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Hypoxic avoidance behaviour in cod (*Gadus morhua* L.): The effect of temperature and haemoglobin genotype

J.E. Skjæraasen [a,*], T. Nilsen [b], J.J. Meager [a], N.A. Herbert [c], O. Moberg [a], V. Tronci [a], T. Johansen [d], A.G.V. Salvanes [a]

[a] *Department of Biology, University of Bergen, N-5020, Bergen, Norway*
[b] *Department of Mathematics, University of Bergen, N-5020, Bergen, Norway*
[c] *Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK*
[d] *Insitute of Marine Research, N-5817, Norway*

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Abstract

Hypoxia can influence fish growth, survival and on larger scales, population structure. These effects may be influenced by water temperature, and may vary intra-specifically with genotype. In Atlantic cod (*Gadus morhua* L.), the two haemoglobin homozygotes (*Hb-I*11* and *Hb-I*22*) vary in oxygen affinity at different temperatures, which is thought to correspond to variation in hypoxia tolerance. We therefore tested if hypoxic avoidance behaviour in cod 1) depends on ambient temperature and 2) is modified by haemoglobin genotype. In a laminar flow choice box, we subjected juvenile cod to an initial phase of non-escapable hypoxia, and a subsequent recovery phase, where one habitat was kept at 20% O_2 saturation while the other was raised in steps to full saturation. The experiment was performed at 5 and 15 °C with *Hb-I*11* and *Hb-I*22* cod. Cod responded to inescapable hypoxia by reducing their overall swimming speed and then, at the initial levels of the recovery phase, avoiding the most hypoxic habitat, irrespective of temperature or genotype. Fish recovered quickly as O_2 levels increased, as evidenced by increased swimming speed and time spent in the most hypoxic habitat. The avoidance response depended strongly on temperature: the relative reduction in speed and avoidance of the most hypoxic habitat was more pronounced at 15 than at 5 °C. During the recovery phase, stressed fish initially maintained a higher swimming speed in the most hypoxic habitat. However, as O_2 increased, swimming speed in both habitats converged. This point of convergence occurred at a lower O_2 saturation at 5 °C. Fish ventilation rate in inescapable hypoxia was also higher at 15 °C. Haemoglobin genotype did not influence either ventilation rates or the nature of the hypoxic avoidance response at either temperature, but *Hb-I*11* cod swam faster than *Hb-I*22* cod in normoxia at 15 °C. Our results indicate that increased temperature limits the ability of cod of both haemoglobin genotypes to exploit hypoxic habitats. This may have negative future consequences for coastal cod stocks in light of increasing global temperatures and eutrophication in coastal waters.

Keywords: Avoidance; Cod; Haemoglobin; Hypoxia; Temperature

1. Introduction

The availability of oxygen in water can limit fish metabolic scope (Schurmann and Steffensen, 1997; Claireaux et al., 2000) and hence growth and performance of fishes (Thetmeyer et al., 1999; Wu et al., 2003). These effects depend on a range of factors including ambient temperature and exposure time (Plante et al., 1998). At the extreme, chronic exposures to hypoxia can result in death (Schurmann and Steffensen, 1992; Plante et al., 1998). However, the availability of dissolved oxygen often varies substantially over small scales in marine environments, hence hypoxic habitats can also be utilised by fish to forage (Pihl, 1994; Neuenfeldt, 2002) or evade predators

* Corresponding author. Department of Biology, University of Bergen, P.O. Box 7800, N-5020, Bergen, Norway. Tel.: +47 55584626; fax: +47 55584450.
E-mail address: jon.skjaeraasen@bio.uib.no (J.E. Skjæraasen).

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(Robb and Abrahams, 2002, 2003) by limiting their visits to short incursions. Fish also have behavioural strategies to increase the duration of their visits to hypoxic habitats, such as reducing swimming speed (e.g. Herbert and Steffensen, 2005) or increasing gill ventilation (Randall, 1982).

Temperature both increases oxygen consumption by increasing the metabolic rate of fish (e.g. Schurmann and Steffensen, 1997) and reduces oxygen availability by decreasing its solubility (Benson and Krause, 1984). The effect on fishes of increases in the severity and frequency of hypoxic events from eutrophication (e.g. Powers et al., 2005), may therefore be exacerbated by concurrent temperature increases from global warming (e.g. Grottoli et al., 2006; Schiermeier, 2006). This may place additional pressure on stocks already at historical lows, such as the Atlantic cod (*Gadus morhua* L.) populations in the North Atlantic (e.g. Hutchings and Baum, 2005).

However, variation in intra-specific tolerance to hypoxia may mean that populations are affected differentially across species ranges. In Atlantic cod, hypoxia tolerance is thought to differ between cod with different haemoglobin genotypes (Brix et al., 2004). Haemoglobin binds oxygen in the gills and transports and distributes it via the blood flow to the tissues. The haemoglobin system of Atlantic cod consists of two different alleles, the *HbI-1* and *HbI-2*, resulting in three main genotypes (*Hb-I*11*, *12* and *22*). Karpov and Novikov (1980) found that the different *Hb-I** genotypes had different temperature optima with regard to oxygen affinity: *Hb-I*22* had the highest affinity at low temperatures (5–10 °C), whereas *Hb-I*11* had the highest affinity at high temperatures (15–20 °C). The *Hb-I*12* genotype was intermediate between the two. These results were more or less reaffirmed by Brix et al. (1998), who further argued that high O_2-affinity correlates to increased hypoxia tolerance. Hence, the behavioural response of cod to hypoxia may vary with both temperature and haemoglobin genotype. So far, no study has tested this hypothesis.

The response of cod to hypoxia, has however, been addressed (e.g. Herbert and Steffensen, 2005). A recent study, using the same experimental set-up as the present study, indicated that cod do not avoid hypoxia at set threshold levels, but rather that avoidance behaviour is controlled by internal physiological state/stress (Herbert et al., unpublished data). It was also found that in heterogeneous oxygen habitats, cod increase their swimming speed in hypoxia, but reduce it again once more favourable O_2-habitats had been found. At higher temperatures, increased swimming speed is likely to exacerbate the effects of hypoxia by increasing oxygen demand (Schurmann and Steffensen, 1997). Conversely, decreased oxygen demand at low temperatures, i.e. slower swimming speeds, lower metabolic rate and more dissolved oxygen in the water, is likely to reduce the behavioural effects of hypoxia.

Our study examined the influence of temperature on the behavioural response of cod to hypoxia. Specifically, we aimed to test if: (1) hypoxic avoidance behaviour is modified by ambient temperature and (2) the effect temperature on hypoxia avoidance depends on the haemoglobin genotype; i.e. *Hb-I*22* cod will show less avoidance behaviour at 5 °C, while *Hb-I*11* will show less avoidance behaviour at 15 °C.

2. Materials and methods

2.1. Experimental fish

Parental cod were collected in the Bergen area (60° 25′ N, 5° 20′ E) in October 2002 and October 2003 and transported to the High Technology Centre in Bergen, where the cod were kept in two separate storage tanks of 7000 L until the experiment started. Prior to spawning, the haemoglobin genotypes of all cod were identified from blood samples obtained from the caudal vein, by agar electrophoresis in Smithies buffer as described by Fyhn et al. (1994). We then selected cod of the two homozygote genotypes, *Hb-I*11* and *Hb-I*22*, and separated them into two spawning groups and allowed them to spawn under a natural photoperiod during the spring of 2004. The *Hb-I*11* group contained 9 females and 12 males, while the *Hb-I*22* group contained 7 females and 7 males. Eggs were collected from the different tanks and incubated simultaneously. Larvae were reared in 500 L tanks on a diet of natural zooplankton and transferred to 500 L tanks as juveniles. Juveniles were fed pellets supplied by a band feed and seawater was introduced from a flow-through system (temperature: 8 to 10 °C). Haemoglobin genotypes were not mixed at any time during this stage.

In November 2004, fish were sedated with Metacaine (0.05 g L^{-1}) and pit-tagged. 24 fish of each genotype were then randomly selected and transferred to two separate 500 L holding tanks. Over a period of four days, the temperature in the tanks was gradually adjusted to either 5 or 15 °C. This temperature was maintained until completion of the experiment. Both genotypes were kept in these mixed groups at each temperature regime. The experiment started in December 2004 after a minimum acclimation period of 5 weeks.

2.2. Laminar flow choice box

The experiment was conducted in a laminar flow-through system that provided fish a free choice of two discretely separated oxygen concentrations without the use of physical barriers (see Kroon and Housefield, 2003). This 'choice box' (Loligo, Denmark) was constructed of Plexiglass (inner dimensions: 120 cm length; 40 cm width; 11 cm height, Fig. 1). To establish a laminar flow, water first passed through a separator and two honeycomb diffusers at 17 L min^{-1}. A further separator was placed downstream of the experimental arena. Dye tracer tests (food colouring) showed that the laminar flow demarcation was sharp and stable with and without fish swimming in the choice box. Oxygen gradients were established to ±2% by bubbling nitrogen or air through the header tank (Fig. 1), and were regulated using oxygen sensors (WTW 340i, Wissenschaftlich-Technicshe Werkstäffen) and a computer-controlled feedback system (Model 5514, Loligo, Denmark) located outside the experimental room.

2.3. Experimental protocol

The experiment tested the effect of temperature, i.e. 5 and 15 °C on hypoxia avoidance behaviour by juvenile cod of the

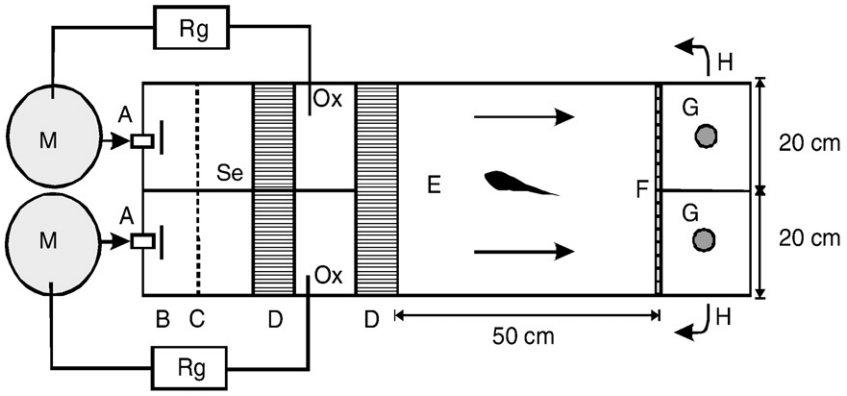

Fig. 1. Laminar flow 'Choice Box': seawater entered the system through the inlets (A) at 17 L min^{-1} and flow separation was established and maintained by using a separator (Se), diffusers (B, C) and honeycomb baffles (D). The test area (E) was separated from the drainage section (G) by a further screen (F). Pumps (H) were used to re-circulate test water to the mixing tanks (M). O_2 levels in each flow stream were maintained to ±2% by computerized regulators (Rg) that used a solenoid valve connected to a compressed bottle of nitrogen to control de-oxygenation in the mixing tanks, based on a signal from the oximeters (Ox). The computerized regulators would release gas, by opening the solenoid valve, into the water stream if the signal received from the oximeter was above a preset O_2 value and conversely close the valve if the signal from the oximeters was below the preset value. Arrows indicate the direction of flow.

two different haemoglobin homozygotes. The temperatures were chosen because, theoretically, *Hb-I*22* fish is expected to be favoured in terms of higher O_2-affinity at 5 °C, whereas as *Hb-I*11* should be favoured at 15 °C (e.g. Brix et al., 1998).

At the start of each experiment, one fish was introduced into the choice box (100% O_2) and allowed approximately 16 h of acclimation. We then monitored fish behaviour for 30 min under control conditions (fully oxygen saturated), before reducing the oxygen saturation of both sides of the choice box to 20% O_2 for 30 min. We chose 20% O_2 saturation as it represents the standard median lethal concentration (LC_{50}) of cod for chronic exposure (i.e. 96 h period, Plante et al., 1998) and occurs in natural cod habitats (e.g. Pihl, 1994; Neuenfeldt, 2002).

Fish were then allowed to choose between one choice box side maintained at 20% and a series of increasing oxygen saturation levels on the other (30, 40, 50, 60, 80 and 100% O_2; Fig. 2). The 20% hypoxic side of the choice box was switched between trials to avoid any tank bias.

Each new steady-state oxygen saturation level (20, 30, 40, 50, 60, 80 and 100% O_2) lasted for 30 min and was preceded by a 15 min unsteady state level where the oxygen was adjusted to the new steady state. The observational period thus lasted for 6.5 h per fish.

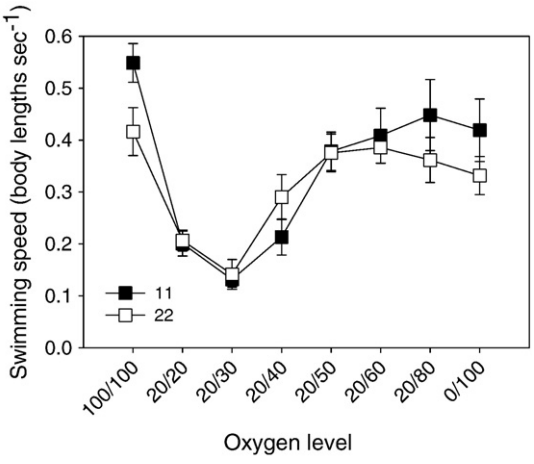

Fig. 2. Swimming speed of the two haemoglobin genotypes at 15 °C. Error bars are the standard error (S.E.).

Eight fish of each genotype were tested at 15 °C. Due to a problem with the 5 °C holding tank, resulting in a limited number of fish, only six fish of each genotype were tested in the 5 °C experiment. A small, but significant difference was found in condition between the *Hb-I*11* and *Hb-I*22* fish selected for the 5 °C experiment (two-tailed *t*-test, $p<0.05$, Table 1). No other genotype difference was found in length, weight or condition at either temperature (two-tailed *t*-tests all p's>0.05, Table 1).

2.4. Analysis of fish behaviour and swimming speed

Fish behaviour was monitored by a CCD camera (WV BP550, Panasonic) placed 0.6 m above the choice box and connected to a PC and VCR via an external frame grabber (Trust VI-3100USB2). The choice box was illuminated from above by a fluorescent lamp, and image contrast was enhanced by an additional fluorescent light source located underneath the choice box. The position of the centre of mass of each fish was tracked digitally at a frequency of 10 Hz (LoliTrack, Loligo, Denmark). Positional data was then converted to average swimming speed in body lengths per second (BLS^{-1}) and the percentage of time spent by fish in the hypoxic environment, i.e. habitat choice. Swimming speed was measured as relative to the choice box and

Table 1
Length, weight and condition (Fulton's $K=W*L^{-3}*100$) of fish selected for the experiment at 5 and 15 °C

Variable	5 °C		15 °C	
	*Hb-I*11* (6)	*Hb-I*22* (6)	*Hb-I*11* (8)	*Hb-I*22* (8)
Weight	33.3 (±2.9)	28.4 (±4.8)	53.1 (±7.0)	43.6 (±8.1)
Length	16.2 (±0.4)	14.7 (±0.7)	17.9 (±0.8)	16.8 (±1.0)
Condition	0.78 (±0.02)	0.86 (±0.02)	0.91 (±0.06)	0.85 (±0.02)

Number of fish of each haemoglobin genotype at each temperature is given in brackets. Values given for the variables are mean±standard error.

not corrected for flow rate. We also measured ventilation rate (opercula-beats min^{-1}) for each fish over a 2 min period at the 20–20% O_2 concentration (the most hypoxic environment) by visual examination of the recorded video footage.

2.5. Data analyses

To quantitatively model the effect of oxygen level on fish behaviour we employed the mixed effect model package *nlme* (Pinheiro and Bates, 2000; Pinheiro et al., 2007) of the statistical software R (R Development Core Team, 2007). The mixed effect modelling approach was used because we had repeated measurements of individual fish over a range of oxygen concentrations. We initially investigated if haemoglobin had any effect on the individual curves for each of the two temperatures separately. This was done by using mixed effect models with a random effect for each fish and oxygen level as a fixed categorical effect as well as expanded models where we added the categorical variable haemoglobin as a fixed effect with and without an interaction with the oxygen factor. Which variables were included in the final models was based on the Bayesian Information Criterion (BIC) (Pinheiro and Bates, 2000).

We separated the behavioural response of fish into two distinct phases: the initial phase, with the first three experimental O_2 levels (Fig. 2), in which swimming speed and the number of visits to the hypoxia area (min^{-1}) decreases and a clear avoidance response is observed. This is followed by a subsequent recovery phase comprising the last six experimental O_2 levels (Fig. 2), where fish increase their swimming speed, the number of visits and the total time spent in the most hypoxic habitat. These two distinct phases were modelled separately with the O_2 levels 20–30% acting as a joint between the two phases.

2.5.1. The initial phase

The percentage of time, y_{ijk}, spent on the focal side, i.e. the side destined to remain at 20% oxygen saturation, was for the initial phase modelled by a mixed effect variance model:

$$y_{ijk} = \mu_0 + a_i + \beta_j + \gamma_k + e_{ijk} \quad (1)$$

where y_{ijk} is the time spent by the i'th fish at the j'th oxygen level at the k'th temperature. μ_0 is the general intercept, a_i denotes a random effect for the i'th fish, β_j a fixed effect describing the j'th oxygen level, γ_k describes the effect of the k'th temperature and e_{ijk} is the error term.

The number of visits to the focal side and swimming speed during the initial phase was modelled by the following mixed effect regression model:

$$y_{ijk} = \mu_0 + \beta_{0k} + b_{0i} + (\mu_1 + \beta_{1k} + b_{1i})*O_j + e_{ijk} \quad (2)$$

where y_{ijk} denotes the measurement of the given variable for the i'th fish at the j'th oxygen level and the k'th temperature. Subscript 0 refers to an intercept value and 1 to a slope. The oxygen levels (O_j), 100–100, 20–20, 20–30, were coded as 0, 1, 2 respectively. A Greek letter denotes a fixed effect and a Latin letter a random effect.

2.5.2. The recovery phase

For the recovery phase we employed a non linear asymptotic regression for each temperature separately:

$$y_{ij} = \phi_1 + (\phi_2 - \phi_1)e^{-e^{\phi_3}(O_j - 30)} + e_{ij} \quad (3)$$

where y_{ij} denotes the measurement of the i'th fish at the j'th oxygen level for the variable in question, i.e. habitat choice, swimming speed and number of visits. $\varnothing_1$ is the asymptotic value, $\varnothing_2$ is the value when the oxygen level of the non-focal side, O_j equals 30 and the decay constant is modelled as $e^{\phi 3}$. It is possible to include random effects in $\varnothing_1$, $\varnothing_2$, $\varnothing_3$. To decide which should have a random component to account for variation between fish we fitted the model to each fish separately by using the function *nlmList*. Plots of individual confidence intervals indicated that only a random effect in the asymptotic term was necessary. This model also gave the lowest BIC value and was chosen for the final analyses.

Post hoc comparisons between the different temperatures and oxygen level combinations were made through the use of Students *t*-tests. For the 7 hypoxic levels from 20/20 to 20/100 we adjusted experiment-wise significance according to Dunn–Sidak's method and assigned significance at a *p*-value of 0.0073 (Ury, 1976).

Recovery of fish was estimated as the oxygen level during the recovery phase where swimming speeds between sides of the choice box converged at $\alpha = 0.05$. We used a paired *t*-test and adjusted for experiment-wise significance according to the Dunn–Sidak's method (Ury, 1976). We base the assumption that convergence represents recovery on the results of Herbert et al. (unpublished data). They found that fish initially maintained a higher swimming speed in the most hypoxic habitat after exposure to inescapable hypoxia, but that this difference diminished and eventually disappeared during the recovery phase. This happened concurrently with the increase in overall swimming speed, and in the time spent and frequency of visits to the most hypoxic habitat. Differences in ventilation rates between genotypes and temperatures were tested with ANCOVAs', using fish weight and swimming speed as covariates.

3. Results

The initial mixed effect analyses with haemoglobin as a categorical variable revealed that no haemoglobin effect was evident for any of the response variables at either temperature (*p* values > 0.05), with only two exceptions: *Hb-1*11* fish swam significantly faster (Fig. 2) and visited the focal side more often in normoxia at 15 °C, and the *Hb-1*22* fish visited the focal side more frequently at normoxia at 5 °C (not shown). On this basis, we have pooled the results of both genotypes for the remainder of the analysis.

3.1. Habitat choice

There was no difference in habitat choice between temperatures at the 100/100 and 20/20 level ($p > 0.05$, mixed effect model, Eq. (1)). At both temperatures, no preference for

the focal side was observed during the normoxic starting level nor the following inescapable hypoxic situation. However, when fish were given a choice between different oxygen concentrations at the 20/30 oxygen levels, fish showed a clear avoidance of the most hypoxic habitat (Fig. 3).

During the recovery phase, the 15 °C fish tended to spend less time than the 5 °C fish in the most hypoxic habitat at the initial 20/30 and 20/40 levels (Fig. 3). Towards the end of the experiment, both 5 and 15 °C fish spent a similar amount of time in the most hypoxic environment ($p=0.33$, Fig. 3, mixed effect model, Eq (3)). The *post hoc* tests, i.e. the Student's *t*-tests, showed that 15 °C fish spent significantly less time in the most hypoxic habitat at the 20/30 level ($p=0.0069$, Fig. 3) and close to significantly less at the 20/40 level ($p=0.016$) at 5 °C. No other oxygen levels differed.

3.2. Number of visits

During the initial phase there was a significant negative effect of decreasing oxygen on the number of visits to the hypoxic area ($t=-2.41$, $df=54$, $p<0.05$, mixed effect model, Eq. (2)). However, this effect was stronger for fish at 15 °C ($t=-4.84$, $df=54$, $p<0.001$, mixed effect model, Eq. (2)).

During the recovery phase, 15 °C cod tended to visit the most hypoxic habitat less initially (Fig. 3), but significantly more at the end ($p<0.001$, mixed effect model, Eq. (3)) (Fig. 4). The *post hoc* tests showed that fish visited the focal side more frequently at 15 than 5 °C at the 100/100 and from the 20/50 oxygen level onwards. In contrast to this there was a trend for 15 °C fish to visit the most hypoxic habitat less frequently at the 20/30 level ($p=0.031$, Fig. 3).

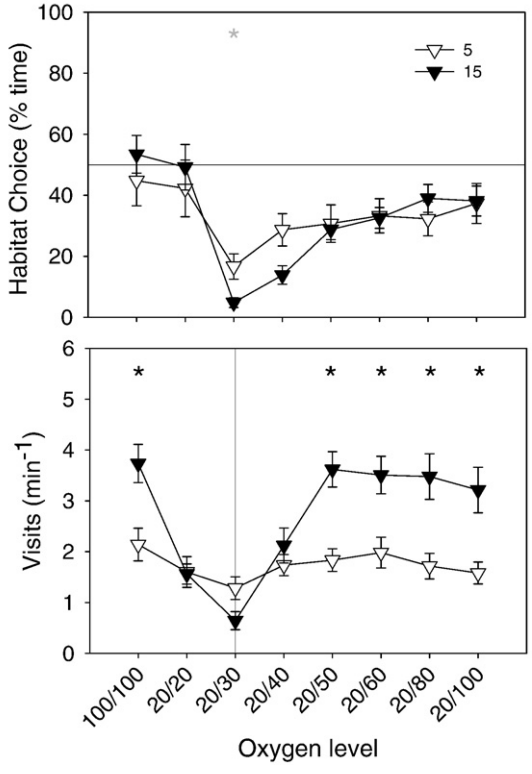

Fig. 3. Habitat choice and number of visits to the hypoxic habitat at 5 and 15 °C. The horizontal line indicates the neutral response i.e. 50% time on the focal side. Error bars are the standard error (S.E.). A dark asterisk indicates a significantly higher value at the respective oxygen level for the 15 °C fish and a grey asterisk indicates a significantly higher value for the 5 °C fish (based on two-tailed *t-post hoc* tests). The vertical line in visits separates the initial phase, to the left of the line, and the recovery phase to the right of the line.

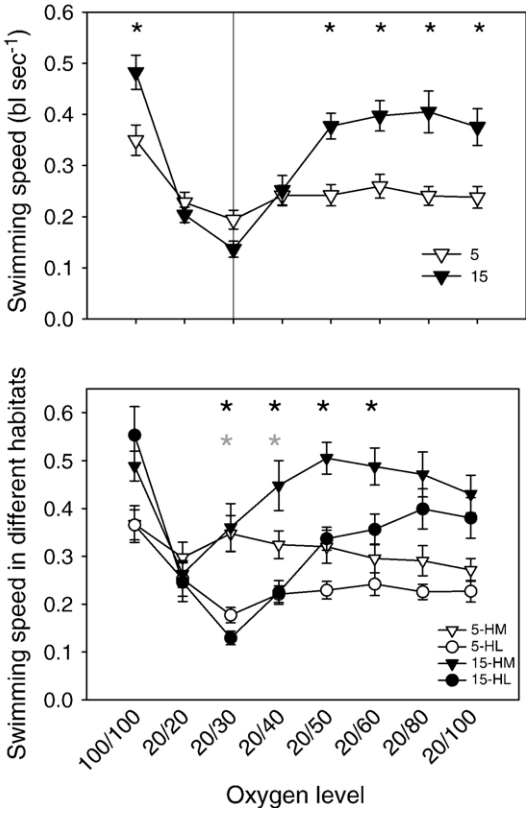

Fig. 4. Overall swimming speed and swimming speed in the least (HL) and most (HM) hypoxic habitat of the choice box at 5 and 15 °C. The vertical line in overall swimming speed separates the initial phase, to the left of the line, and the recovery phase to the right of the line. For overall swimming speed, a dark asterisk indicates a significantly higher value at the respective oxygen level for the 15 °C fish and a grey asterisk indicates a significantly higher value for the 5 °C fish. For swimming speed in each habitat of the choice box a dark asterisk indicates a significantly higher value for 15 °C fish in the most hypoxic compared to the least hypoxic environment of individual fish, whereas a grey asterisk indicates a significantly higher value for 5 °C fish in the most hypoxic compared to the least hypoxic environment (based on the *post hoc* paired *t*-tests). Error bars are the standard error (S.E.).

3.3. Swimming speed

The results for swimming speed closely reciprocated the results for number of visits. During the initial phase there was a strong negative effect of decreasing oxygen on overall swimming speed ($t=-4.98$, $df=54$, $p<0.001$, mixed effect model, Eq. (2)), however this effect was again more pronounced for fish at 15 °C ($t=-4.65$, $df=54$, $p<0.001$, mixed effect model, Eq. (2)).

During the recovery phase, 5 °C fish initially tended to swim faster, but 15 °C fish swam significantly faster than 5 °C fish at

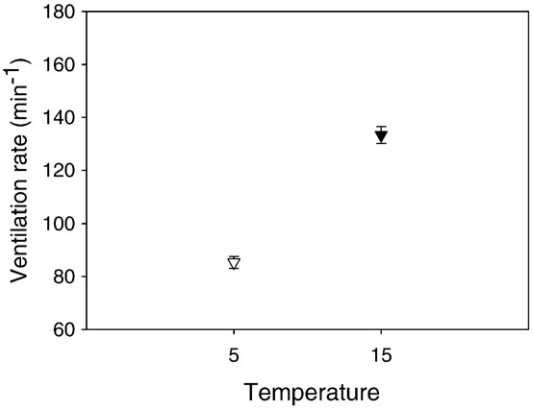

Fig. 5. Ventilation rate of fish at the 20/20 oxygen level. Error bars are the standard error (S.E.).

the end of the experiment ($p<0.001$, Fig. 4, mixed effect model, Eq. (3)). The *post hoc* tests showed that fish swam faster at 15 °C at the 100/100 level and from the 20/50 oxygen level onwards. Again, in contrast to this there was a clear trend for 15 °C fish to swim slower at the 20/30 level ($p=0.025$, Fig. 4).

The paired *t*-test comparing the swimming speed in different habitats for individual fish showed that fish maintained the same swimming speed in "both habitats" at the two initial levels. The fish then swam faster in the hypoxic habitats at both temperatures at the 20/30 and 20/40 levels. However, whereas 5 °C fish maintained the same swimming speed in both habitats at both oxygen levels from 20/50 onwards, 15 °C swam faster in the most hypoxic environment until the 20/80 oxygen level (Fig. 4).

3.4. Ventilation rate

Ventilation rates in the 20/20 hypoxia level were significantly higher at 15 than at 5 °C (one-way *ANCOVA*, $F_{1,24}=175.4$, $p<0.001$, Fig. 5). Overall ventilation rate was also inversely related to weight ($F_{1,24}=8.79$, $p<0.01$), but unaffected by swimming speed ($F_{1,24}=1.84$, $p=0.19$). There was no difference between haemoglobin genotypes at either temperature.

4. Discussion

Cod responded to inescapable hypoxia by reducing their overall swimming speed and then, at the initial levels of the recovery phase, by avoiding the most hypoxic habitat, irrespective of temperature or genotype. Recovery was quick when a partial oxygen refuge was available in one part of the tank, as evidenced by an overall increased swimming speed, more frequent visits and more time spent in the most hypoxic habitat (Figs. 3 and 4, mixed effect models (Eqs. (1–3)).

In the current experiment, we show that the nature of these behavioural avoidance responses is strongly temperature dependent, with more severe effects at 15 than 5 °C. Haemoglobin genotype had no effect on the behavioural response of cod to hypoxia at either temperature. However, *Hb-I*11* cod swam faster than *Hb-I*22* cod at 15 °C in normoxia.

4.1. Hypoxia and temperature

At higher temperatures, there is both an increased oxygen demand due to increased metabolism exacerbated by faster swimming speeds (Fig. 4) and less dissolved oxygen in the water (e.g. Benson and Krause, 1984; Schurmann and Steffensen, 1997). Hence, theoretically, these factors act together to increase the physiological stress of fish exposed to a given O_2 saturation at higher temperatures. Our results clearly support such an interpretation. Temperature modified the strength of the behavioural response of cod, both to inescapable hypoxia and to the hypoxia gradient (Figs. 3 and 4). On average, hypoxia reduced swimming speed by 79% at 15 °C (from normoxia to 20/30 O_2 level, Fig. 4), compared to only 33% for cod at 5 °C. At 15 °C, avoidance of the most hypoxic habitat also tended to be stronger at the 20/30 and 20/40 O_2 levels (Fig. 3), than at 5 °C. The observed decrease in swimming speed by cod in inescapable hypoxia has previously been attributed to a strategy that enables fish to conserve energy and/or offset metabolic stress (e.g. Nilsson et al., 1993; Schurmann and Steffensen, 1994; Claireaux et al., 2000).

Further, if the convergence of swimming speeds between habitats represents partial recovery from hypoxic stress, this only occurred at 15 °C when the least hypoxic habitat reached an oxygen saturation of 80%, although they spent a similar proportion of time in hypoxia as the 5 °C fish from the 20/50 level onwards (Fig. 3). It is worth noting here, however, that even though swimming speed converged earlier among the 5 °C than the 15 °C fish, swimming speed had not reached the initial normoxic levels at either temperature (paired *t*-test, both *p*'s<0.01) at the final 20/100 level (Fig. 4). This indicates that a partial effect of the hypoxic exposure was still present at both temperatures.

Increased swimming speed in the least favourable O_2-habitat is advantageous in that it enables stressed cod to quickly escape this habitat. However, fish reduce their swimming speed when encountering a more favourable O_2-habitat (Fig. 4). This probably has a dual advantage in it that reduces activity and thus facilitates quick recovery and increases the chance of remaining in the most favourable habitat. As fish recover and the physiological stress effects decrease, they are able to begin to explore the most hypoxic habitat and their initial avoidance response disappears.

Cod swam faster in normoxia at the highest temperature (Fig. 4). Hence, oxygen consumption at 15 °C was even higher than would be expected from a higher standard metabolic rate alone (Schurmann and Steffensen, 1997). At the 20/30 oxygen level, the swimming speed of 15 °C fish' had decreased from significantly faster to almost significantly slower than the 5 °C fish ($p=0.025$, Fig. 4). This reduction occurred even though cod pumped more water over their gills in hypoxia at the higher temperature (i.e. higher ventilation rates, Fig. 5).

Interestingly, cod rarely spent all their time on one side of the choice box (Figs. 3 and 4). Rather, cod continued to sample both

sides. A similar behavioural response to hypoxic gradients has been described by Claireaux et al. (1995). Completely restricting their search paths to a small area in response to acute hypoxia is likely to be maladaptive as it limits the opportunity of finding a habitat with higher oxygen levels. Such behaviour may also indicate the possibility for cod to engage in short foraging excursions into hypoxia (e.g. Neuenfeldt, 2002).

Another trait that is likely to facilitate short excursions into hypoxia is the ability of fish to recover from hypoxic stress. Stress will limit the duration that fish can remain in a hypoxic habitat, and their behavioural and physiological performance within this habitat (Claireaux et al., 1995; Lefrançois et al., 2005). Our study has shown that the ability of cod to recover from hypoxic experiences is strongly temperature dependent. Hence, temperature is likely to limit the availability of hypoxic habitat for foraging and to avoid predators and should be taken into account in models of predator–prey overlaps (e.g. Neuenfeldt and Beyer, 2006).

The link between hypoxia and temperature is particularly relevant today with global warming (Grottoli et al., 2006; Schiermeier, 2006) and increased eutrophication in coastal areas (Nielsen, 1984; Turner and Rabalais, 1994). On broader scales, ecosystem effects of hypoxia have been shown. For example, reduced occurrence of Ophiuroids following an hypoxic event caused reduced growth and lowered condition for flathead flounder, *Hippoglossoides dubius*, in Funka Bay, Japan (Kimura et al., 2004) and eutrophication of the Neuse River Estuary, US caused a switch in the diet of Atlantic croaker, *Micropagonia undulates*, from clams to less nutritional items such as plants and detritus (Powers et al., 2005). Similarly, Pihl (1994) found that cod ate less crustaceans and more polychaetes and echinoderms under hypoxic conditions in the Baltic Sea.

4.2. Haemoglobin effects

This is the first study to examine potential behavioural differences in oxygen gradients between the two haemoglobin homozygotes. The possibility of such a difference has been suggested based on studies of population structure, combined with the electrolytic characteristics of stripped haemoglobins (Brix et al., 2004). However, a more realistic test of differences between haemoglobin genotypes is the present scenario where cod can choose between different O_2-environments, given that cod are often found in heterogeneous O_2-environments (e.g. D'Amours, 1993; Pihl, 1994; Neuenfeldt, 2002). In our study, the only difference between genotypes was the higher activity levels by *Hb-I*11* cod at 15 °C under the initial normoxic control levels (Fig. 2). We do not consider the "choice" of the focal side at normoxia of *Hb-I*22* fish at 5 °C as a difference since it was not accompanied by a difference in swimming speed. Although we have found no evidence that the response to hypoxia differs between haemoglobin genotypes, our results do support earlier studies suggesting a genetic component of individual differences in cod behaviour (Salvanes and Hart, 2000).

High activity levels may be important for successful foraging, for example by increasing the probability of encountering prey or alternatively in food scramble competition. The higher swimming speed of *Hb-I*11* cod at 15 °C may therefore be interpreted as better performance of this genotype at higher temperatures in accordance with the results of Petersen and Steffensen (2003).

Only the study of Petersen and Steffensen (2003) on Baltic cod has examined a possible link between haemoglobin genotype and hypoxic tolerance previously. In 30% inescapable hypoxia they found that *Hb-I*11* cod lowered their preferred temperature to a similar temperature as the *Hb-I*22* cod, whereas the preferred temperature of *Hb-I*22* cod did not change. This was interpreted as the *Hb-I*22* genotype being more tolerant to hypoxic conditions. However, an alternative interpretation is that while *Hb-I*11* fish perform better at higher temperatures during normoxic conditions, there is no difference between the haemoglobin homozygotes under hypoxia. This corresponds well with the present results.

One explanation for the lack of differences between haemoglobin genotypes in hypoxia avoidance is potential acclimatisation effects. The distribution of the two alleles *Hb-I*1* and *Hb-I*2* varies among the populations of cod in the North Sea and North Atlantic Ocean. For instance, *Hb-I*1* is typically only observed at frequencies of 0.10–0.15 in the fjords of Northern Norway, whereas this frequency increases southwards along the Norwegian coast to approximately 0.6 in the North Sea (Frydenberg et al., 1965). The previously reported differences in haemoglobin affinities (e.g. Karpov and Novikov, 1980; Brix et al., 1998) were found in fish from the same population, so arguably, haemoglobin related differences should have been detectable in the present study. However, the acclimatisation of at least 6 weeks to the 5 and 15 °C temperature prior to the experiment may have minimised the difference between genotypes, as cod can modulate the oxygen binding properties of their genotypes by changing the concentrations of their haemoglobin components (Brix et al., 2004). This could potentially have obscured differences between haemoglobin genotypes. This is an interesting question which would, however, require a different experimental set-up than the present study, which focused on the biologically relevant scenario of a mixed population with a similar temperature history, exposed to a heterogeneous O_2-environment.

Finally, it should be noted that behavioural responses to hypoxia may not necessarily be a direct manifestation of physiological state, and hence may mask underlying physiological differences between haemoglobin genotypes in hypoxia tolerance. Alternatively, differences in oxygen affinity between haemoglobin genotype may not be sufficient to modify the nature and timing of avoidance responses to hypoxia.

5. Conclusion

Our study shows that cod will detect and actively avoid the most hypoxic environment after initial exposure to inescapable hypoxia. The behavioural response in our study was more pronounced at the highest temperature, making the situation in many coastal cod habitats today with the predicted increase in global warming and increased hypoxia one of particular

concern. No difference in hypoxia tolerance was found between haemoglobin genotypes. Studies on more populations are needed, but at present we find no support for the hypothesis that haemoglobin genotype influences avoidance of hypoxia by cod.

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