



Correlations between hemoglobin type and temperature preference of juvenile Atlantic cod *Gadus morhua*

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ABSTRACT

Atlantic cod (*Gadus morhua* L.) exhibits polymorphic hemoglobin variants with the Hbl locus showing a strong North-South geographic cline in frequency distribution of three main types (1/1, 1/2 and 2/2). This may indicate selective advantages of the different Hbl types under various temperature regimes. Despite this only one study has directly examined the temperature preference of the two homozygous types, Hbl-1/1 and Hbl-2/2, whereas the preference of the heterozygote (Hbl-1/2) has never previously been addressed. By exposing fish to a 4–19 °C temperature gradient in an annular preference chamber we recorded the preferred temperature of wild juvenile *G. morhua* of all three main Hbl types originating from an area where they co-exist. Hbl-2/2 *G. morhua* preferred significantly cooler water (8.9 ± 0.2 °C) compared to the Hbl-1/1 group (11 ± 0.6 °C), this difference, however, not being as distinct as previously reported. There was pronounced inter-individual variation in the temperature preference of the Hbl-1/2 *G. morhua* ranging between 6.7 and 13.8 °C, and their overall preference (10.5 ± 0.9 °C) did not differ significantly from either of the homozygous Hbl types. Notably, the mean range of utilized temperature (temperature span between 1st and 3rd quartile) was very similar between all 3 Hbl types with 3.2–3.5 °C. Considering the complexity of a trait like temperature preference, there are clearly many other factors besides Hbl type that influence the thermal biology of cod, and therefore we also investigated possible associations between genotype and temperature preference for 12 variable candidate gene single nucleotide polymorphisms (SNPs) a priori expected to be related to growth and reproduction. There were, however, no significant correlations between temperature preference and any of the candidate gene SNPs indicating that none of these polymorphisms strongly associates with thermal behavior. Considering however the high-throughput genotyping methods becoming increasingly accessible there is great potential for association studies involving many more genetic markers to identify additional genetic polymorphisms that are important for temperature preference in *G. morhua*. In conclusion, we support the notion of a 'warm' (Hbl-2/2) and a 'cold' (Hbl-1/1) Hb type, although we suggest the difference to be more subtle than previously reported. Furthermore Hbl-1/2 *G. morhua* shows rather inconsistent thermoregulatory behavior. To obtain a more definitive picture of the extent to which thermal niches are realized under natural conditions field observations in areas where the 3 Hbl types co-exist should be performed.

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1. Introduction

The body of most fish has the same temperature as the surroundings and their main means of controlling body temperature is by thermoregulatory movement behavior. To aid digestion Atlantic stingray *Dasyatis sabina* and Bear Lake sculpin *Cottus extensus* for example move into warmer water after feeding (Wallman and Bennett, 2006; Wurtsbaugh and Neverman, 1988). Due to this inherent property of fishes, and other ectothermic aquatic animals, the ongoing shift in water temperature

regimes has led to increased awareness of the importance of determining how individuals and populations respond to, and perform under, changes in environmental temperature.

Atlantic cod *Gadus morhua* (Linnaeus, 1758) is a typical inhabitant of the continental shelf throughout the North Atlantic (Hutchings, 2004; Rose, 2004) and is predicted to be affected by future rising sea temperatures (e.g. Perry et al., 2005). Temperature tolerance and preference likely varies between different cod populations. Over forty years ago Sick (1961), showed that this species exhibits polymorphisms in hemoglobin (Hb) proteins with three main types that he termed Hbl-1/1, Hbl-1/2, and Hbl-2/2. The Hbl-2/2 type is more prevalent in northern cooler regions, while the Hbl-1/1 type is more prevalent in warmer waters (Frydenberg et al., 1965; Sick, 1965a,b).

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The geographical distributions include an apparent cline along the Norwegian coast (Petersen and Steffensen, 2003). Intuitively such strong cline patterns over distances where neutral genetic markers show much more limited differentiation (e.g. Nielsen et al., 2009; O'Leary et al., 2007) could indicate selective advantages of the different types under various environmental conditions. This question has mainly been addressed from the viewpoint of optimality and with focus on maximal growth e.g. aiming to improve the cost-efficiency of production in aquaculture. However, while some studies support the notion that the geographical differentiation in Hbl type indicates that Hbl-1/1 should do better at warm temperatures as compared to fish possessing the Hbl-2/2 type (Brix et al., 2004; Nævdal et al., 1992), others on the contrary either found that Hbl-1/1 cod grew faster at cold temperatures (7 °C) (Imsland et al., 2004) or that there was no significant effect of Hbl type on growth when fish were reared at 6 or 14 °C (Jordan et al., 2006). Furthermore, Gamperl et al. (2009) found no correlation between upper temperature tolerance and Hbl type, including the heterozygote Hbl-1/2. The one study to date addressing the correlation between Hbl type and temperature preference found that juvenile Hbl-2/2 fish preferred 8.2 °C whereas Hbl-1/1 preferred 15.4 °C (Petersen and Steffensen, 2003). The apparent inconsistency between these results and the comparative assessments of physiological performance could suggest that the temperature preference may not provide a good indicator of optimum growth temperature, or vice versa.

Better insight into the thermal biology of cod will advance our understanding of the species ecology and distribution (Brown et al., 2004) and further improve our predictions of how climate change may affect geographic distributions and population dynamics (Harley et al., 2006; Wikelski and Cook, 2006; Young et al., 2006). There is a clear need for additional empirical data on how this species behaves in heterogeneous thermal environments. Ideally field studies should provide information on the actual temperatures experienced, but such studies are logistically challenging, time consuming and expensive. So far only one study has reported direct observations of habitat, and temperature, occupation of cod by means of electronic data storage tags employed throughout the north-east Atlantic (Righton et al., 2010). If designed properly, laboratory experiments that allow for a more rigid test of hypotheses can provide supplementary useful information on thermal preferences, thermal niches and limits to thermal tolerances, enabling predictions on individual performance and resilience in a natural environment.

Traditional thermal preference apparatuses for fishes however have significant drawbacks; shuttleboxes (producing a horizontal gradient controlled by the movement of the organisms) have features (e.g. corners) that provide available or perceived cover and water currents, both potentially affecting the behavior of the animal, whereas the outcome of thermal response experiments using vertical chambers may be biased by differential pressure and light intensities. Myrick and colleagues (Myrick et al., 2004) first presented an annular-shaped chamber which is believed to be an improved design for preference studies as it minimizes the potential biases of the external influences inherent in other preference systems while maximizing the span of the gradient. The design has since been used by other research groups (Chen et al., 2008; Gräns et al., 2010; McMahon et al., 2008). In the present study juvenile Atlantic cod were allowed to move freely in a horizontal temperature gradient within an annular-shaped chamber. The aim was to obtain a better understanding of the influence of Hbl type on the preferred temperature and the range of temperatures most frequently utilized by the fish. As no previous records exist on temperature preference of the Hbl-1/2 animals, and as these constitute around half of the cod population in the study area (Øresund; Sick, 1965a), they were included in the experiment together with the two homozygous types.

Although most previous efforts to understand the genetic basis of temperature preference and tolerance in Atlantic cod have focused on

Hbl polymorphisms, it is obvious that many other genes may also influence such a complex trait that integrates the effects of many physiological processes. Recently, several studies have revealed strong correlations between temperature and the distribution of alleles at multiple other genetic loci in cod (Bradbury et al., 2010; Nielsen et al., 2009), but the effects of these have not been assessed at the individual level. For this reason, we also genotyped 31 single nucleotide polymorphisms (SNP markers) in candidate genes for growth and reproduction – some of which display apparent temperature-associated spatial distribution (Hemmer-Hansen, pers. com.) – to assess whether the variation in any of these were associated with different behaviors in the experiment.

2. Materials and methods

2.1. Fish

Juvenile Atlantic cod were caught by gill net in the vicinity of the Marine Biological Laboratory in the northern part of Øresund, Denmark. Post-capture, fish were transferred to the laboratory where they were kept in 10 °C re-circulated, full strength aerated seawater and light/dark conditions of 13 h:11 h. The fish were fed ad libitum two times a week with chopped herring *Clupea harengus* or sandeel *Ammodytidae* sp. For later identification of the fish an individual PIT tag (12 × 2 mm, ISO standard 11784/11785, FDX-B) was placed subcutaneously just beneath the dorsal fin of each fish. After two weeks of acclimation to laboratory conditions 40 fish (TL 16–19 cm) exhibiting different Hbl types were transported to the University of Gothenburg in a 200 L thermo-isolated tank with 10 °C water that was continuously oxygenated. In Gothenburg the fish were left to recover and acclimatize for two additional weeks under water conditions comparable to those at the Marine Biological Laboratory, Denmark, before the temperature preference experiments were initiated.

2.2. Molecular analysis

We determined the Hbl type of all experimental animals through DNA sequencing of two variable positions in the β 1 hemoglobin gene: the Met55Val and the Lys62Ala substitutions that have been shown to unambiguously correlate with the Hbl polymorphisms observed through gel electrophoresis (Andersen et al., 2009; Borza et al., 2009) DNA was extracted from fin tissue of each individual using an E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek, USA). A 279 bp fragment spanning the relevant β 1 hemoglobin polymorphisms was amplified with polymerase chain reaction using the primers 5'-ACAGAGGGTCTGCAGACAT-3' and 5'-GACGTGAGTTTCTCGGAGT-3' and the amplified fragments were sequenced to identify the genotypes at the variable sites correlating with Hbl types. Furthermore, each individual was genotyped for a total of 31 candidate gene SNPs using an assay developed by Hemmer-Hansen et al. (2011) based on single-base extension implemented in the SNaPshot protocol (Applied Biosystems, USA). Primer sequences and reaction details are available in Hemmer-Hansen et al. (2011).

2.3. Temperature preference chamber

The temperature preference chamber used was a copy of that described in detail by Myrick et al. (2004). The total diameter was 1 m and the swimming channel was 10 cm wide with a water depth of 15 cm. The channel dimensions did not prevent the fish from changing swimming direction. Water was distributed via 16 feeding lines from 4 reservoirs containing cooled (4 °C), warmed (19 °C), or ambient (10 °C) water. To measure temperature in the setup, 16 thermocouple temperature probes were placed around the inner wall of the swimming channel at mid-water depth. The cool water was obtained using a customized 5 kW cooling unit with build in titanium heat exchanger;

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