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RESEARCH ARTICLE

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The response of Atlantic cod, *Gadus morhua*, to progressive hypoxia: fish swimming speed and physiological stress

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Abstract Atlantic cod, *Gadus morhua*, were exposed to a progressive stepwise decline in water oxygen pressure (19.9, 13.2, 10.5, 8.4, 6.2 and 4.3 kPa P_{O_2}). Fish swimming speed and indicators of primary and secondary stress (e.g. blood cortisol and lactate) were measured to assess whether a severe shift in physiological homeostasis (i.e. stress) preceded any change in behaviour or vice versa. Swimming speed increased by 18% when P_{O_2} was reduced rapidly from 19.9 kPa to 13.2 kPa and was interpreted as an initial avoidance response. However, swimming speed was reduced by 21% at a moderate level of steady P_{O_2} (8.4 kPa) and continued to drop by 41% under progressively deep hypoxia (4.3 kPa). Elevations in plasma cortisol and blood lactate indicated major physiological stress but only at 4.3 kPa, which corresponds to the critical oxygen tension of this species. We propose that the drop in speed during hypoxia aids to offset major stress and is adaptive for the survival of cod in extensive areas of low oxygen.

Abbreviations P_{O_2}: Partial pressure of oxygen

Introduction

Oxygen has a vital role in aerobic metabolism yet is subject to large fluctuations within the aquatic environment. Many coastal areas are highly sensitive to eutrophication and severe oxygen depletion events (i.e. hypoxia) have become increasingly common over the last century (Johannessen and Dahl 1996). For water breathing fishes, hypoxia impacts on many ecologically important variables (Poulin et al. 1987; Pihl et al. 1991; Chabot and Dutil 1999; Pichavant et al. 2000; Eby and Crowder 2002; Ærtebjerg et al. 2003) and is undoubtedly a critical factor influencing the evolution and life history of many fish species (Randall et al. 1981).

Hypoxia can result in behavioural and physiological stress, but it is difficult to generalize on how adaptations are expressed because fish inhabit a wide range of environmental conditions and are subject to different metabolic demands. Indeed, the physiological response of fish is variable at set levels of water oxygen pressure (P_{O_2}) and different species exhibit varying degrees of tolerance to hypoxia (e.g. Burton and Heath 1980; Van Raaij et al. 1996b; Ishibashi et al. 2002; Pichavant et al. 2002). Interspecific differences do exist but the hypoxic physiological response of fish consists typically of a primary hormonal reaction (e.g. catecholamines and/or cortisol) and a suite of linked, secondary responses (e.g. metabolic, ionoregulatory and cardio-ventilatory adjustments)(Mazeaud et al. 1977; Pickering and Pottinger 1995). As progressively deep hypoxia constrains aerobic metabolism, any change in metabolite concentration (i.e. lactate, glucose, fee fatty acids, etc.) usually reflects the resultant shift in energy supply-demand dynamics (Burton and Heath 1980; Dalla Via et al. 1994; Van Raaij et al. 1996a, b; Muusze et al. 1998; Ishibashi et al. 2002). Hypoxic stress responses are generally considered adaptive, but some secondary responses, such as a shift in ionoregulatory status, may be the unfortunate result of primary and/or secondary adjustments (e.g. the ''osmo-respiratory compromise''. Mazeaud and Mazeaud 1981). The behavioural response to hypoxia is also variable since fish either exhibit no change in behavioural activity or (depending on species, age and environmental factors) adjust their swimming with an increase or a decrease in speed (Dizon 1977;

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Metcalfe and Butler 1984; Bejda et al. 1987; Nilsson et al. 1993; Schurmann and Steffensen 1994; Waller et al. 2000; Domenici et al. 2000). The activity state of fish in hypoxia is clearly complex and, with the ultimate need to survive, reflects a (species- and context-dependent) balance between energy conservation and low O_2 avoidance.

Although many behavioural and physiological studies have examined independently the hypoxic responses of fish, few studies have attempted to integrate these two areas of research and establish a link between hypoxic stress and fish swimming behaviour. For example, hypoxia-induced increases in fish swimming speed have been interpreted as a behavioural alarm reaction to respiratory distress (Domenici et al. 2000), yet physiological samples have rarely been extracted during behavioural studies. The few studies that have simultaneously monitored the physiology and behaviour of fish during hypoxia have generally suggested that reduced activity (or quiescent behaviour) occurs with the conservation of energy and/or the avoidance of major metabolic stress (Fischer et al. 1992; Van Raaij et al. 1996a; Dalla Via et al. 1998; Waller et al. 2000; Herbert and Wells 2001). While the context-dependent nature of fish responses is becoming increasingly well documented, our overall understanding would be greatly increased if hypoxic stress and its potential link with behavioural change are examined over a wider range of species and conditions.

Atlantic cod, *Gadus morhua*, inhabit several coastal areas where hypoxia is a common phenomenon; examples include cod in the Baltic Sea (Nielsen and Gargas 1984; Neuenfeldt 2002; Ærtebjerg et al. 2003) and the Canadian Gulf of St. Lawrence (D'Amours 1993; Plante et al. 1998). *G. morhua* depress their swimming speed by as much as 72% during progressive hypoxia (Schurmann and Steffensen 1994), but the physiological stress response of this species has not been addressed during such transition in behaviour. Perry et al. (1991), Claireaux and Dutil (1992) and Plante et al. (1998) have documented the physiological response of cod to hypoxia but, unfortunately, incompatible protocols prevent us from comparing their observations with existing behavioural data.

The current study aims to examine the level of P_{O_2} that prompts a shift in swimming speed and physiological homeostasis under standardised conditions in cod. Blood cortisol, lactate, glucose and osmolality have been monitored as valid indicators of primary and secondary stress and are ultimately used to quantify major (vs subtle) changes in hormonal, metabolic and ionoregulatory homeostasis. It is particularly important to assess whether a rise in lactate precedes a change in behaviour because this secondary anaerobic metabolite is known to serve a behavioural signalling function in various animal groups (Pörtner et al. 1994; De Wachter et al. 1997) but its role has never been examined in fish. For a species of fish that regularly encounters hypoxia, however, it does not seem adaptive that lactate, nor any other indicator of major stress, would serve as a trigger for behavioural change. We hypothesise therefore that cod will decrease its swimming speed before the onset of major physiological stress, as an adaptive form of energy-conserving behaviour.

Materials and methods

Fish capture and initial acclimation

Forty-five Atlantic cod, (mean ± SD) 332.6 ± 127.0 g; 34.3 ± 3.6 cm TL, were caught in the Øresund by trawling and transferred immediately to the Marine Biological Laboratory, Helsingør. All cod were held under a 12-h dark: 12 h light regime in 450-l tanks, recirculated with near-fully air-saturated seawater at 10.0°C, for at least 2 weeks prior to experimentation. Cod fed readily on herring and sandeel.

Experimental tank

A diagrammatic representation of the experimental apparatus is given in Fig. 1. The response of *G. morhua* to a progressive, stepwise decline in P_{O_2} was assessed in a circular (1.29-m diameter, 60-cm deep, 785 l) experimental tank. Near-fully air-saturated seawater (10.0 ± 0.2°C) was supplied to the experimental tank at a rate of 5 l min^{-1} and exited via a central standpipe. Light was supplied during a 06:00–18:00-h period on a 12-h light dark:12-h dark regime. The entire area around the tank was screened off with black plastic sheeting to minimize the impact of general laboratory disturbances and maintain a controlled lighting regime for the satisfactory tracking of fish movements (see below).

Behavioural monitoring

A CCD video camera was mounted 1.3 m above the circular tank and connected to a computer equipped with a frame grabber (Visionetics VFG-512 BC) that digitizes and analyses single video frames with a resolution of 256×256 pixels at 10 frames s^{-1}. The geometric center of a flat, black (1.5 cm×2 cm) "target", sutured to the dorsal nasal region of a single fish in a group, was determined using a customized software programme and its x, y coordinate transmitted, via the RS-232 port, to a data acquisition package (Labtech Notebook). Fish positional (x, y) data was stored on the hard drive for later calculations of swimming speed (i.e. the cumulative distance swum in body lengths per second). The black "target" was sutured securely onto fish anaesthetized with benzocaine (40 mg l^{-1}) in bicarbonate buffered seawater.

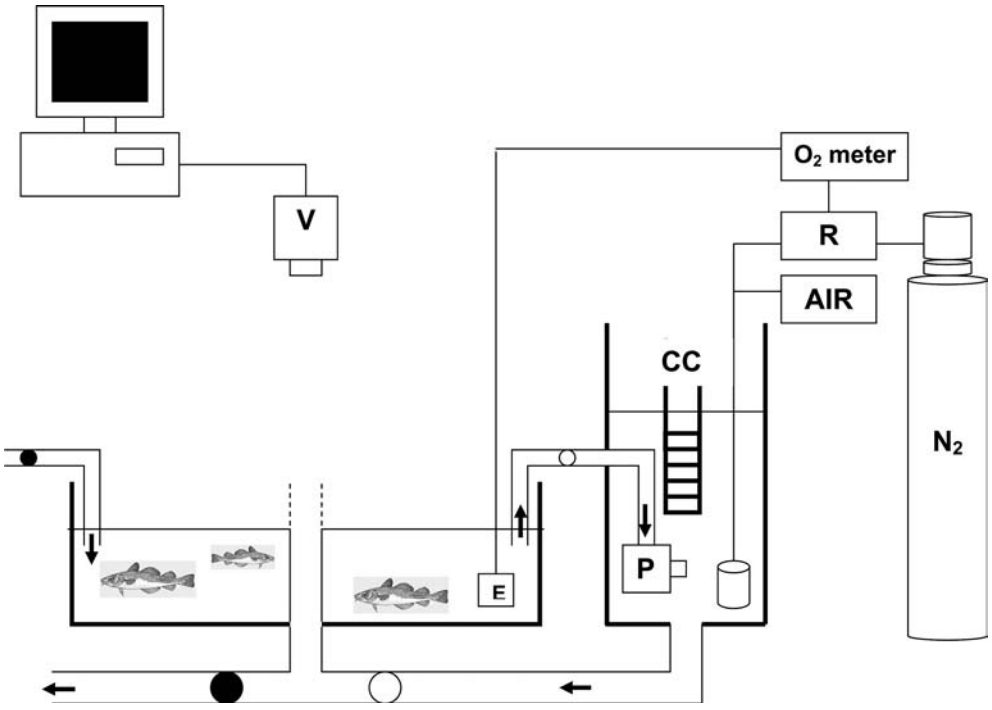

Fig. 1 Diagrammatic representation of the experimental system (see Materials and methods section for full description). The swimming behaviour of cod in a 1.29-m diameter (circular) tank was monitored using a CCD video camera (*V*) connected to a computer with a behavioural tracking system. *Open* and *closed* circulation was achieved with paired water-tight valves (represented by the black and white circles). *Open* circulation was maintained when the black valves were open and the white valves were closed; water was not recirculated through the tall mixing tower in this state. *Closed* circulation was achieved by shutting off the black valves, starting the pump (*P*), and opening the white valves for a flow of water through the mixing tower. The pump (*P*) sucked water into the tall mixing tower and, by creating a relative head of pressure, water was returned to the experimental tank by gravity and via the central standpipe. Water oxygen pressure in the experimental tank was monitored by an oxygen electrode (*E*) connected to an oxygen meter. Water oxygen pressure was manipulated during closed recirculation by bubbling air or N_2 through an air-stone in the mixing tower. For controlled deoxygenation, the oxygen meter was connected to an O_2 regulating unit (*R*), equipped with a solenoid valve, and a compressed bottle of N_2. *CC* indicates the presence of cooling coils in the mixing tower. *NB* Not to scale

Control of water oxygen levels

The continuous supply of near-fully air-saturated seawater maintained the tank in *open* circulation at all times, except during the main experimental periods when oxygen levels were regulated. A *closed* system was maintained during the experimental periods by closing off the main laboratory supply and recycling water through a separate 930 l (oxygenating/deoxygenating) mixing chamber. A pump (Eheim 1060, Germany, 2,280 l h^{-1}) in the mixing chamber was used to transfer water from the experimental tank and, by generating a mild head of pressure, water was passed back to the main tank under gravity (via the central standpipe) without disturbing the water surface and hence the efficacy of the fish tracking programme. By switching from open to closed circulation and using the central standpipe for the removal and delivery of water respectively, we were able to (1) acclimate fish for prolonged periods with the continuous supply of high quality water and (2) deoxygenate the *large* experimental tank in a controlled and highly repeatable manner. There is no reason to assume that the switch from open to closed circulation disturbed the fish because the pump was not located in the experimental tank and the surface of water was not disturbed by either flow regime.

Water P_{O_2} in the experimental tank was manipulated by bubbling air (for oxygenation) or nitrogen (for deoxygenation) through the separate mixing chamber. P_{O_2} was monitored with a WTW microprocessor oximeter (OXI 196) equipped with an O_2 probe located at one side of the main experimental tank. For controlled deoxygenation, an O_2 regulating unit received the tank P_{O_2} signal and, via a solenoid valve, controlled the flow of gas from a compressed bottle of nitrogen. Rapid mixing ensured that oxygen levels were uniform throughout the experimental tank. Recirculating water (5°C) was periodically passed over cooling coils in the separate mixing chamber to ensure water temperature was maintained at 10.0 ± 0.2°C throughout the closed experimental period.

Experimental design

Each experiment was conducted over a 6-day period as follows:

Day 0 A single "targeted" cod and a group of 5–8 unmarked fish were introduced to the experimental tank and left undisturbed for 2 days to recover from anaesthesia and resume routine activity patterns.

Day 3 The behavioural response of the single "targeted" cod in near-fully oxygenated water (i.e. 19.8 ± 0.4 kPa P_{O_2}) was assessed, between 10:00 h and 17:00 h, to establish routine (i.e. background) activity. We considered the routine response of fish because the experimental period was prolonged and, based on the results of other studies, a change in activity was expected (Metcalfe and Butler 1984; Nilsson et al. 1993).

Day 4 The behavioural response of the single "targeted" cod to progressive, stepwise hypoxia (19.9, 13.2, 10.5, 8.4, 6.2 and 4.3 kPa P_{O_2}) was assessed between 10:00 h and 16:30 h. The grouped fish were subsequently allowed 41 h to recover under normoxic conditions.

Day 6 The same progressive, stepwise decline in oxygen level was induced and a sample of blood was extracted sequentially and rapidly from all fish in the group, but only at the end of one of the six steady-state periods (i.e. either 19.9, 13.2, 10.5, 8.4, 6.2 and 4.3 kPa P_{O_2}). The entire 6-day experiment was therefore replicated six times so that the physiological stress of cod was assessed over all six O_2 levels (total $N = 40$) and the behaviour of six "targeted" fish was quantified. The time to sequentially sample blood from all fish in a group was recorded (total time = 3.5–7.5 min) and used to evaluate the effect of sampling time on each of the physiological variables.

The switch from open to closed circulation on Days 3, 4 and 6 always occurred at 09:00 h (i.e. 1 h before the start of behavioural assessments). "Steady" state is used to describe the period in which the designated P_{O_2} was in a constant, unchanging state and was controlled by the O_2 comparator. "Unsteady" state is used to describe the 30-min period during which P_{O_2} was progressively reduced to the next steady level.

Blood sampling and analysis

Fish were killed with a blow to the head and a sample of blood (approximately 0.5–0.8 ml) extracted rapidly by caudal venepuncture using a heparinised syringe. Fifty microlitres of whole blood was deproteinised in 100 µl 0.6 mol l^{-1} perchloric acid, spun at 12,000 *g* and 4°C for 5 min and stored at −20°C for later analysis of blood lactate. Blood lactate was measured spectrophotometrically (Pharmacia Ultrospec 2000) at 340 nm with an enzymatic test kit (Sigma 826-UV). The remaining sample of whole blood was spun at 12,000 *g* and 4°C for 5 min and, if not analysed immediately, the supernatent was stored overnight at −20°C and analysed for plasma cortisol, glucose and osmolality the following day. Plasma cortisol was measured spectrophotometrically with a microtiter plate reader (Labsystems Multiscan RC) at 450 nm using an enzyme-linked immunoassay test kit (ADALTIS EIAgen Cortisol L14003K). Plasma glucose was measured with a spectrophotometer (Pharmacia Ultrospec 2000) at 340 nm using an enzymatic test kit (Sigma 16-UV). Plasma osmolality was measured using a Wesco osmometer (Vapro 5520).

Physiological reference group

To estimate the maximal level of stress in cod, a separate group of cod were given a severe and strenuous bout of exercise in normoxia. The physiological response of cod in hypoxia was therefore compared against this strenuously exercised group for the sole purpose of "scaling" the relative level of stress at each P_{O_2}. This method does not imply that the metabolic impact of hypoxia and strenuous exercise is identical. Individual cod ($N = 5$) were subjected to stressful strenuous exercise by manually chasing them to exhaustion for 5 min in a 110-l container. Fish were left undisturbed in fully oxygenated water for a 30-min period and a sample of blood was then extracted and analysed for cortisol, lactate, glucose and osmolality (as above). The 30 min post-exercise period was selected because (1) it corresponds temporally to the steady- and unsteady-state periods and (2) represents a time post-stress when primary and secondary indicators of stress are close to maximal levels of disturbance in cod (N.A. Herbert, J.F. Steffensen, unpublished) and other fish species (Milligan and Wood 1987).

Statistics

Non-linear regression analysis was performed on cod swimming speed to examine the effect of experimental time. Analysis of cod swimming speed (in both steady and unsteady P_{O_2}) was based on the repeated measures ANOVA (Statistica 5.5) and followed by a priori planned comparisons to determine whether hypoxic responses differed from expected routine activity.

It is difficult to visualize the absolute effect of hypoxia on cod swimming speed when routine activity shifts over time. For this reason, a second method of analysis was carried out for graphical presentations. A non-linear (second order inverse polynomial) regression was used to analyze the routine swimming speed of cod at a P_{O_2} of 19.8 ± 0.4 kPa. The expected swimming speed of cod was calculated from this regression and compared against observed speed during hypoxia, with the difference denoted as differential speed in BL s^{-1} . A negative differential speed value indicates that the observed swimming speeds are below the expected level of activity and vice versa.

All statistics on physiological data were performed using Sigmastat version. 2.03. Differences in blood physiology between the six oxygen levels (19.9, 13.2, 10.5, 8.4, 6.2 and 4.3 kPa P_{O_2}) were examined using one-way ANOVA and followed by Tukey post hoc tests for specific pair-wise comparisons. Kruskall–Wallis ANOVA was performed when data was not normally distributed and was followed by specific

pair-wise comparisons using Dunn's Method. Either *t* tests for independent samples or Mann–Whitney *U* tests (was used to compare the physiology of the 30 min post-exercise group with that of relevant P_{O_2} groups: (1) the 19.9-kPa control group and (2) groups exhibiting significant post-hoc differences during hypoxia. Significance was accepted at $P < 0.05$.

The percentage O_2 saturation values of Schurmann and Steffensen (1994), Claireaux et al. (2000) and Plante et al. (1998) were converted to P_{O_2} assuming a standardised total air pressure of 101 kPa.

Results

Behavioural activity

The routine swimming speed of cod in normoxia declined gradually over the 420-min experiment (Fig. 2a); 95% of the variability in speed was attributed to a difference in time ($R^2 = 0.95$, $P < 0.01$) according to the following equation:

$$\mathrm{BL\,s^{-1}} = -0.000001T^2 + 0.00002T + 0.5,$$

where T is time in minutes. The swimming speed of cod in progressive hypoxia also declined over the experimental period (Fig. 2b); 87% of the variability in speed was attributed to a difference in time ($R^2 = 0.87$, $P < 0.01$) according to the following equation:

$$\mathrm{BL\,s^{-1}} = -0.000003T^2 + 0.0004T + 0.49$$

To accurately assess the effect of hypoxia on cod swimming speed, both routine and hypoxic activity was incorporated into a repeated measures analysis. The results of the repeated measures analysis and the relative change in speed are summarized in a plot of differential swimming speed (Fig. 3). Steady levels of low oxygen reduced the swimming speed of cod by 21, 28 and 41% at 8.4, 6.2 and 4.3 kPa, respectively ($P < 0.05$). Unsteady P_{O_2} increased the swimming speed of cod by 18%, but only when oxygen was reduced from 19.9 kPa to 13.2 kPa ($P < 0.05$).

Stress physiology

Although blood was sequentially sampled from each fish in the group, there was no significant effect of sampling time on any physiological variable and effectively enabled physiological data to be presented as mean values. It is important to note that the progressive, stepwise decline in water oxygen pressure was identical between the behavioural and physiological experiments.

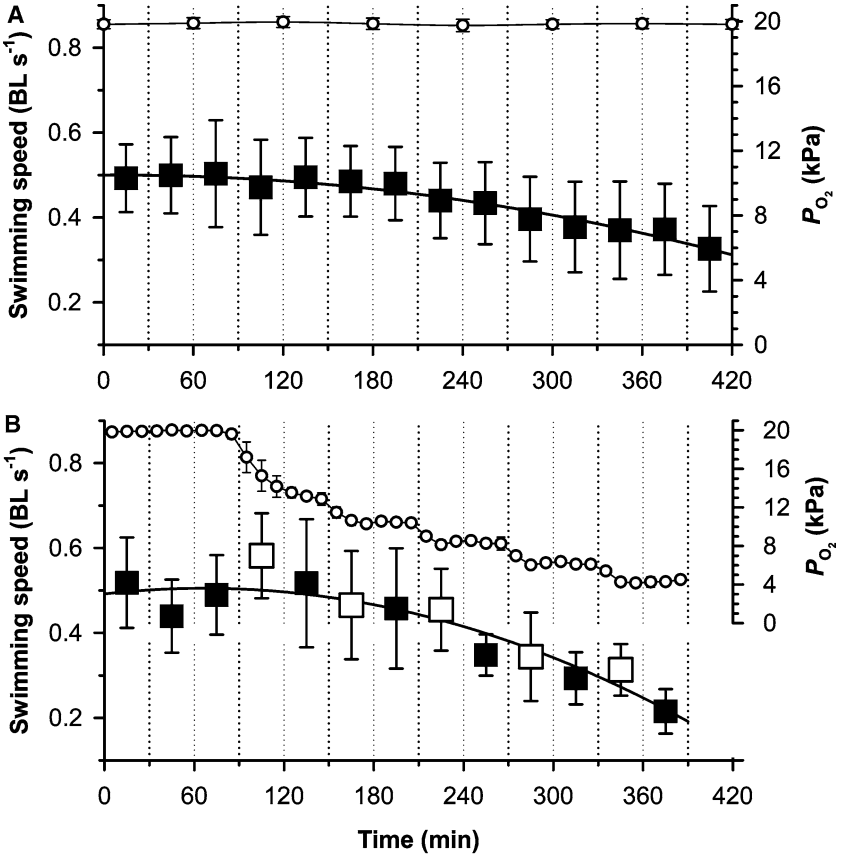

Fig. 2 The swimming speed of cod. **a** Routine swimming speed in near-fully oxygenated water on Day 3. **b** Swimming speed during a progressive stepwise decline in oxygen on Day 4. "Steady" state is used to describe the period in which the designated P_{O_2} was in a constant, unchanging state. "Unsteady" state describes the 30-min period in which P_{O_2} was progressively reduced to the next steady level. *Closed squares* indicate swimming speeds during steady P_{O_2} whereas, *open squares* represent swimming speed during unsteady P_{O_2}. P_{O_2} is indicated by *small open circles*. *Error bars* represent 95% CIs ($N = 6$)

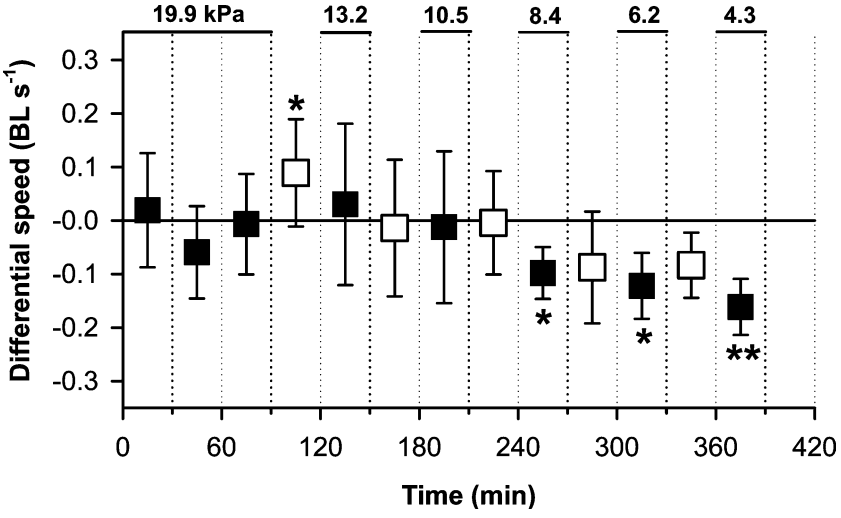

Fig. 3 The differential swimming speed of cod. *Closed squares* indicate swimming speed during steady P_{O_2} whereas, *open squares* represent speed during unsteady P_{O_2}. The 30 min steady-state periods are marked with a *horizontal bar* and its corresponding level of mean P_{O_2}. *Error bars* represent 95% CIs ($N = 6$). *Values marked with an asterisk* indicates that the 30-min mean value is significantly different from the (expected) background swimming response (repeated measures ANOVA: $*P < 0.05$; $**P < 0.01$)

The level of blood lactate and plasma cortisol in normoxia (i.e. 19.9 kPa) were significantly lower than the 30 min post-exercise group ($P < 0.05$, Fig. 4a, b) and represented baseline values. Declining P_{O_2} increased plasma cortisol and blood lactate above the normoxic baseline level but only at 4.3 kPa ($P < 0.01$). At 4.3 kPa, blood lactate levels were significantly less ($P < 0.01$) than, but cortisol levels comparable ($P > 0.05$) to those measured in fish after strenuous exercise.

Plasma glucose at the control level of P_{O_2} was not significantly different from the 30 min post-exercise group ($P > 0.05$. Fig. 4c) and was not adjusted by hypoxia at any level of saturation ($P > 0.05$). Plasma osmolality at 19.9 kPa was significantly less than the 30 min post-exercise group ($P < 0.05$. Fig. 4d) and represented a basal level. Declining P_{O_2} had a significant effect on plasma osmolality ($P < 0.01$) by stimulating a transient rise at 10.5 kPa that decreased back to control levels between 8.4 kPa and 4.3 kPa. The transient rise in osmolality at 10.5 kPa was not significantly different from the highly stressed, 30 min post-exercise group ($P > 0.05$).

Discussion

Cod behaviour during hypoxia

Cod swimming speed was reduced at low P_{O_2} and, in this regard, is consistent with the results of Schurmann and Steffensen (1994) and Chabot and Dutil (1999). However, contrary to the results of Schurmann and Steffensen (1994), we show that the swimming speed of cod is first reduced at 8.4 kPa (vs 10.9 kPa) and is reduced by only 41% (vs 63%) at 4.3 kPa (Figs. 2, 3). This discrepancy can possibly be explained by differences in methodology since we utilized a larger holding facility, considered the response of fish in a group (vs those held individually) and included the routine swimming response of fish within our statistical analysis. Consideration of routine activity is clearly important during prolonged experimentation because a progressive decline in background speed has been reported previously (Nilsson et al. 1993) and presumably occurred as a result of an endogenous activity rhythm (Metcalfe and Butler 1984).

The swimming response of cod to hypoxia is surprisingly sensitive given that a sudden decline in P_{O_2}, from 19.9 kPa to 13.2 kPa, results in a modest (18%) increase in swimming speed. To validate this observation in cod and other fish species, we have recorded the same response in *G. morhua* during a separate acute hypoxia trial (J.L. Johansen, NA Herbert, J.F. Steffensen, unpublished) and schooling northern anchovy increase their speed in response to a rapid, but not slow, decline in P_{O_2} (Moss and McFarland 1970). It is unclear why changes in speed were not observed at low unsteady pressures but, since positive and negative changes in speed appear to depend upon the stability and absolute level of P_{O_2} respectively (Fig. 3), the possibility exists that activity remained unadjusted due to counteracting signals from low and unsteady P_{O_2}.

Cod in a heterogeneous O_2 environment make directed movements from discrete patches of hypoxia but this species avoids both moderate and low levels of P_{O_2} (< 9 kPa at 5°C. Claireaux et al. 1995; < 3 kPa at < 9°C. Neuenfeldt 2002) and its O_2 avoidance threshold is therefore unclear. Our observed increase in activity during the initial drop in P_{O_2} (19.9–13.2 kPa) is probably an additional component of avoidance, and suggests that cod have the capacity to "sense" and avoid hypoxia, not only well in advance of physiological stress but also at surprisingly high levels of P_{O_2}. We therefore propose that the avoidance response of cod is highly context-dependent and varies according to the duration and rate of hypoxic exposure. Indeed, active avoidance would be maladaptive if the hypoxic region is extensive or progressive because an increased amount of oxygen would be required for the increased level of activity. In

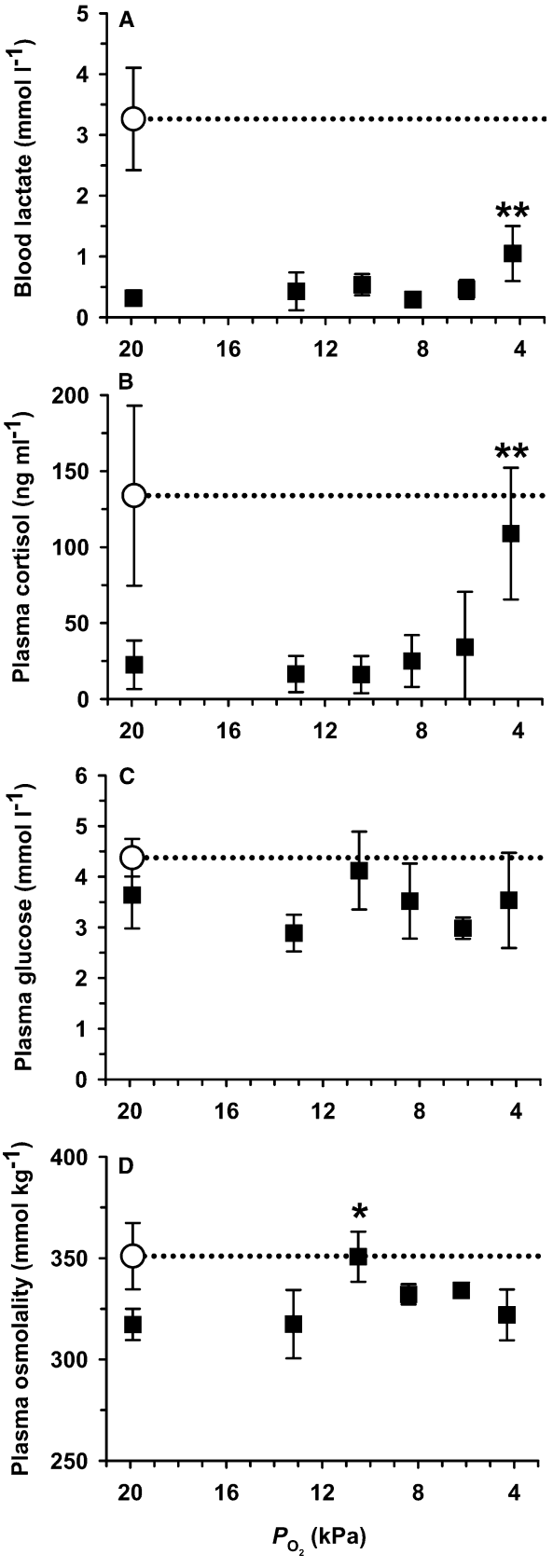

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Fig. 4 The physiological response of cod to progressive stepwise hypoxia (*closed squares*) and 30 min post-exercise (*open circle*); **a** Blood lactate, **b** Plasma cortisol, **c** Plasma glucose and **d** Plasma osmolality. Values are mean ± 95% CIs. *Values marked with an asterisk* indicate a significant difference from the 19.9-kPa (control) group (*$P < 0.05$; **$P < 0.01$). Total $N = 40$

accordance with this hypothesis, cod clearly reverse their behavioural strategy during progressively deep hypoxia (Figs. 2, 3). With a (21–41%) drop in swimming speed and an associated reduction in metabolic O_2 demand (Soofiani and Priede 1985; Schurmann and Steffensen 1997), this change in behaviour would presumably translate into improved hypoxia tolerance.

Physiological stress of cod during hypoxia

Cod do not exhibit significant levels of primary and secondary stress until exposed to a P_{O_2} of 4.3 kPa. At this level of hypoxia, a rapid manifestation of stress is apparent as plasma cortisol surges to a level observed in cod following exhaustive exercise (Fig. 4b) and stressful, high-density transport (Staurnes et al. 1994). The modest 1 mmol l^{-1} rise in blood lactate at 4.3 kPa also shows that cod approached their aerobic metabolic limit, especially because an unusually high level of lactate is not retained in the white muscle of cod (Claireaux and Dutil 1992) and swimming activity was reduced greatly at $P_{O_2} \leqslant 8.4$ kPa. Plasma glucose was not altered significantly at any steady level of P_{O_2}, indicating some degree of insensitivity to hypoxia. Glucose may not, however, be the best indicator of short-term metabolic stress as metabolite levels do not necessarily reflect a change in glucose turnover (Haman et al. 1997) and the application of various stressors in excess of 24 h leads to significant deviations in cod plasma glucose levels (Staurnes et al. 1994; Chabot and Dutil 1999). While we manipulated P_{O_2} over 30 min steps, cod may only adjust blood glucose with exposures >1 h at severely deep levels of hypoxia (Claireaux and Dutil 1992).

There is nothing unusual about the sudden increase in cortisol and lactate at a P_{O_2} of 4.3 kPa; These parameters are often elevated in other fish species during hypoxia (Tetens and Christensen 1987; Dalla Via et al. 1994; van den Thillart et al. 1994; van Raaij et al. 1996a, 1996b; Muusze et al. 1998) and exemplify the primary and secondary stress response (Pickering and Pottinger 1995). Our ability to compare the current (4.3 kPa) threshold value against other species is hampered slightly by different experimental conditions (e.g. temperature, number of progressive P_{O_2} steps etc.) but, when appropriate comparisons are made, cod appear to be moderately tolerant of hypoxia. Rainbow trout is considered generally as a hypoxia intolerant species and exhibits a shift in lactate and cortisol homeostasis at pressures in excess of 4.3 kPa (Tetens and Christensen, 1987; van Raaij et al. 1996a, 1996b). Conversely, flatfish, such as sole *Solea solea*, and Amazonian species are noted for their extreme tolerance of O_2 deficient waters

and exhibit a similar change in lactate and cortisol at very low O_2 pressures (approximately 1.6 kPa)(Dalla Via et al. 1994; van den Thillart et al. 1994; Muusze et al. 1998).

Critical and lethal levels of P_{O_2}

Pre-conditioning to hypoxia (on Day 4) is not likely to have compromised the physiological response of cod (on Day 6) because fish only received a relatively short pulse of deep hypoxia in between prolonged periods of normoxic acclimation (versus the study of Routley et al. 2002). We are confident therefore that cod experience major stress at 4.3 kPa P_{O_2} and this corresponds well with the known critical oxygen level of this species (Schurmann and Steffensen 1997; Claireaux et al. 2000). Since a significant physiological rhythm is not likely for cod over our 11:30–16:30 h sampling period (Pickering and Pottinger 1983; Lankford et al. 2003), we are also confident that our hypoxic physiological thresholds have been accurately assessed from "typical" basal levels. The level of hypoxia where the rate of basal metabolism is no longer maintained (S_{crit}) occurs at 4.8 kPa at 10°C (Schurmann and Steffensen 1997; Claireaux et al. 2000) and explains why both plasma cortisol and lactate concentrations increased markedly at our slightly lower P_{O_2}. Given that the modest (1 mmol l^{-1}) rise in lactate is non-maximal, we suspect that exposure time is the predominant factor influencing the survival of cod under these conditions. Indeed, the P_{O_2} that is required to kill 50% of cod over 96 h is 4.0 kPa at 6°C (Plante et al. 1998) and therefore either above or in the region of 4.3 kPa at 10°C. Similarly, Schurmann and Steffensen (1992) report the absolute lethal O_2 levels for cod and show that fish held in 10°C seawater will all die rapidly at 2.9 kPa P_{O_2}.

Behavioural signalling functions

In accordance with our main hypothesis, cod exposed to progressive hypoxia reduce their swimming speed prior to the manifestation of major physiological stress. Although a rise in lactate has been shown to prompt a change in the behaviour of various animals (Pörtner et al. 1994; De Wachter et al. 1997), our data demonstrates that lactate is not necessarily a prerequisite for a shift in the swimming speed of cod during hypoxia. Future infusion studies may still reveal that lactate has a behavioural signaling function in fish but our data challenges whether lactate plays a useful role for cod in this process. For a species that regularly encounters hypoxia, we argue that any parameter associated with major aerobic stress is not likely to serve as an *adaptive* behavioural signal. To promote hypoxia tolerance, energy-preserving strategies should be activated well in advance of aerobic metabolic stress and presumably by non-critical (and maybe even synergistic) shifts in physiology. Candidate signals may originate from peripheral chemoreceptors but, although they are known to drive adaptive physiological responses in cod (Fritsche and Nilsson 1989), their role in the behaviour of fish has not yet been examined. Alternatively, catecholamines are implemented as a causal agent in fish anapyrexia (i.e. behavioural hypo-thermoregulation. Wollmuth et al. 1987 1988) and may also be involved in our hypoxic hypo-activity response (Fig. 3). In favour of this latter signal, Perry et al. (1991) observed a significant rise in adrenaline at 10 kPa and our transient rise in osmolality at 10.4 kPa (Fig. 4) could be the result of a catecholamine surge and the known "osmo-respiratory compromise" (Mazeaud and Mazeaud, 1981). However, since Perry et al. (1991) exposed cod to 10 kPa within 15–20 min (vs 180 min in the current study) and an increased rate of hypoxia induction heightens the stress response of this species (J.L. Johansen, N.A. Herbert, J.F. Steffensen, unpublished data), further investigations are clearly required to demonstrate that catecholamines have a universal behavioural signalling function in fish.

Metabolic scope in hypoxia

Previous studies suggest that reduced activity during hypoxia is an active strategy enabling fish to conserve energy and/or offset metabolic stress (Fischer et al. 1992; Nilsson et al. 1993; Schurmann and Steffensen 1994; Van Raaij et al. 1996a; Dalla Via et al. 1998; Herbert and Wells 2001). Claireaux et al. (2000) hypothesise further that cod regulate behavioural activity in hypoxia to remain safely within the maximal limit of their available metabolic scope. The aerobic metabolic scope of cod at 10°C is compressed by 80, 52, 34 and 19% at a P_{O_2} of 6.2, 8.4, 10.5 and 13.2 kPa, respectively (Claireaux et al. 2000); maximal rates of aerobic metabolism, and hence swimming activity, should also be reduced if homeostasis is to be maintained during hypoxia. On the basis that cod show no change in distribution of velocity or turning angles (Schurmann and Steffensen 1994), we are led to assume that hypoxia affects only total mean speed rather than the proportion of burst and steady swimming. We are unable to assign metabolic rate estimates to our measures of complex activity but, since physiological stress was apparent only at a critical P_{O_2}, it seems unlikely that the maximal metabolic scope of cod was surpassed at pressures ≥6.2 kPa.

Consideration of alternative behaviours and Hb genotype

Coastal marine areas are typically heterothermic (D'Amours 1993) and, while we have examined behavioural and physiological responses under thermally stable conditions, we acknowledge that wild cod may further extend their survival by preferentially selecting cooler water as part of their behavioural response to hypoxia (Schurmann and Steffensen 1992;

Petersen and Steffensen 2003). Cod have polymorphic haemoglobin and, depending on whether they are homozygous for "warm" type 1 or "cool" type 2 haemoglobin, are shown to select different temperatures under normoxia but not hypoxia (Petersen and Steffensen 2003). We have not examined the effect of haemoglobin polymorphism in the present study because there is no significant difference in whole blood Hb–O_2 binding between cod type 1 and 2 at 10°C (N.A. Herbert, P.V. Skov, J.F. Steffensen, unpublished data).

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