the body temperature during hypoxia, including lower metabolic rate, higher oxygen affinity for haemoglobin and higher oxygen solubility in the water (Jobling, 1994). The disadvantages of preferring lower water temperatures during hypoxia are a reduction in swimming speed as well as a reduction in food intake and digestion rate, which results in decreased growth (Brett, 1971). Changes in enzyme conformation, membrane structure and acid–base regulation are also consequences of a sudden decrease in temperature (Reynolds and Casterlin, 1980;

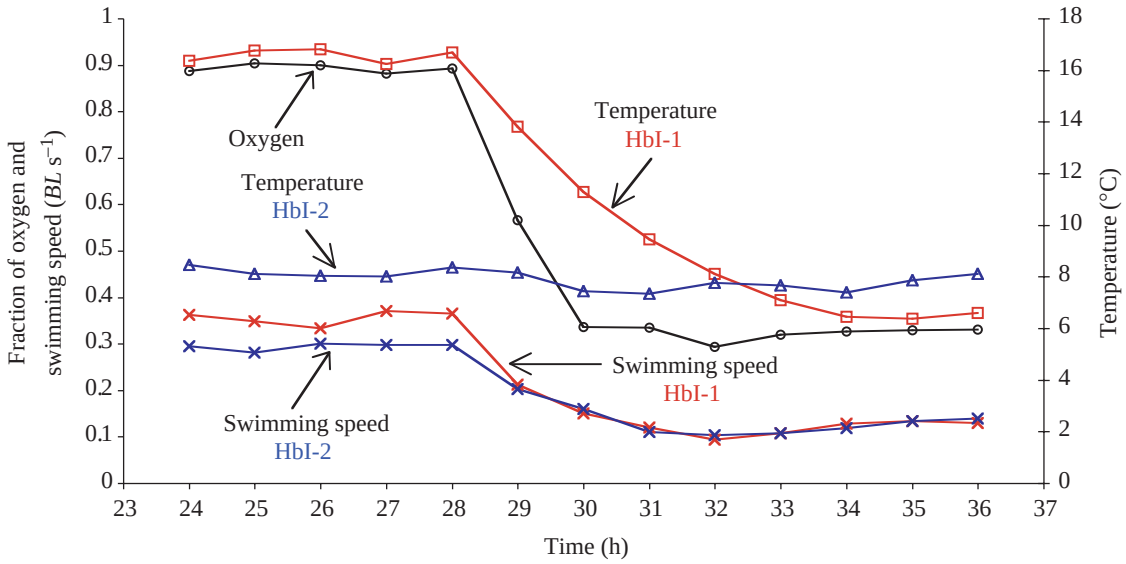

Fig. 1. Oxygen saturation, swimming speed and water temperature measured for cod with haemoglobin type HbI-1 and HbI-2. When the oxygen saturation is lowered to 35% the preferred temperature of HbI-1 cod decreases, while this tendency is absent for HbI-2 cod. For both the haemoglobin types the swimming speed decreases during hypoxia. Measurements were made every second and each point is a mean value calculated for 1 h.

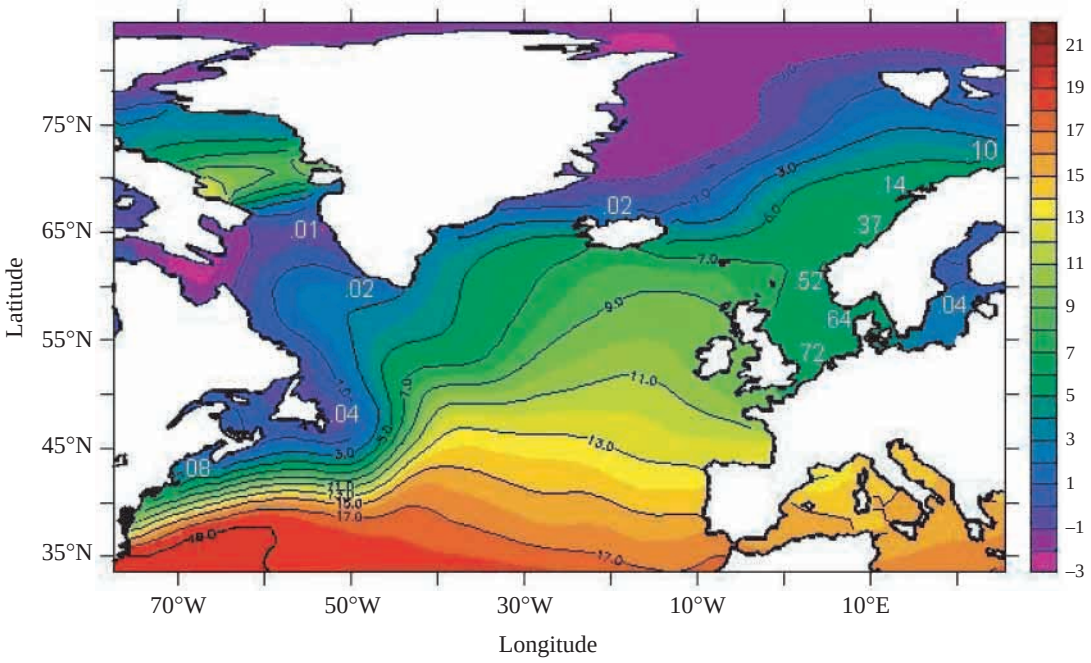

Fig. 2. Sea surface temperatures (spring) and the frequency distribution of the HbI^1 allele throughout the range of the Atlantic cod (Sick, 1965a,b; Frydenberg et al., 1965). The frequency of the HbI^1 allele is low in cold water regions and become more dominant in warmer areas. A clear cline is found along the Norwegian coast.

Jobling, 1994). A decrease in the preferred temperature as a consequence of hypoxia has both advantages and disadvantages for the fish. Hypoxia, however, seems to be nothing but a disadvantage for the fish. Chabot and Dutil (1999) found that the growth rate of Atlantic cod at 10°C decreased significantly when the oxygen fell below 56% saturation. In the same study it was observed that spontaneous activity also decreases during hypoxia.

The observation that the preferred temperature of HbI-2 cod did not decrease as a consequence of hypoxia (8.2±1.5–8.0±2.9°C) indicates that the energy saving advantages of an even lower temperature was not necessary for the cod to survive. This conclusion makes sense, since the preferred temperature of HbI-1 cod never went below this temperature during hypoxia. Schurmann and Steffensen (1992) found that the lowest preferred temperature correlated with the lowest oxygen saturation (15%). If the HbI-2 genotypes had been exposed to the same low level of hypoxia, possibly the preferred temperature would have been significantly lower.

In the present study swimming speeds for HbI-1 and HbI-2

cod at normoxia were 0.38 and 0.29 $BL\,s^{-1}$, respectively. These swimming speeds correspond with values obtained in previous studies (Schurmann and Steffensen, 1994). Whether the haemoglobin type actually affects the swimming speed is not known, but since the preferred temperature is thought to be the zone where physiological processes are optimised or maximized (Jobling, 1994), the question is relevant.

Hypoxia (35% oxygen saturation) significantly lowered the swimming speed for both haemoglobin types (0.17 and 0.16 $BL\,s^{-1}$). The preferred temperature also decreased for HbI-1 exposed to hypoxia. Since Schurmann and Steffensen (1994) found a tendency of a higher swimming speed at 10°C compared to 15°C, the decrease in swimming speed for HbI-1 cod in the present study was probably caused by hypoxia and not by the temperature. The levels of swimming speed at 35% oxygen saturation correspond to previous measurement for Atlantic cod; 0.18 $BL\,s^{-1}$ was the average swimming speed at 30% oxygen saturation measured at 5°C and 10°C by Schurmann and Steffensen (1994). The reduction in swimming speed during hypoxia will not only decrease the oxygen requirement of the Atlantic cod, but also reduce the chance of reaching a more favourable environment. Another strategy for fish during hypoxia is to increase the activity level, which enhances the probability of encountering better oxygen conditions. The latter strategy is observed for the skipjack tuna (*Katsuwonus pelamis*) (Dizon, 1977) and the red hake (*Urophycis chuss*) (Bejda et al., 1987).

The consequence of the difference in the preferred temperature of HbI-1 and HbI-2 cod should be a differentiation of physiological processes (Jobling, 1994). So far only a limited number of studies have distinguished between the haemoglobin types when studying physiology and behaviour of Atlantic cod. An exeption was McFarland (1998) who differentiated between the haemoglobin types when measuring the metabolism, and found that HbI-2 cod had a significantly lower standard metabolic rate at 4°C compared with HbI-1 cod. Feeding behaviour has also been examined and the result showed that the competitive performance was highest for HbI-2 cod measured at 6°C (Salvanes and Hart, 2000). The advantageous feeding behaviour for HbI-2 cod at low water temperatures is also reflected in growth-related parameters; HbI-2 cod from mid to northerly Norwegian coastal waters has a higher growth rate and earlier age of first spawning than HbI-1 cod (Jørstad and Nævdal, 1994; Mork et al., 1983, 1984). It seems that the theory of optimised physiological performances at the preferred temperature fits HbI-2 cod perfectly, whereas the advantages of HbI-1 cod at high water temperatures are more blurred and less well known. We predict that there are more physiological and behavioural differentiations between the haemoglobin types due to their different preferred temperatures. These results suggest that for physiological and behavioural studies as well as for aquacultural use, Atlantic cod should be subdivided according to its haemoglobin type.

The coupling between temperature and the occurrence of cod with different haemoglobin genotypes is of interest when discussing the biological consequences of increased water temperatures due to global warming. The results from the present study indicate that increasing water temperatures will result in an increased frequency of HbI-1 cod, because this haemoglobin type prefers a higher temperature. Furthermore, Atlantic cod stocks will move north if the seawater temperature exceeds the preferred temperature of the HbI-1 genotype. If a combination of increased water temperature and hypoxia should occur, the predicted superiority of HbI-1 cod as a consequence of increased water temperatures, and the fact that HbI-1 cod prefers a lower temperature during hypoxia, will cause an unfavourable situation for the HbI-1 cod. This situation is especially relevant in coastal regions where hypoxia is common, and could cause extensive damage to the Baltic cod stock, for example.

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