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RESEARCH ARTICLE

Anaemia adjusts the aerobic physiology of snapper (*Pagrus auratus*) and modulates hypoxia avoidance behaviour during oxygen choice presentations

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SUMMARY

The effect of altered oxygen transport potential on behavioural responses to environmental hypoxia was tested experimentally in snapper, *Pagrus auratus*, treated with a haemolytic agent (phenylhydrazine) or a sham protocol. Standard metabolic rate was not different between anaemic and normocythaemic snapper (Hct=6.7 and 25.7 g dl^{-1}, respectively), whereas maximum metabolic rate, and hence aerobic scope (AS), was consistently reduced in anaemic groups at all levels of water P_{O_2} investigated (P<0.01). This reduction of AS conferred a higher critical oxygen limit (P_{crit}) to anaemic fish (8.6±0.6 kPa) compared with normocythaemic fish (5.3±0.4 kPa), thus demonstrating reduced hypoxic tolerance in anaemic groups. In behavioural choice experiments, the critical avoidance P_{O_2} in anaemic fish was 6.6±2.5 kPa compared with 2.9±0.5 kPa for controls (P<0.01). Behavioural avoidance was not associated with modulation of swimming speed. Despite differences in physiological and behavioural parameters, both groups avoided low P_{O_2} just below their P_{crit}, indicating that avoidance was triggered consistently when AS limits were reached and anaerobic metabolism was unavoidable. This was confirmed by high levels of plasma lactate in both treatments at the point of avoidance. This is the first experimental demonstration of avoidance behaviour being modulated by internal physiological state. From an ecological perspective, fish with disturbed oxygen delivery potential arising from anaemia, pollution or stress are likely to avoid environmental hypoxia at a higher P_{O_2} than normal fish.

Key words: avoidance, hypoxia, anaemia, metabolic scope, haemoglobin, P_{crit}.

INTRODUCTION

Aquatic animals reside in environments characterised by low oxygen solubility and some face the continual threat of environmental hypoxia (Graham, 1990). Metabolic demand for oxygen has therefore necessitated adequate functional designs that are challenged further by increasingly prevalent episodes of environmental hypoxia (Diaz and Rosenberg, 2008; Gilbert et al., 2010), with negative effects manifested from the cellular level through to the scale of populations (for a review, see Wu, 2002). Impaired oxygen extraction may limit opportunities for fish exposed to waters below fully saturated (normoxic) levels (for a review, see Chabot and Claireaux, 2008a). While the rate of oxygen consumption required for maintenance, measured as standard metabolic rate (SMR), remains relatively fixed and stable at low-to-moderate oxygen partial pressures (P_{O_2}), maximal rates of oxygen consumption (MMR) are constrained heavily during hypoxia (Claireaux and Lagardère, 1999). The progressive drop in MMR during progressive hypoxia subsequently reduces an animal's aerobic scope (AS=MMR–SMR) to a point where MMR is only sufficient to maintain stable SMR (and AS is equal to zero), a point referred to as the critical oxygen tension, or P_{crit}. If aquatic P_{O_2} drops toward P_{crit}, the resulting hypoxia-induced drop in AS may cause important eco-physiological functions such as growth, activity and reproduction to be compromised (Chabot and Claireaux, 2008a). Major life-threatening processes are challenged at P_{O_2}<P_{crit} as the fish is forced into an oxygen-conforming state (McKenzie et al., 2007).

Hypoxia undoubtedly presents a physiological challenge to fish but hypoxic episodes are often patchy, ephemeral (Rabalais et al., 2002) and, in the case of stratification events, vertically defined (Breitburg, 1994). When hypoxia is spatially limited and fish have the option to move between different areas, complete low-O_2 avoidance would naturally represent an adaptive response as a result of its immediate benefit to basic aerobic functions at moderate P_{O_2} and survival at extremely low P_{O_2}. However, and perhaps surprisingly, numerous studies have identified that fish enter severe hypoxia voluntarily (Jones, 1952; Höglund, 1961; Claireaux et al., 1995; Wannamaker and Rice, 2000; Kaartvedt et al., 2009; Neuenfeldt et al., 2009; Herbert et al., 2011). With some evidence of larger predatory species being less hypoxia tolerant than smaller prey species, deliberate excursions into hypoxic water have proven beneficial to some fish as a form of predator avoidance (Robb and Abrahams, 2003; Kaartvedt et al., 2009). It is thought that other species, such as Atlantic cod (*Gadus morhua*), are also able to exploit a rich feeding ground by 'diving' into deep hypoxic layers on a voluntary basis (Neuenfeldt et al., 2009). These observations provide fine examples of behavioural trade-offs in the field but when studies on the same species are compared under more experimental conditions it is also evident that avoidance rarely occurs at a fixed threshold, leading authors to speculate that the physiological state of the animal influences hypoxia avoidance behaviour. For example, vague systemic signals such as 'respiratory distress' (Jones, 1952; Höglund, 1961) and 'systemic stress' (Herbert et al., 2011) have been quoted as triggers of avoidance behaviour, rather than the actual

level of aquatic P_{O_2}. The association between physiological state and avoidance behaviour has also been questioned by Farrell and Richards, who asked whether fish avoidance behaviours were performed anaerobically as their P_{crit} level was approached, or whether avoidance is conservative, occurring with a fraction of aerobic scope remaining (Farrell and Richards, 2009). Despite the critical importance of hypoxia to aquatic animals, an understanding of the physiological basis of hypoxia avoidance behaviour is lacking and requires urgent attention.

To address these concerns we sought to probe basic physiological mechanisms and their association with the avoidance behaviour of juvenile *Pagrus auratus* (Sparidae; common name snapper or red bream). Firstly, we investigated whether disturbed oxygen transport potential has an effect on the aerobic physiology of the fish, and whether altered aerobic state influences avoidance behaviour during hypoxic exposures. Secondly, the question of whether fish avoid hypoxia at P_{O2} above or below their P_{crit} was addressed to resolve the level of safety within the hypoxia avoidance response. If impaired oxygen transport potential has a bearing on hypoxia avoidance behaviour, we hypothesised that fish will avoid hypoxia at higher levels of P_{O_2}. We also hypothesised that fish should avoid hypoxia at $P_{O2}>P_{crit}$ as part of a safe and adaptive avoidance strategy. Accordingly, a comparison was made between two experimental treatments involving (i) a group exposed to induced anaemia and (ii) a normocythaemic (sham) treatment group. This enabled us to compare fish with reduced blood oxygen-carrying capacity invoked by the reduced erythrocyte volume, and a hypothesised drop in aerobic metabolic capacity, with controls. Snapper were selected for the study owing to the wide distribution of the Sparidae family throughout global temperate and tropical coastal environments, as well as their significance and abundance within the waters of northern New Zealand.

MATERIALS AND METHODS

Fish handling and anaemia induction

Juvenile *P. auratus* (Forster 1801) 20±2 cm fork length (FL), were caught by line and barbless hook in the inshore waters in the vicinity of Leigh Harbour (36°19′S, 174°48′E, Northland, New Zealand). Fish were housed at the Leigh Marine Laboratory in a 2000 l circular tank supplied with high quality, 200 µm filtered, flow-through seawater. Fish were kept for a minimum of 6 weeks before experimentation and fed with a diet of frozen squid and pilchard. Prior to experimentation, anaemia was induced using a haemolytic agent (Gilmour and Perry, 1996). Briefly, fish were transferred to an anaesthetic tank containing 22 p.p.m. AQUI-S (AQUI-S NZ Ltd, Lower Hutt, New Zealand) and left to progress to Stage II-2 anaesthesia (Ross and Ross, 2008). Fish were then weighed and given an intraperitoneal injection of (i) phenylhydrazine hydrochloride (Phz) (Sigma Aldrich, Auckland, New Zealand) or (ii) a sham saline injection. Phz was administered at a dose of 10 mg kg^{-1} (fish mass). To achieve this doseage, Phz was dissolved in a marine teleost modified Cortland saline (Milligan and McDonald, 1988) at a concentration of 2 mg ml^{-1}, with a distribution volume of 5.0 ml kg^{-1} fish mass. Similarly, sham-treated fish were administered the physiological saline at 5.0 ml kg^{-1} fish mass. Following treatment, fish were placed in holding tanks for 4–7 days. Separate experimental groups, each involving an anaemic and normocythaemic treatment, were utilised for behavioural analysis and respirometry (N=32 and N=52, respectively). Fish destined for behavioural analysis were netted carefully from the holding tanks and transferred in water to the experimental choice box (described below) 16–20 h prior to experimental recording.

All capture, holding and experimental techniques were performed under the approval of The University of Auckland Animal Ethics Committee (approval: R711).

Respirometry

All measures of oxygen consumption were obtained using automated, intermittent flow respirometry (Steffensen, 1989). Fish were transferred to a 5 l acrylic respirometry chamber, contained within a larger, 25 l reservoir. After the fish had been sealed in the respirometer, a programme of flush, wait and measurement phases (5, 1 and 4 min, respectively) was initiated by customised software coupled to a DAQ (in-house design, Leigh Marine Laboratory) control unit. Water temperature was held constant (18.0±0.1°C) by conducting experiments in a temperature-controlled room, with additional stability provided by an on/off relay supplying a flow of refrigerated water through the respirometry system. Filtered (5 µm) water circulating through the external reservoir was passed continuously through a UV steriliser, then through a 40 l cylindrical gassing tower that maintained desired oxygen saturation by the addition of compressed nitrogen or air through a fine bubble diffuser. Control of hypoxic P_{O_2} set points was provided by an Oxyreg controller (Loligo Systems, Tjele, Denmark).

P_{O_2} within the respirometer chamber was recorded using a Cellox galvanic probe connected to a WTW 3310 meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). The decline in P_{O_2} was used to calculate the mass-specific rate of oxygen consumption ($\dot{M}_{O_2}$) using standard methods described elsewhere (Steffensen, 1989; Svendsen et al., 2010). $\dot{M}_{O_2}$ values were used to calculate SMR, MMR and AS at multiple levels of P_{O_2}. Respirometry performed at ~21 kPa was continued for 40 h for multiple measures of $\dot{M}_{O_2}$ under normoxic conditions. SMR was subsequently defined according to the quantile method (Chabot and Claireaux, 2008b; Dupont-Prinet et al., 2010). A 15% quantile value was selected as this value typically coincided with the modal value of $\dot{M}_{O_2}$ when all data points were plotted in a frequency distribution. Once SMR was resolved, the peak value of $\dot{M}_{O_2}$ following chasing/exhaustion was adopted to resolve the MMR (Reidy et al., 1995). Accordingly, fish were chased within a 1500 l circular tank for periods exceeding 5 min until the fish would not escape when constrained against the side of the tank. The fish was then transferred to the respirometer chamber and 5–6 measurement cycles initiated immediately, with MMR typically observed within the first three cycles. MMR was determined at five points of P_{O_2} (~21, 14, 10, 6 and 3 kPa; N=8 for each regimen). Following each respirometric determination, fish were removed from the chamber and killed by a percussive stun followed by brain ablation using the iki-jime technique. Blood was sampled *via* caudal venepuncture for haematological analysis (see below). AS was calculated as the difference between MMR and SMR at equivalent P_{O_2}.

Behavioural apparatus

Hypoxia avoidance behaviour was examined within a laminar flow choice chamber (Fig. 1). A behavioural arena (length×width×depth, 800×600×300 mm) consisting of two parallel, laminar, flows of water separated by a central divider with a square hole (100×100 mm) provided fish free passage between the two discrete bodies of water. Water from each of the two channels was circulated through a sump before being pumped back into respective gassing towers. Water flowing from the gassing towers passed through a series of baffles and honeycomb diffusers, generating laminar flow, prior to re-entering the behavioural arena.

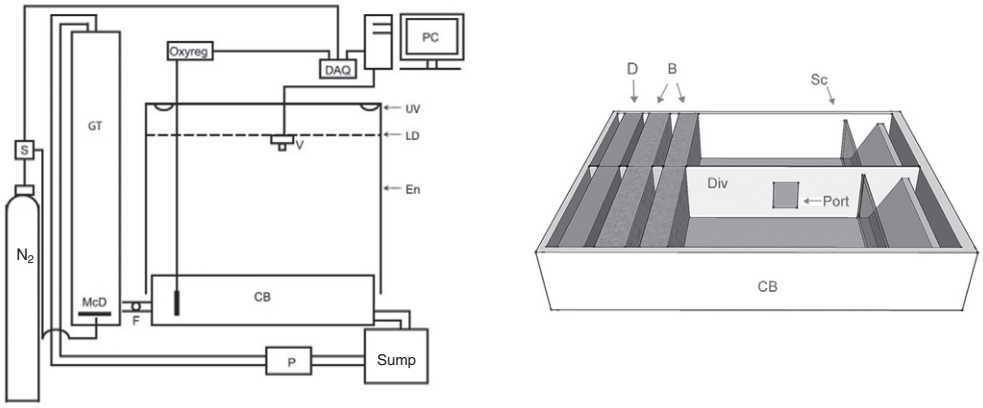

Fig. 1. Elevation view of experimental apparatus with accompanying 3D representation of the choice box setup. Water passing through gassing towers (GT) was aerated or deoxygenated by gas passing through the micro-diffuser (McD) before flowing, *via* flow control valves (F), into the choice box (CB). Water was initially passed through coarse diffusers (D) before flowing through honeycomb baffles (B), creating strictly laminar flow. The two bodies of water passing through the choice box were separated by a divider (Div) with a small port (Port) allowing fish movement between flows. A screen (Sc) prevented the fish from moving towards the outlet. Water would pass through the outlets into a twin channel sump before being returned with a pump (P) back to the gassing towers. The choice box apparatus was enclosed with blockout material (En). Additional light was provided from UV lights (UV) set behind diffuser panels (LD). Real-time tracking was enabled by a Fire-I camera (V) connected directly to a PC running SwisTrack software. Measurement of water P_{O_2} was performed by an Oxyreg control system, with output to a data acquisition card (DAQ). Oxygen probes were positioned between baffles to measure water P_{O_2} before it entered the behavioural arena. Control of deoxygenation setpoints was provided by a Labview script actuating a solenoid (S), enabling proportional nitrogen flow.

Flow rates within the tank were 2,400 l h^{-1} per side (4,800 l h^{-1} combined flow rate). Within the gassing towers, water P_{O_2} was held at normoxic levels with compressed air, or, in the case of hypoxia induction, P_{O_2} was altered by an intermittent flow of nitrogen. Control of nitrogen flow (and hence rate of hypoxia induction) was provided by a custom-designed software program written in Labview (v8.6, National Instruments, Austin, TX, USA). P_{O_2} measurements from either side of the chamber were acquired from the analog output of two Oxyreg instruments (Loligo Systems). A proportional control algorithm actuating the solenoids controlled the rate of de-oxygenation within the channel destined for hypoxia. A Minilab 1008 (Measurement Computing, Norton, MA, USA) served as the data acquisition (DAQ) module. The temperature of the circulating water was monitored by a thermistor coupled to the Labview software. Temperature set-point control was achieved within the Labview application by an on/off relay actuating a flow of refrigerated water through aluminium heat transfer coils set within the oxygenation towers. A constant temperature of 18.0±0.2°C was maintained. The behavioural arena was screened with (light-proof) block-out material to prevent visual disturbance by investigators. Two diffuse 8 W fluorescent UV white lights, set behind diffuser panels, provided a low level of illumination.

Fish activity within the behavioural arena was quantified in real-time in two dimensions using SwisTrack tracking software (Correll et al., 2006). A modified digital Fire-I digital camera (Unibrain Inc., San Ramon, CA, USA; 640×480 pixels, 15 frames s^{-1}) was fitted above the behavioural arena and connected to a PC *via* an IEEE-1394 cable. Components were applied to the tracking software, which tracked the geometrical centre of the swimming fish. Activity variables including side preference, hypoxic residence time, and swimming speed were calculated from the output of *x*- and *y*-coordinates. For analysis, raw swimming speed values of individual fish were normalised (as a differential value) using the speed of individual fish in the control period as the starting reference (Herbert and Steffensen, 2005).

Characterisation of the hypoxia avoidance response was performed by a progressive hypoxia induction in one of the two flow channels, while the other channel was maintained at normoxic P_{O_2} (Fig. 2). After transfer of fish to the behavioural arena and 16 h acclimation, experimentation began at 12:00 h with activity being tracked at ~21 kPa for a 1 h control period. Over this period routine swimming speed and side preference were determined. The experiment was continued only if fish showed a strong (>80%) side preference, and excursions into the alternative channel did not exceed 15 s duration. Ultimately, 40% of fish from the anaemic treatment and 60% of fish from the normocythaemic treatment preferred the left-hand side channel, identifying no major bias to one particular side of the tank. The fact that fish showed a random preference for one particular side was viewed as routine formation (a component of any normal animal personality/coping syndrome) (Coppens et

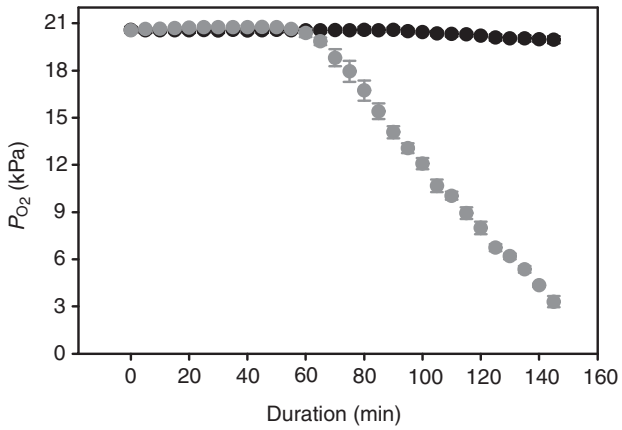

Fig. 2. Experimental hypoxia induction protocol showing the P_{O_2} of the normoxic channel (black symbols) and P_{O_2} of the channel destined for hypoxia (grey symbols). The channel within which the fish chose to reside was then deoxygenated progressively at a constant rate of 1 kPa 5 min^{-1}. Data are means ±95% confidence interval (CI).

al., 2010) and was not considered a problem as neither side of the behavioural arena was considered disadvantageous. In fact, by 'driving' fish from their preferred side with progressive hypoxia, the obvious side preference of fish was exploited in the design of our experiments, allowing us to observe clear avoidance thresholds. After the 1 h control period, the preferred side within the behavioural arena was deoxygenated progressively at a constant rate of 1 kPa 5 min^{-1}. The oxygen pressure at which the fish avoided the preferred (hypoxic) channel for greater than 30 s was deemed the avoidance P_{O_2}. Immediately after this avoidance event, the fish was netted from the chamber and killed. Blood was sampled immediately and placed on ice for haematological analysis. For comparison, routine swimming speed and haematological parameters were investigated in an unexposed control group that received the sham and anaemic treatments but never experienced hypoxia. After acclimation to the choice box, unexposed controls were left in normoxia for 150 min with behavioural recording and blood sampling carried out as above.

Blood and tissue analysis techniques

Haematocrit (Hct) and haemoglobin (Hb) analyses were performed on freshly drawn heparinised blood. Hct was measured in 75 mm capillary tubes, spun within a haemofuge (Haemocentaur, MSE, London, UK) for 10 min. Hb concentration of whole blood was quantified spectrophotometrically at 540 nm using modified Drabkin's reagent (Wells et al., 2007). Lactate analysis was performed on thawed plasma samples and was based on the standard lactate dehydrogenase-coupled assay with spectrophotometric absorbance readings at 340 nm using a 96-well multiplate reader (Multiskan, Thermoscientific, Vantaa, Finland).

Statistical techniques

Statistical analysis of haematological values, SMR values and hypoxic avoidance P_{O_2} was performed using Students *t*-tests. Between-treatment comparisons of a variety of measures against P_{O_2} were made using two-way ANOVA. These included swimming speed, excursion duration and values of MMR. *Post hoc* Bonferroni comparisons were performed where applicable. Analyses were performed in Sigmaplot (v 11, Systat Software Inc., www.systat.com). The effect of routine swimming speed on the avoidance response of the two treatments was investigated using one-way ANCOVA in Statistica (v9, Statsoft Inc., www.statsoft.co.uk). Curve fitting of MMR data points within the AS determination was carried out using a modified hyperbolic curve (Eqn 1) with a forced *y*-intercept of zero:

$$y = y_0 + \frac{ax}{(b + x)}\ , \qquad (1)$$

where *a* and *b* are constants.

The curve through points of MMR represents the limiting oxygen curve (LOC). The fitting of a LOC to points of MMR over the measured P_{O_2} range enabled us to resolve the point of intercept with SMR. This point of intercept was defined as the P_{crit} value. In all statistical analyses, significance was accepted at $P<0.05$.

RESULTS

Respirometry

SMR was not significantly different between the normocythaemic and anaemic treatments (Fig. 3; $t=1.75$, $P>0.05$), with mean values (±95% confidence intervals) of 133.6±9.6 and 122.1±8.6 mg O_2 kg^{-1} h^{-1} for the normocythaemic and anaemia treatments, respectively ($N=12$ per treatment). The MMR of normocythaemic fish was generally higher at all levels of P_{O_2} ($F=327.7$, $P<0.01$) but a significant interaction effect

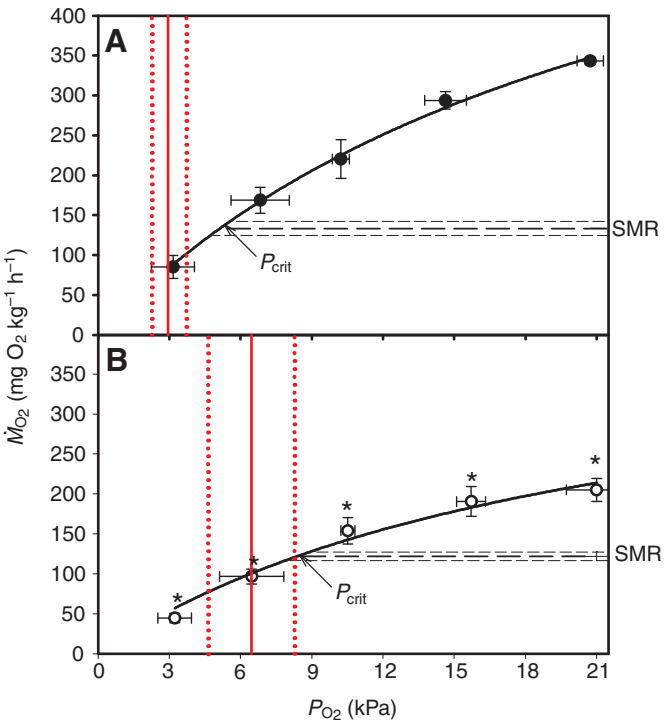

Fig. 3. Metabolic characterisation of (A) the normocythaemic treatment and (B) the anaemic treatment, both at 18°C. Thick dashed lines represent the standard metabolic rate (SMR) extrapolated across the relevant P_{O_2} range, with thin dashed lines representing the 95% CI. Circles indicate points of maximum metabolic rate (MMR) ±95% CI at measured P_{O_2}. The vertical solid red line represents the avoidance P_{O_2} of the treatment, with broken red lines representing the 95% CI. *Significant difference from the corresponding normocythaemic value. Mathematical constants describing the limiting oxygen curve (LOC) were $a=738.01$ and $b=23.31$ for the normocythaemic treatment and $a=427.42$ and $b=20.98$ for the anaemic treatment. P_{crit}, critical oxygen partial pressure; $\dot{M}_{O_2}$, metabolic oxygen consumption.

indicated that the magnitude of this difference was reduced under increasingly low P_{O_2} ($F=13.6$, $P<0.01$). Taken together, the lack of difference in SMR but higher MMR in the normocythaemic fish led to two additional observations: (i) AS was consistently higher in the normocythaemic fish than in the anaemic fish ($F=183.6$, $P<0.01$) and (ii) a significant difference in P_{crit} was observed between the normocythaemic (5.3±0.4 kPa) and anaemic (8.6±0.6 kPa) treatments ($t=-8.9$, $P<0.01$), as governed by the rate of decline in AS and the eventual crossover of SMR with MMR.

Behavioural choice and avoidance

Anaemic fish avoided hypoxia at significantly higher P_{O_2} (6.6±2.5 kPa) than did fish from the normocythaemic treatment (2.9±0.5 kPa) ($F=10.195$, $P<0.01$) (Fig. 3). Within the preferred channel (i.e. the channel destined for progressive de-oxygenation), residence time was 96.5% (±4.2%) in the normocythaemic treatment and 93.0% (±8.3%) in the anaemic treatment with no statistically significant differences ($t=0.49$, $P>0.05$). Even during hypoxic exposures, short excursions from the preferred channel into the normoxic channel (always <15 s) were considered too short for fish to repay any anaerobic debt. Throughout the study period, three fish (two normocythaemic and one anaemic fish) did not develop a side preference during the control measurement period and were therefore excluded from the study.

Table 1. Haematological parameters and routine behaviour of unexposed control and hypoxia-exposed (post-avoidance) snapper from the two different anaemia treatments

	Unexposed controls		Post-avoidance	
	Normocythaemic	Anaemic	Normocythaemic	Anaemic
Routine swimming speed (BL s^{-1})	0.44 (0.08)	0.39 (0.12)		
Hct (%)	25.7 (1.1)	6.7 (0.9)*	31.8 (2.5)‡	7.7 (1.7)†
Hb (g dl^{-1})	5.7 (0.4)	1.8 (0.2)*	7.47 (0.9)‡	1.9 (0.2)†
MCHC (g dl^{-1})	25.7 (1.1)	28.5 (2.1)	23.46 (1.1)	26.0 (3.7)
Plasma lactate (mmol l^{-1})	0.6 (0.2)	0.5 (0.2)	5.49 (1.0)‡	6.3 (1.4)‡
N	6	6	10	10

Values are means with 95% CI presented in parentheses.
Significant differences (P<0.05) between unexposed controls and post-avoidance values are indicated: *comparisons between normocythaemic and anaemic unexposed controls; †comparisons between normocythaemic and anaemic fish post-avoidance; and ‡comparisons between unexposed control and post-avoidance fish from the respective treatments (normocythaemic or anaemic).
BL, body length; Hct, haematocrit; Hb, haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

Behavioural activity

No significant difference in the mean routine swimming speed of fish was observed between normocythaemic and anaemic treatment groups during the 1 h control measurement period (t=–1.27, P>0.05) (Table 1). However, routine speed varied within treatments and a positive link between routine swimming speed and avoidance P_{O_2} was observed for both groups (one-way ANCOVA, F=30.3, P<0.01; Fig. 4). This indicated that faster swimming fish avoided hypoxia earlier at higher P_{O_2}. By taking routine swimming speed into consideration as a covariate, between-treatment differences were confirmed, whereby anaemic fish did indeed avoid hypoxia earlier at higher P_{O_2} (one-way ANCOVA, F=14.7, P<0.01). Because initial routine swimming speed in normoxia varied between individuals, swimming speed during hypoxia was normalised as a differential (Herbert and Steffensen, 2005). Such normalisations revealed that neither anaemic nor normocythaemic fish adjusted their swimming speed during hypoxia (F=0.7, P>0.05 and F=1.7, P>0.05, respectively; Fig. 5).

Blood parameters

Haematological parameters (Table 1) of the normocythaemic and anaemic control treatments were significantly different, with anaemic fish showing lower Hct (t=32.2, P<0.01) and lower Hb concentrations (t=16.7, P<0.01). As expected, circulating lactate concentrations were low and not significantly different between unexposed control fish. After hypoxic exposure and avoidance, normocythaemic fish had elevated Hct (t=21.0, P<0.01) and Hb concentrations (t=25.0, P<0.01) when compared with unexposed normocythaemic controls. In contrast, fish from the anaemic treatment did not demonstrate elevated Hct and Hb concentrations after being exposed to hypoxia. After avoidance, circulating plasma lactate concentrations were significantly elevated in both normocythaemic (t=6.0, P<0.01) and anaemic (t=7.7, P<0.01) treatment groups with no significant differences between the two treatments (t=–1.0, P>0.05).

DISCUSSION

Effects of anaemia on aerobic physiology and avoidance behaviour

According to our measures of haematological O_2-carrying capacities and rates of O_2 consumption ($\dot{M}_{O_2}$), normocythaemic and anaemic snapper differ in their aerobic physiology, and this in turn appears to exert a strong influence over hypoxia avoidance behaviour. SMR was comparable between the two treatments, implying that within the anaemic treatment group remaining erythrocytes in circulation are sufficient to support standard metabolic function, but all other measures of aerobic potential showed marked differences. Haemolytic anaemia has been widely employed in other studies on fish to address the consequences of reduced O_2-carrying capacities (Smith et al., 1971; Houston et al., 1988; Gallaugher et al., 1995; Gilmour and Perry, 1996). Of these, the study of Gallaugher and colleagues (Gallaugher et al., 1995) investigated changes in respiratory character, and showed an approximate 70% drop in maximum rate of O_2 uptake ($\dot{V}_{O_2,max}$) when anaemic (10% Hct) and normocythaemic (30% Hct) rainbow trout were compared under normoxic conditions. This compares qualitatively, but not quantitatively, with the current study in which Phz-treated snapper were highly anaemic (6.7% Hct, Table 1) yet showed only a 45% drop in MMR compared with normocythaemic fish with normal erythrocytic volumes (25.7% Hct) in normoxia. However, our experiments give further insight into the aerobic capacity of anaemic fish during hypoxia. Anaemic fish had depressed MMR and showed a concomitant drop in the AS at all P_{O_2} values, which, combined with an unchanging SMR, shifted the P_{crit} of the anaemic group upwards from 5.3 to 8.6 kPa, a remarkably large difference of 3.3 kPa (Fig. 3). The main objective of this study was to understand how aerobic state influences hypoxia avoidance behaviour, and we found that the 3.3 kPa P_{crit} shift of the anaemic fish was paralleled by an increase in the behavioural avoidance threshold. Normocythaemic

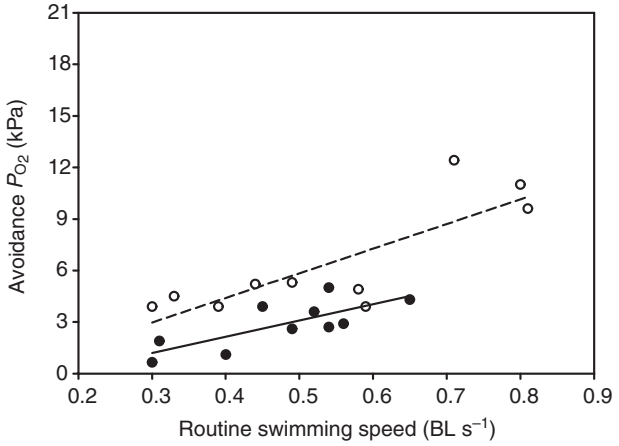

Fig. 4. Correlation between avoidance P_{O_2} and routine swimming speed for the normocythaemic (filled symbols) and anaemic (open symbols) treatments. Regression lines show significant linear correlation in the normocythaemic (t=3.34, r^2=0.76, P<0.01) and anaemic (t=3.06, r^2=0.76, P<0.05) treatments. BL, body length.

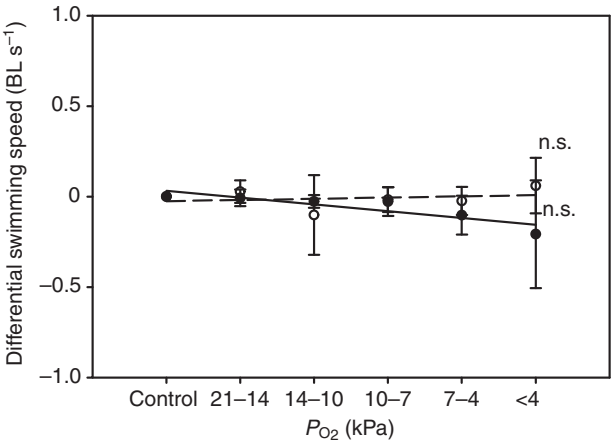

Fig. 5. The differential swimming speed of the normocythaemic (filled symbols) and anaemic treatments (open symbols) at different P_{O_2} over the course of the experimental protocol. Data are means ±95% CI. n.s., not significant.

fish avoided low O_2 at 2.9±0.5 kPa while anaemic fish avoided hypoxia at the higher level of 6.6±2.5 kPa, a difference of 3.7 kPa. That the behavioural avoidance threshold shifted slightly more than P_{crit} in the anaemic fish may be explained by the lack of capacity to release additional erythrocytes from the spleen that would otherwise boost O_2-carrying capacity during hypoxia (Table 1). Indeed, oxygen-carrying capacity was observed to increase in normocythaemic snapper during hypoxia, as indicated by an increase in Hct and Hb concentration with no evidence of red blood cell swelling. It is therefore assumed that the observed change of behaviour was driven directly by the decrease in oxygen-carrying capacity but we do also acknowledge that unknown non-target effects of Phz administration could potentially have a role in the interaction between physiology and behaviour. Further evidence that physiological state might affect avoidance is provided by our observation of faster routine swimmers avoiding hypoxia at a higher P_{O_2} than slower swimmers (Fig. 4). Moreover, at any given routine swimming speed, the avoidance P_{O_2} threshold of anaemic fish was consistently higher than that of normocythaemic fish.

A number of studies have observed fish entering potentially dangerous levels of hypoxia on a voluntary basis (Jones, 1952; Höglund, 1961; Herbert et al., 2011; Claireaux et al., 1995; Kaartvedt et al., 2009; Neuenfeldt et al., 2009; Wannamaker and Rice, 2000) implying that residence time under low levels of oxygen is a key driver of avoidance. Recently, Herbert and colleagues have shown that cod (*G. morhua*) voluntarily swim into critical P_{O_2} without any change in their behaviour but only when they have access to an O_2 refuge (Herbert et al., 2011). This response was clearly regulated by residence time in hypoxia because avoidance did occur when the safety (i.e. P_{O_2}) of the O_2 refuge was reduced. Based on their observations, Jones (Jones, 1952), Höglund (Höglund, 1961) and Herbert and colleagues (Herbert et al., 2011) have all speculated that systemic signals may be responsible for the initiation of hypoxia avoidance behaviour, as opposed to the actual level of P_{O_2} being encountered during escapable hypoxia. Because fish in hypoxia often exhibit sudden erratic behaviour prior to avoidance, Jones (Jones, 1952) and Höglund (Höglund, 1961) proposed 'respiratory distress' as the driver of the avoidance reaction but the authors did not clarify whether they refer to ventilatory oxygen diffusion limitations and resulting hypoxaemia or cellular substrate limitations. The results from the current study therefore present the first set of data linking aerobic state with hypoxia avoidance, with evidence of reduced oxygen-carrying capacity and a shift in the P_{crit} having a profound effect on avoidance thresholds. We achieved this by using only fish exhibiting an initial preference of side in the behavioural choice box, which was included in our design as a means of standardising residence time. This effectively allowed us to 'drive' fish from hypoxia under standardised conditions such that our experiment was free of behavioural variability that typically occurs when fish roam on a routine basis between normoxia and hypoxia. This is an important point because residence time in hypoxia appears to be a potent regulator of avoidance (see Herbert et al., 2011) and this is should always be considered in studies addressing avoidance reactions.

By standardising the residence time of snapper in hypoxic water we were also allowed an insight into the avoidance response of fish with respect to physiological limitations. Interestingly, the avoidance responses of both anaemic and normocythaemic snapper were not triggered until P_{O_2} values below their treatment-dependent P_{crit} had been surpassed and aerobic scope had been totally exhausted (Fig. 3). The fact that blood lactate was comparably high in the two treatments certainly supports the view that physiological aerobic limits were surpassed in a comparable way and that anaerobic metabolism may somehow be involved in the initiation of avoidance. Further examples of the hypoxia avoidance behaviours of fish and their relationship with physiologically relevant thresholds are surprisingly limited (see Table 2). Furthermore, physiological and behavioural measurements are often not reported within the same

Table 2. Summary values of physiological tolerance matched to hypoxia avoidance behaviour of various marine and freshwater fish species

Species	P_{crit}	12 h LC_{50}	Avoidance P_{O_2}
Oncorhynchus mykiss	2.9 kPa (15°C)[a]		2.6 kPa (13°C)[b]
	3.6 kPa (20°C)[a]		7.3 kPa (20°C)[b]
Brevoortia tyrannus		3.3 kPa (25°C)[c]	6.0–3.0 kPa (25°C)[d]
Gadus morhua	3.4 kPa (5°C)[e]		7.2 kPa (5°C)[f]
Gadus morhua	3.4 kPa (5°C)[e]		9.0–3.0 kPa (5°C)[g,*]
Gadus morhua	4.8 kPa (10°C)[e]		4.3–12.8 kPa (11°C)[h,**]
Pagrus auratus	5.3 kPa (18°C)[g]		2.9 kPa (18°C)[i]

Literature values reported in Torr were converted to partial pressure values (kPa). Values originally reported in concentration units (providing temperature and salinity values are also reported) were converted to partial pressure (kPa) units assuming a standard atmospheric pressure of 101.3 kPa. LC_{50}, lethal concentration for 50% of sample population.

*Cod generally avoided oxygen levels below 9 kPa but feeding excursions into hypoxic waters equal to 3 kPa were observed. **Avoidance behaviour of Atlantic cod was identified to be variable depending on the provision and magnitude of a normoxic refuge.

Cited references: [a]Ott et al., 1980; [b]Jones, 1952; [c]Shimps et al., 2005; [d]Wannamaker and Rice, 2000; [e]Schurmann and Steffensen,1996; [f]D'Amours, 1993; [g]Claireaux et al., 1995; [h]Herbert et al., 2011; and [i]current study.

study, meaning that we have collated the data from disparate experiments. Avoidance behaviours are observed to occur across a range of P_{O_2} values ranging from 'safe' avoidance (avoidance threshold $>P_{crit}$) through to 'dangerous' avoidance strategies (avoidance threshold $<P_{crit}$). With reference to fish in the current study, avoidance thresholds are consistently less than P_{crit}, indicating that this species possibly employs a high-risk and dangerous avoidance strategy. Avoidance in this case is heavily reliant on the animal's ability to function anaerobically and, by virtue of the maximal level of lactate observed in the blood of snapper at the point of avoidance (5.5–6.3 mmol l^{-1}) (Wells and Dunphy, 2009), our fish presumably had little time left before being overwhelmed by disorientation and death. Further support for the dangerous nature of the snapper avoidance strategy comes from the study of Janssen and colleagues (Janssen et al., 2010). In anaesthetised snapper, cardiac function was impaired suddenly below 3.1 kPa with evidence of synchronous bradycardia, decreased ventricular depolarisation (reduced QRS amplitude) and arrhythmias (Janssen et al., 2010). Dangerous avoidance strategies resulting in severe physiological perturbations and fatigue therefore lead us to question the fitness of fish that are unable to relocate to an oxygen refuge. Indeed, although difficult to infer from the current experiment, we doubt that snapper would knowingly use this strategy to perform hypoxic dives to feed in the wild, or as a form of predator refuge. However, before reaching this conclusion, we should also consider whether fish in the current study were simply naive through lack of previous experience of hypoxia. Incorporating hypoxic pre-conditioning protocols within future experimental designs may help to resolve this uncertainty.

Behavioural activity

One of the assumptions of the aerobic metabolic scope framework is that as oxygen becomes limiting, fish will reduce behavioural activity to limit energetic expenditure (Chabot and Claireaux, 2008a). This down-regulation in activity has been demonstrated in species such as cod *G. morhua* (Schurmann and Steffensen, 1994; Herbert and Steffensen, 2005), crucian carp *Carassius carassius* (Nilsson et al., 1993), sole *Solea solea* (Dalla Via et al., 1998) and eelpout *Zoarces viviparous* (Fischer et al., 1992) but was not shown in herring (Domenici et al., 2000; Herbert and Steffensen, 2006), red hake *Urophycis chuss* (Bejda et al., 1987), tuna *Katsuwonus pelamis* and *Thunnus albacares* (Dizon, 1977; Bushnell and Brill, 1991) and weakfish *Cynoscion regalis* (Brady et al., 2009). Differences between species appear to be associated with their ecotype, where more active, pelagic species generally up-regulate swimming activity in an attempt to reduce the residence time within the hypoxic environment, while less active, benthic species down-regulate activity to conserve energy. An intriguing observation from the current study was that swimming speed was not adjusted throughout progressive hypoxia and avoidance, even when fish had clearly surpassed the aerobic limits set by the LOC of the AS model. A modulation of swimming activity clearly did not assist avoidance behaviour, further suggesting that snapper do not have a hypoxia avoidance strategy, potentially as a result of naivety and inexperience of hypoxia in their natural environment.

CONCLUSIONS

By experimentally manipulating the oxygen-carrying capacity, aerobic scope and critical O_2 limit of *P. auratus*, and exposing them to range of P_{O_2} choices, we have provided compelling evidence that physiological state has a strong influence over the hypoxic avoidance response of fish. Thus, anaemic fish avoided aquatic hypoxia at higher P_{O_2} than did normocythaemic fish, and these behaviours corresponded closely with a comparable shift in P_{crit} driven by compressed aerobic scope. The additional observation that anaemic and normocythaemic fish did not avoid hypoxia until ambient P_{O_2} values were below their respective P_{crit} values (where AS is exhausted and anaerobic pathways activated) represents a dangerous avoidance strategy. From a more general ecological perspective, our data suggest that fish with disturbed oxygen delivery potential, arising from anaemia, pollution or stress, are more likely to avoid hypoxia earlier, which may have an impact on the distribution of important finfish species.

LIST OF ABBREVIATIONS

AS	aerobic metabolic scope
Hb	haemoglobin
Hct	haematocrit
LOC	limiting oxygen curve
$\dot{M}_{O_2}$	metabolic oxygen consumption
MMR	maximum aerobic metabolic rate post-exercise
P_{crit}	critical oxygen partial pressure
Phz	phenylhydrazine hydrochloride
P_{O_2}	partial pressure of oxygen
SMR	standard metabolic rate

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REFERENCES

Bejda, A. J., Studholme, A. L. and Olla, B. L. (1987). Behavioural responses of red hake, *Urophycis chuss*, to decreasing concentrations of dissolved oxygen. *Environ. Biol. Fishes* **19**, 261-268.

Brady, D. C., Targett, T. E. and Tuzzolino, D. M. (2009). Behavioral responses of juvenile weakfish (*Cynoscion regalis*) to diel-cycling hypoxia: swimming speed, angular correlation, expected displacement, and effects of hypoxia acclimation. *Can. J. Fish. Aquat. Sci.* **66**, 415-424.

Breitburg, D. L. (1994). Behavioural response of fish larvae to low dissolved oxygen concentrations in a stratified water column. *Mar. Biol.* **120**, 615-625.

Bushnell, P. G. and Brill, R. W. (1991). Responses of swimming skipjack (*Katsuwonus pelamis*) and yellowfin (*Thunnus albacares*) tunas to acute hypoxia, and a model of their cardiorespiratory function. *Physiol. Zool.* **64**, 787-811.

Chabot, D. and Claireaux, G. (2008a). Environmental hypoxia as a metabolic constraint on fish: the case of Atlantic cod, *Gadus morhua*. *Mar. Pollut. Bull.* **57**, 287-294.

Chabot, D. and Claireaux, G. (2008b). Quantification of SMR and SDA in aquatic animals using quantiles and non-linear quantile regression. *Comp. Biochem. Physiol.* **150A**, S99.

Claireaux, G. and Lagardère, J. P. (1999). Influence of temperature, oxygen and salinity on the metabolism of the European sea bass. *J. Sea Res.* **42**, 157-168.

Claireaux, G., Webber, D. M., Kerr, S. R. and Boutilier, R. G. (1995). Physiology and behaviour of free-swimming Atlantic cod (*Gadus morhua*) facing fluctuating salinity and oxygenation conditions. *J. Exp. Biol.* **198**, 61-69.

Coppens, C. M., de Boer, S. F. and Koolhaas, J. M. (2010). Coping styles and behavioural flexibility: towards underlying mechanisms. *Philos. Trans. R. Soc. B* **365**, 4021-4028.

Correll, N., Sempo, G., de Meneses, Y. L., Halloy, J., Deneubourg, J. L., Martinoli, A. and IEEE (2006). *SwisTrack: A Tracking Tool for Multi-Unit Robotic and Biological Systems*. New York: IEEE.

D'Amours, D. (1993). The distribution of cod (*Gadus morhua*) in relation to temperature and oxygen level in the Gulf of St. Lawrence. *Fish. Oceanogr.* **2**, 24-29.

Dalla Via, J., Van den Thillart, G., Cattani, O. and Cortesi, P. (1998). Behavioural responses and biochemical correlates in *Solea solea* to gradual hypoxic exposure. *Can. J. Zool.* **76**, 2108-2113.

Diaz, R. J. and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* **321**, 926-929.

Dizon, A. E. (1977). Effects of dissolved oxygen concentration and salinity on swimming speed of 2 species of tunas. *Fish. Bull.* **75**, 649-653.

Domenici, P., Steffensen, J. F. and Batty, R. S. (2000). The effect of progressive hypoxia on swimming activity and schooling in Atlantic herring. *J. Fish Biol.* **57**, 1526-1538.

Dupont-Prinet, A., Chatain, B., Grima, L., Vandeputte, M., Claireaux, G. and McKenzie, D. J. (2010). Physiological mechanisms underlying a trade-off between

growth rate and tolerance of feed deprivation in the european sea bass (*Dicentrarchus labrax*). *J. Exp. Biol.* **213**, 1143-1152.
Farrell, A. P. and Richards, J. G. (2009). Defining hypoxia: an integrative synthesis of the responses of fish to hypoxia. In *Hypoxia*, Vol. 27 (ed. J. G. Richards, A. P. Farrell and C. J. Brauner), pp. 488-504. London: Academic Press.
Fischer, P., Rademacher, K. and Kils, U. (1992). *In situ* Investigations on the respiration and behavior of the eelpout *Zoarces viviparus* under short-term hypoxia. *Mar. Ecol. Prog. Ser.* **88**, 181-184.
Gallaugher, P., Thorarensen, H. and Farrell, A. P. (1995). Hematocrit in oxygen transport and swimming in rainbow trout (*Oncorhynchus mykiss*). *Respir. Physiol.* **102**, 279-292.
Gilbert, D., Rabalais, N. N., Diaz, R. J. and Zhang, J. (2010). Evidence for greater oxygen decline rates in the coastal ocean than in the open ocean. *Biogeosciences* **7**, 2283-2296.
Gilmour, K. M. and Perry, S. F. (1996). The effects of experimental anaemia on CO_2 excretion in vitro in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **15**, 83-94.
Graham, J. B. (1990). Ecological, evolutionary and physical factors influencing aquatic animal respiration. *Am. Zool.* **30**, 137-146.
Herbert, N. A. and Steffensen, J. F. (2005). The response of Atlantic cod, *Gadus morhua*, to progressive hypoxia: fish swimming speed and physiological stress. *Mar. Biol.* **147**, 1403-1412.
Herbert, N. A. and Steffensen, J. F. (2006). Hypoxia increases the behavioural activity of schooling herring: a response to physiological stress or respiratory distress? *Mar. Biol.* **149**, 1217-1225.
Herbert, N. A., Skjæraasen, J. E., Nilsen, T., Salvanes, A. G. V. and Steffensen, J. F. (2011). The hypoxia avoidance behaviour of juvenile Atlantic cod (*Gadus morhua* L.) depends on the provision and pressure level of an O_2 refuge *Mar. Biol.* **158**, 737-746.
Höglund, L. B. (1961). The reactions of fish in concentration gradients. *Fish Board Swed. Rep.* **43**, 1-147.
Houston, A. H., Murad, A. and Gray, J. D. (1988). Induction of anemia in goldfish, *Carassius auratus* L, by immersion in phenylhydrazine hydrochloride. *Can. J. Zool.* **66**, 729-736.
Janssen, G. J. A., Jerrett, A. R., Black, S. E. and Forster, M. E. (2010). The effects of progressive hypoxia and re-oxygenation on cardiac function, white muscle perfusion and haemoglobin saturation in anaesthetised snapper (*Pagrus auratus*). *J. Comp. Physiol. B* **180**, 503-510.
Jones, J. R. E. (1952). The reactions of fish to water of low oxygen concentration. *J. Exp Biol.* **29**, 403-415.
Kaartvedt, S., Røstad, A. and Klevjer, T. A. (2009). Sprat *Sprattus sprattus* can exploit low oxygen waters for overwintering. *Mar. Ecol. Prog. Ser.* **390**, 237-249.
McKenzie, D. J., Steffensen, J. F., Korsmeyer, K., Whiteley, N. M., Bronzi, P. and Taylor, E. W. (2007). Swimming alters responses to hypoxia in the Adriatic sturgeon *Acipenser naccarii*. *J. Fish Biol.* **70**, 651-658.
Milligan, C. L. and McDonald, D. G. (1988). Invivo lactate kinetics at rest and during recovery from exhaustive exercise in coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*). *J. Exp. Biol.* **135**, 119-131.
Neuenfeldt, S., Andersen, K. H. and Hinrichsen, H. H. (2009). Some Atlantic cod *Gadus morhua* in the Baltic Sea visit hypoxic water briefly but often. *J. Fish Biol.* **75**, 290-294.
Nilsson, G. E., Rosen, P. and Johansson, D. (1993). Anoxic depression of spontaneous locomotor-activity in crucian carp quantified by a computerized imaging technique. *J. Exp. Biol.* **180**, 153-162.
Ott, M. E., Heisler, N. and Ultsch, G. R. (1980). A re-evaluation of the relationship between temperature and the critical oxygen tension in freshwater fishes. *Comp. Biochem. Physiol.* **67**, 337-340.
Rabalais, N. N., Turner, R. E. and Scavia, D. (2002). Beyond science into policy: Gulf of Mexico hypoxia and the Mississippi River. *Bioscience* **52**, 129-142.
Reidy, S. P., Nelson, J. A., Tang, Y. and Kerr, S. R. (1995). Post-exercise metabolic rate in Atlantic cod and its dependence upon the method of exhaustion. *J. Fish Biol.* **47**, 377-386.
Robb, T. and Abrahams, M. V. (2003). Variation in tolerance to hypoxia in a predator and prey species: an ecological advantage of being small? *J. Fish Biol.* **62**, 1067-1081.
Ross, L. G. and Ross, B. (2008). *Anaesthetic and Sedative Techniques for Aquatic Animals*. Oxford: Blackwell Publishing Limited.
Schurmann, H. and Steffensen, J. F. (1994). Spontaneous swimming activity of Atlantic Cod *Gadus morhua* exposed to graded hypoxia at 3 temperatures. *J. Exp. Biol.* **197**, 129-142.
Shimps, E. L., Rice, J. A. and Osborne, J. A. (2005). Hypoxia tolerance in two juvenile estuary-dependent fishes. *J. Exp. Mar. Biol. Ecol.* **325**, 146-162.
Smith, C. E., McLain, L. R. and Zaugg, W. S. (1971). Phenylhydrazine-induced anemia in chinook salmon. *Toxicol. Appl. Pharmacol.* **20**, 73-81.
Steffensen, J. F. (1989). Some errors in respirometry of aquatic breathers: how to avoid and correct for them. *Fish Physiol. Biochem.* **6**, 49-59.
Svendsen, J. C., Tudorache, C., Jordan, A. D., Steffensen, J. F., Aarestrup, K. and Domenici, P. (2010). Partition of aerobic and anaerobic swimming costs related to gait transitions in a labriform swimmer. *J. Exp. Biol.* **213**, 2177-2183.
Wannamaker, C. M. and Rice, J. A. (2000). Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern United States. *J. Exp. Mar. Biol. Ecol.* **249**, 145-163.
Wells, R. M. G. and Dunphy, B. J. (2009). Potential impact of metabolic acidosis on the fixed-acid Bohr effect in snapper (*Pagrus auratus*) following angling stress. *Comp. Biochem. Physiol.* **154A**, 56-60.
Wells, R. M. G., Baldwin, J., Seymour, R. S., Christian, K. A. and Farrell, A. P. (2007). Air breathing minimizes post-exercise lactate load in the tropical Pacific tarpon, *Megalops cyprinoides* Broussonet 1782 but oxygen debt is repaid by aquatic breathing. *J. Fish Biol.* **71**, 1649-1661.
Wu, R. S. S. (2002). Hypoxia: from molecular responses to ecosystem responses. *Mar. Pollut. Bull.* **45**, 35-45.