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Prolonged SDA and reduced digestive efficiency under elevated CO_2 may explain reduced growth in Atlantic cod (*Gadus morhua*)

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ABSTRACT

Land-based aquaculture systems expose fish to elevated dissolved CO_2 levels, a factor that is correlated with reduced growth, feed conversion efficiency and body condition index. The physiological basis underlying the pathological effects of environmental hypercapnia is poorly understood, in particular for marine fish species. We investigated whether changes in energy expenditure and the specific dynamic action (SDA) of digestion and assimilation could account for the lower growth of adult Atlantic cod (*Gadus morhua*) under environmental hypercapnia. Fish acclimated to a partial pressure of 800 μatm CO_2 (0.6 mmHg, 1.5 mg/L) and 9200 μatm CO_2 (7 mmHg, 18.7 mg/L) exhibited no difference in maintenance metabolic rates, which concurs with previous research for this species and other fish species. At 9200 μatm CO_2 Atlantic cod had a significantly diminished (14%) maximum aerobic capacity. While hypercapnia did not affect the amount of oxygen required for the SDA process, it did prolong the SDA duration by 23%. The longer SDA process time may offer an explanation for the observation of lower feed intake, growth and condition factor in long-term hypercapnia studies. Comparison of aerobic scope and cardiac performance during digestion suggested that reduced oxygen delivery capacity under hypercapnia could be one mechanism by which CO_2 prolongs SDA, although our results could not definitively demonstrate this effect.

1. Introduction

Marine fish have evolved in an aqueous environment conducive to the transport of respired CO_2 away from the blood and gills, and are accustomed to relatively low partial pressures of CO_2 (pCO_2) in body fluids. The plasma pCO_2 level in fish (1300–2600 μatm) is relatively close to the ambient CO_2 level (~400 μatm), whereas terrestrial animals have evolved to live with much higher plasma pCO_2 (e.g. 55–59,000 μatm in humans) (Dejours, 1975) and are therefore not as susceptible as fish to relatively minor changes in ambient CO_2 levels (i.e. within an order of magnitude). The effect of elevated pCO_2 levels (termed hypercapnia) on fish physiology is of growing interest due to the continuing global increase in atmospheric and oceanic CO_2 levels (ICES, 2010), and also because the rapid expansion of farming in land-based recirculated aquaculture systems exposes fish to chronically elevated CO_2 levels (Timmons et al., 2010). There is a substantial difference in the range of CO_2 concentrations fish are exposed to under these two hypercapnic conditions (400–1000 μatm for the oceans versus ~2000–25,000 μatm for aquaculture), and accordingly, different research focuses for each field. In this study, we measured the metabolic and digestive response of Atlantic cod (*Gadus morhua* Linnaeus, 1758) held at two pCO_2 levels relevant to land-based aquaculture. Studies of this kind can be used to elucidate the physiological basis underlying decreased growth performance and feed conversion observed under elevated CO_2 culture conditions, and are also important for the development of informed water quality guidelines to ensure the welfare of farmed stock.

Fish have physiological and behavioural mechanisms to cope with the extra H^+ generated in tissues from exposure to pCO_2 levels well in excess of 30 times current atmospheric concentrations (Brauner and Baker, 2009; Claiborne and Heisler, 1984, 1986;

Abbreviations: MS, metabolic scope for activity; t_{peak}, time from feeding to SDA_{scope}; SDA_{scope}, the maximum $\dot{M}O_2$ value recorded during the SDA process – SMR; SDA_{50}, oxygen required for digestion; $SDA_{coefficient}$, SDA_{50} dived by meal energy (%); t_{50}, time from feeding to $\dot{M}O_2$ = SMR + 50% SDA_{scope}; t_{100}, time from feeding to $\dot{M}O_2$ = SMR; DE_{50}, digestion efficiency calculated from feeding to t_{50} (%); DE_{100}, digestion efficiency calculated from feeding to t_{100} (%); *Q*, cardiac output; *f*H, heart rate; Vs, stroke volume.

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Esbaugh et al., 2012; Hayashi et al., 2004; Larsen et al., 1997; Lee et al., 2003). While many fish species can survive relatively high pCO_2 levels (Crocker and Cech, 1996; McKenzie et al., 2003), long-term exposure to sub-lethal environmental hypercapnia can affect a range of attributes including growth performance (Fivelstad et al., 1998; Hosfeld et al., 2008; Moran and Støttrup, 2011), feed intake (Cecchini et al., 2001; Smart, 1981), feed conversion efficiency (Foss et al., 2003) and aerobic performance (Heuer and Grosell, 2014; Methling et al., 2013; Munday et al., 2009). The physiological basis for lowered growth, feed intake and feed conversion under chronic hypercapnia is not well understood, especially for marine fish species.

Digestion is a particularly important process to study as it represents a significant fraction of daily energy expenditure (Soofiani and Hawkins, 1982), and any change in feed conversion dynamics due to CO_2 toxicity will have consequences for growth, dietary demand and the reserves available for movement and reproduction. The major contributor to the increase in energy expenditure following ingestion of food is the biochemical transformation of food and de novo protein synthesis occurring in the post-absorptive state, leading to the deposition and turnover of tissue components (Brown and Cameron, 1991; Bureau et al., 2002). Digestion and assimilation of nutrients is associated with elevated blood flow to the gut resulting from an increased allocation of blood flow to the gastrointestinal tract via changes in vascular resistance (Fara, 1984) and higher cardiac output (Axelsson and Fritsche, 1991), which together provide nutrients to gut tissues and transports absorbed nutrients to the liver for further processing. In the present study, cardiac performance during the digestive process was monitored via implanted blood flow probes.

The substantial increase in energy expenditure during ingestion, digestion, absorption and assimilation of a meal is commonly termed the specific dynamic action (SDA) (Brett and Groves, 1979; Jobling, 1985), and changes in whole body oxygen consumption over time can be used to infer how the SDA is affected by factors such as meal size (Fu et al., 2005a,b; Jordan and Steffensen, 2007) and water oxygen tension (Eliason and Farrell, 2014; Jordan and Steffensen, 2007). The effect of hypercapnia on fish SDA has not been widely investigated, but it is likely that chronic exposure to significantly elevated CO_2 has an effect given our knowledge of acid–base regulation (Deigweiher et al., 2008; Heuer and Grosell, 2014; Melzner et al., 2009; Perry and Gilmour, 2006) and blood oxygen carrying capacity (Pelster and Decker, 2004). Also, long-term studies have recorded lower growth, dietary intake, feed conversion efficiency and condition factor in fish reared in high CO_2 environments (Cecchini et al., 2001; Fivelstad et al., 1998; Hosfeld et al., 2008; Moran and Støttrup, 2011; Smart, 1981). In the present study the whole body oxygen consumption of individual Atlantic cod was monitored over 72 h following a standardized meal to assess how the SDA varied at two dissolved CO_2 levels. The profile of oxygen consumption over time was used to evaluate whether the pre-feeding metabolic rate, SDA cost and SDA duration differed between the CO_2 treatments. The pre-feeding metabolic rate was used as a measure of the non-digestive maintenance energy costs, and may be predicted to vary between CO_2 treatments if there is a significant cost to the acid-based regulation required to acclimate to the acidification of body tissues. The reduction in blood oxygen carrying capacity associated with hypercapnia (Pelster and Decker, 2004) may affect the ability of Atlantic cod to supply oxygen for digestion, thereby lengthening the digestive process and reducing the rate of gut clearance and digestive efficiency. A recent study by Methling et al. (2013) on European eel (*Anguilla anguilla*) reported such an effect under aquaculture-like CO_2 conditions. In addition to measuring minimal and digestive metabolic rate, we also compared the maximum metabolic rate following an intense period of exercise to evaluate whether hypercapnia had an effect on the metabolic scope of Atlantic cod.

In the present study we exposed two groups of Atlantic cod to dissolved CO_2 levels of approximately 800 μatm (water pH of 7.7) and 9200 μatm (pH of 6.6). These levels are higher than present atmospheric conditions (circa 400 μatm) (Mauna Loa Observatory, United States National Oceanic and Atmospheric Administration) but would be considered relatively low in land-based aquaculture farms (Timmons et al., 2010). The comparatively mild increases in pCO_2 on the scale expected to occur in the atmosphere over the next century have been reported to have a range of negative or neutral effects on fish physiology (reviewed by Heuer and Grosell (2014)), or there might even be an enhancement of oxygen offload into fish tissue (Randall et al., 2014; Rummer et al., 2013a). However, the upper CO_2 test level in the current study was chosen on the basis that it would likely generate insights into the pathological effects of hypercapnia on digestive physiology at substantially higher CO_2 concentrations. A 12-month study by Melzner et al. (2009) of adult Atlantic cod found no effect of CO_2 on growth at 5800 μatm, therefore, we decided to set our upper CO_2 test level above this concentration. Previous research has reported that juvenile Atlantic cod reared at 8300 μatm CO_2 exhibit decreased size-specific growth rate and condition factor (Moran and Støttrup, 2011) and a markedly increased rate of cataract formation (Moran et al., 2012) compared to a lower CO_2 level. The toxicological effect threshold reported by Moran and Støttrup (2011) is lower than the CO_2 effect threshold reported in growth studies for other marine fish species (Fivelstad et al., 1998; Ishimatsu et al., 2008; Petochi et al., 2011), therefore, while 9200 μatm CO_2 is not an ecologically relevant level, it is meaningful in terms of fish welfare and growth efficiency in aquaculture systems, and is a relatively low concentration in the context of reported threshold effect concentrations.

2. Materials and methods

2.1. Fish and experimental setup

Atlantic cod were caught by trawling in the northern part of the Øresund Sound, near Helsingør, Denmark, and transported to the Marine Biological Section, University of Copenhagen, Denmark. Fish were acclimated in 700 L holding tanks (10 °C, 31‰ salinity) for at least three weeks before experimentation and fed three times a week with herring (*Clupea harengus*) fillets.

We determined the effects of hypercapnia on metabolism and digestion by two different experiments. All experiments were carried out in accordance with directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. In Experiment 1 we tested the effects of hypercapnia on specific dynamic action (SDA) and cardiac performance, and in Experiment 2 the effect of hypercapnia on the maximum metabolic rate (MMR). The acclimation period, pCO_2 levels, and experimental setup were the same for the two experiments. Two respirometry setups were used. One was for pre-experimental acclimation and no data were collected from this unit, while the other was for physiological measurements and contained an oxygen and blood flow meter. The acclimation and respirometry system each consisted of two 75 L tanks, one containing the respirometer and one serving as a sump for aeration, water changes, temperature control and CO_2 control (Fig. 1). Water was continuously exchanged between the two tanks via pumping. An inline biofilter and UV disinfection unit (Tetra pond UV1000, Melle, Germany) maintained water quality, while temperature was kept at 10.0 ± 0.1 °C via a programmable digital indicator (pr5714, PR electronics 2704, Rønde, Denmark) and Hetotherm thermostat bath (Heto, Denmark). Water quality was additionally maintained via a gradual exchange of

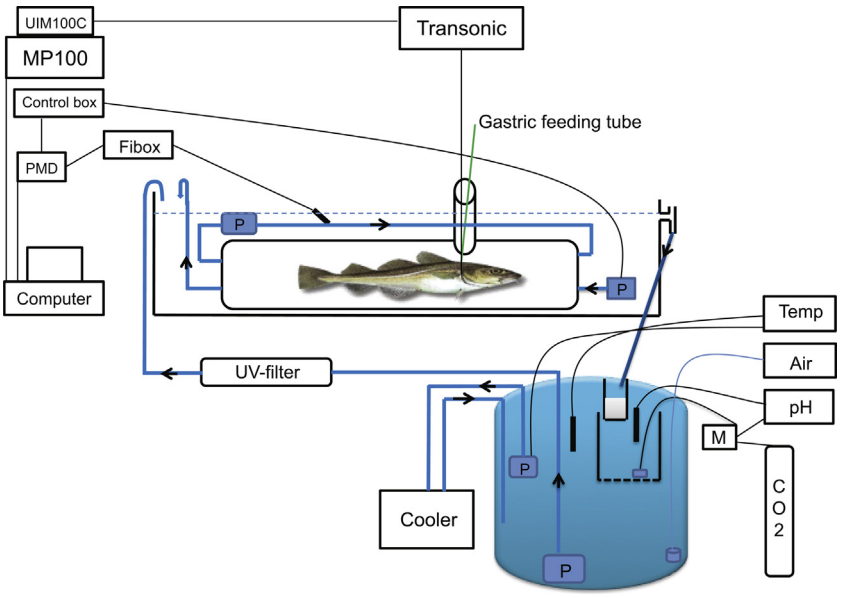

Fig. 1. Schematic of the experimental setup used to measure oxygen consumption and cardiac performance in Atlantic cod at 10 °C. Oxygen consumption was measured continuously in cycles of 10 min (5 min flush period, 1 min closed mixing period and 4 min closed measuring period). A constant pCO_2 level was maintained by a pH-meter controlling the injection of CO_2 into the pump sump. Food was administered through a gastric feeding tube. P = water pump. M = solenoid magnetic valve. See text for a detailed description.

water, equivalent to approximately 2% of total system volume per hour. This was done via a relay box (Unic XT recycler, Brodersen Control Systems, Denmark) controlling a pump that drew water from a clean water tank every hour.

Measurements of oxygen consumption rate were carried out in a 7.4 L cylindrical Plexiglas respirometer (internal diameter 12 cm, length 54 cm) with two tubes at each end for a flushing loop and recirculation loop (5 L min^{-1}, Eheim, Deizisau, Germany, to maintain water circulation during closed vessel respirometry). A 5 cm long vertical chimney allowed access for the flow probe cable and the gastric feeding tube. The length of the chimney minimized the passive diffusion of oxygen during closed respirometry. Oxygen tension (pO_2) was measured in the recirculation loop using a fiber optic oxygen sensor (PreSens, Regensburg, Germany) at a frequency of 1 Hz. The oxygen sensor was connected to a computer via a Fibox 3 (PreSens) and the mass of oxygen consumed over time ($\dot{M}O_2$) determined via computerized intermittent-flow respirometry, as described by Steffensen (1989). $\dot{M}O_2$ was measured continuously in loops of 10 min, which were composed of a 5 min flush period and 5 min closed respirometry. The first minute of closed respirometry was not analysed in order to allow for proper mixing, meaning that oxygen consumption rate was calculated from the remaining 4 min period. Autoresp software (Loligo Systems Aps, Tjele, Denmark) controlled the respirometer flushing times and calculated the $\dot{M}O_2$ online according to Steffensen et al. (1984). Corrections of background respiration (i.e. microbial respiration) followed Capossela et al. (2012).

2.2. Carbon dioxide control

The lowest pCO_2 level tested represented the steady state pCO_2 in the aquaria systems without deliberate CO_2 ingassing. The CO_2 was elevated above atmospheric concentrations due to respired CO_2, and corresponded to a level of 800 μatm. The pCO_2 in the acclimation and respirometry systems were monitored and controlled by pH probes (acting as a proxy for pCO_2) coupled to a gaseous CO_2 injector. pH_{NBS} was measured in the pump sump by WTW pH 340i meters and SenTix HWS pH electrodes (Wissenschaftlich-Technicsche Werkstätten GmbH, Weilheim, Germany) calibrated in NBS buffers. The correlation between pH and pCO_2 for the water used in the experiment were determined by measurement of pH at five pCO_2 levels produced by a gas-mixing pump (Wösthoff Digamix 5, H Wösthoff Messtechnik GmbH, Bochum, Germany). The mechanical gas mixing pump produced accurate gas mixes (checked using LiCor Li820 CO2 gas analyser, LiCor Inc., Nebraska, USA) and reduced the reliance of the study on pH measurements alone to derive pCO_2 test levels. This is important as it is difficult to measure seawater pH accurately and precisely (to better than 0.1 pH units) with glass pH electrodes (Illingworth, 1981). The five pCO_2 levels used to derive a pH/CO_2 relationship for the test water and pH meter ranged between 2500 μatm and 12,600 μatm, and gave a pH/CO_2 relationship of: ($r^2 = 1.00$, $N = 5$)

$$\mathrm{pH} = 11.833 pCO_2{}^{-0.064} \quad (1)$$

A pH_{NBS} of 6.6 and 7.7 corresponded to a pCO_2 of 9200 and 800 μatm, respectively (the upper and lower test levels used in experimentation). The pCO_2 test levels were cross checked during experimentation with an OxyGuard CO_2 meter (CO_2 5145, OxyGuard International A/S, Birkerød, Denmark) according to the recommended operating practices outlined by Moran et al. (2010), and the meter values agreed with the target CO_2 treatment levels. The pH was calculated using knowledge of pCO_2 and total alkalinity (1.709 mEq kg $water^{-1}$, measured via titration) and was reasonably close to the measured value (within 0.08–0.14 pH units, Table 1). The pH-meter was connected via a galvanic isolation

Table 1
Comparison of carbonate chemistry parameters (10 °C, 31‰ salinity).

	800 μatm CO_2	9200 μatm CO_2	
pH_{NBS}	7.7	6.6	pH meter
CO_2 (mg/L)	1–2	19	OxyGuard CO_2 meter
Total alkalinity (mmol kg S W^{-1})	1.709	1.709	Titration
Total CO_2 (mmol kg S W^{-1})	1.702	2.117	Computed from TA and pCO_2 [a]
pH_{NBS}	7.78	6.74	Computed from TA and pCO_2 [a]

[a] Computed using CO_2calc 1.2.9 (Robbins et al., 2010).

amplifier (PR electronics A/S, Rønde, Denmark) to a universal indicator/controller instrument (Preview 5511, PR electronics), controlling the CO_2 injection to the pump sump by opening and closing of a solenoid magnetic valve (Bürkert-contromatic A/S, Herlev, Denmark), according to the pH reading. The measured and calculated carbonate chemistry parameters are given in Table 1.

2.3. Experiment 1: effects of hypercapnia on specific dynamic action

Fish were fasted for three days prior to experimentation. Following anaesthetization in a 20 mM Benzocain bath individuals were measured for total weight, and transferred to the surgical table. The fish was placed on its side on a wetted sponge and aerated saline water containing 10 mM Benzocain continuously irrigated the gills. The ventral aorta (Va) was exposed via a small incision (1–2 cm) ventral to the opercula cavity, and connective tissue was carefully removed without disrupting the pericardium. Next, a 2.5 mm ultrasonic transit-time flow probe (model 2.5S, Transonic Systems, Ithaca, USA) was carefully mounted around the Va. The incision was then closed with small sutures (USP 3/0, Kruuse, Denmark), and the flow probe and cable fixed by five skin sutures. To ensure a known bolus of food was ingested and processed, a gastric feeding tube, (60 cm, 2.7 mm outer diameter, CH8, Unomedical A/S, Conva-Tec, Denmark) was placed into the stomach via the oesophagus and exited through the opercular opening. The flexible gastric feeding tube was fixed to the operculum with three sutures and two sutures to the skin next to the dorsal fin. The feeding tube did impair the movement of the operculum but previous research in our laboratory has shown this technique does not have a measurable effect on SMR compared to voluntary feeding (Jordan and Steffensen, 2007; Schurmann and Steffensen, 1997). After surgery, fish were placed in a respirometry chamber within the recovery setup and left 3–4 days to recover and acclimate to the test CO_2 level. After the acclimatization period, the chamber containing the fish was transferred to the measurement setup and the oxygen optode and blood flow meter connected to the closed respirometry loop and blood flow probe, respectively. Following a 1–2 h settlement period the fish were almost always still in the respirometry chamber facing into the low velocity water current (circa 1 cm s^{-1}). The blood flow probe was coupled to a flow meter (model T206, Transonic Systems, New York, USA) interfaced with a MP100 data acquisition system (Biopac Systems Inc., California, USA) and AcqKnowledge 3.8.1 software (Biopac Systems Inc.) recording continuously with a frequency of 200 Hz. Fish were left for 24 h to settle while cardiac performance and $\dot{M}O_2$ were continuously monitored to provide baseline data prior to feeding. Any fish not observed to have a low steady metabolic rate and cardiac output were kept for an additional 24 h before feeding. Following this settling period, fish were administered a meal of blended herring fillet (7.8 kJ g^{-1}) through the gastric feeding tube, equivalent to 5% wet body mass. After feeding, fish were left undisturbed for 72 h while cardiac performance and $\dot{M}O_2$ were continuously measuring. Five fish were excluded from the dataset due to vomiting during feeding resulting in an effective sample size of seven fish for the low CO_2 level and six fish for the high CO_2 level.

2.4. Experiment 2: effects of hypercapnia on maximum metabolic rate

To measure the effect of hypercapnia on maximum metabolic rate (MMR) seven Atlantic cod (674.1 ± 38.5 g) were acclimatized to the low pCO_2 treatment and eight (576.8 ± 60.9 g) to the high treatment. Following a four-day acclimation period the fish were removed from the acclimation system and exercised to exhaustion by continuous chasing for 5 min (i.e. fish no longer attempted to evade touch and remained stationary) in a circular 50 L tank along with frequent short-term periods of air exposure. A chasing procedure was chosen rather than a critical swimming test as the chasing procedure has been shown to be superior in eliciting maximal metabolic rate in the study species (Reidy et al., 1995). Immediately after chasing, the fish was transferred to a respirometry chamber in the respirometer setup and after a 1 min mixing period the $\dot{M}O_2$ measurements were started. $\dot{M}O_2$ was initially measured in 2 min periods (without flushing) to generate high frequency $\dot{M}O_2$ measurements. When water pO_2 had declined to 70% (after 3–4 measurements) the respirometer was flushed and $\dot{M}O_2$ was measured in 10 min cycles as described in the SDA experiment. Measurements were continued for 1 h, and the maximum rate of oxygen consumption recorded was used as the MMR for an individual.

2.5. Data handling and analysis

To control for the effects of body mass on metabolic rate all $\dot{M}O_2$ measurements were scaled to an average individual mass of 650 g, using the following equation:

$$\dot{M}O_2(650) = \dot{M}O_2 \times \left(\frac{M}{650}\right)^{(1-A)} \quad (2)$$

where $\dot{M}O_2(650)$ is the predicted oxygen consumption (mg O_2 kg^{-1} h^{-1}) of an individual scaled to a weight of 650 g, $\dot{M}O_2$ is the measured oxygen consumption of a fish with body mass = M and A = the mass exponent describing the relationship between metabolic rate and body mass. A mass exponent of 0.8 was chosen because it describes the allometric relationship between standard metabolic rate (SMR), an approximation of maintenance metabolism (Fry and Hart, 1948) and the mass of 69 species of teleost fish (Clarke and Johnston, 1999).

The respirometry data for each individual was fitted with functions describing the pre- and post-feeding pattern of oxygen consumption, which allowed for the derivation of attributes such as peak oxygen consumption and the SDA time course (Fig. 2A). The functions were generated in R (Team, 2012) using the quantreg package (Koenker, 2005, 2011). A nonparametric quantile regression (function rqss) was fitted to both data sets using the same τ value (set to a quantile of 0.25), which ensured that the function fit was comparable for pre- and post-feeding relative to the spread of data points (Dorcas et al., 2004; Dupont-Prinet et al., 2010, 2013). For many individuals the $\dot{M}O_2$ had not returned to pre-feeding levels even after 72 h of recording, therefore, a number of descriptive statistics such as SDA duration and SDA cost were calculated at midpoints of the SDA process. Ten variables were calculated to describe the respirometry data for an individual:

(1) SMR (calculated from the 5 h prior to feeding);
(2) MMR (the maximum $\dot{M}O_2$ recorded after exhaustive exercise);
(3) MS (aerobic scope for activity, MMR–SMR);
(4) % decrease of MS due to hypercapnia (MS_{9200}/MS_{800});
(5) SDA_{scope} (the maximum $\dot{M}O_2$ value recorded during the SDA process – SMR);
(6) % decrease in MS due to oxygen demand of SDA (SDA_{scope}/MS);
(7) t_{peak} (the time from feeding to SDA_{scope});
(8) t_{50} (a measure of time taken for the SDA to progress, time at which $\dot{M}O_2 = SMR + 50\% SDA_{scope}$;
(9) SDA_{50} (the oxygen demand required for SDA and not including SMR cost, measured as area under curve from feeding to t_{50}, mg O_2 kg^{-1});
(10) $SDA_{coefficient}$ (%, the energetic cost of SDA relative to energetic value of meal, calculated as the SDA_{50} multiplied by the oxycalorific coefficient 14.06 kJ g O_2^{-1} (Gnaiger, 1983), then

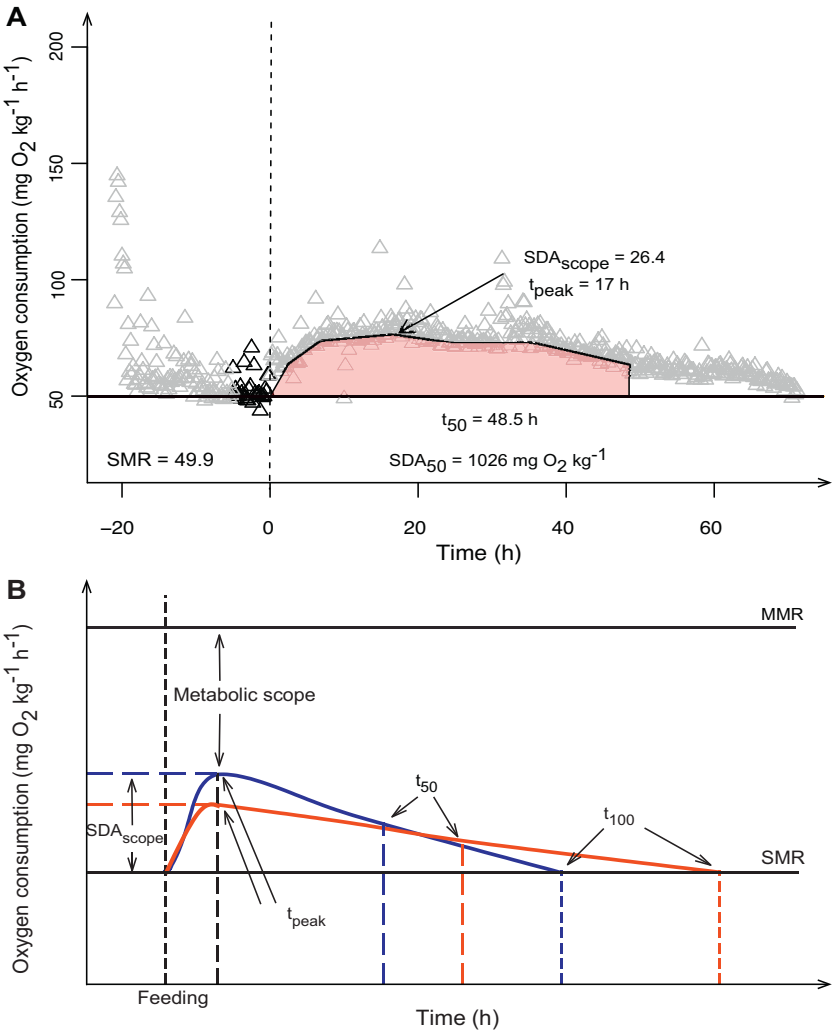

Fig. 2. (A) An example of the data analysis output file for a 721 g fish and (B) an illustration of the terms used in the present study. (A) The example illustrates the algorithm fit that allowed for derivation of standard metabolic rate (SMR) and specific dynamic action (SDA) variables. The small dashed vertical line at time 0 h indicates feeding. SMR was determined from mean $\dot{M}O_2$ in the 5 h pre-feeding (black data points and black vertical dotted line). The SDA curve was determined by $\dot{M}O_2$ measured post-feeding (grey data points). (B) Solid black lines represent SMR (pre-feeding $\dot{M}O_2$) and MMR; and the black small dashed vertical line feeding time (0 h). The solid blue (normocapnia) and red (hypercapnia) lines represent possible SDA scenarios and how respective SDA variables would change. SDA_{scope} (the maximum $\dot{M}O_2$ value recorded during the SDA process – SMR); t_{peak} (the time from feeding to SDA_{scope}); t_{50} (the time at which $\dot{M}O_2$ = SMR + 50% SDA_{scope}); t_{100} (the time taken for the $\dot{M}O_2$ to return to pre-feeding level); SDA_{50} (the oxygen demand required for SDA and not including SMR cost, measured as area under curve from feeding to t_{50}, mg O_2 kg^{-1}). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

divided by the energy content of the meal (estimated using meal mass and 7.8 kJ g^{-1}).

The aforementioned variables are summarized in graphical form in Fig. 2B. In addition, two measures of energetic digestive efficiency were calculated as follows:

(11) DE_{50} (digestion efficiency, %, the amount of ingested energy left when subtracting the energy used for SMR and SDA, measured from feeding to t_{50});

(12) DE_{100} (%, digestion efficiency calculated from feeding to t_{100}). In the DE_{100} calculation, SMR + SDA was calculated as the sum of the mean $\dot{M}O_2$ per hour values measured until 72 h and the predicted $\dot{M}O_2$ values from 72 h until SMR (Fig. 3)).

Differences in the SDA profile of each CO_2 treatment were visualized by plotting mean $\dot{M}O_2$ in hourly time bins. The time taken to return to pre-feeding $\dot{M}O_2$ was extrapolated from 30 h post-feeding, a time point after which the rate of decrease in $\dot{M}O_2$ was highly linear (r^2 = 0.89 and 0.75 for 9200 and 800 μatm CO_2, respectively). The pre-feeding value was taken as the mean $\dot{M}O_2$ for the 5 h prior to feeding. These extrapolations were used to estimate t_{100} (SDA duration, time taken for the $\dot{M}O_2$ to return to pre-feeding levels).

2.6. Cardiac performance

Cardiac output (Q), heart rate (f_H), and stroke volume (Vs) for a given time point were calculated as the mean of three 1 min measurement samples. The aforementioned variables were measured at −2, 6, 10, 17, 24, 30, 36, 48, 62, and 72 h post-feeding. To ensure that we did not sample cardiac performance during spontaneous activity, the $\dot{M}O_2$ during the cardiac sampling had to be within 10% of the $\dot{M}O_2$ value determined by quantile regression analysis. If $\dot{M}O_2$ deviated more than 10%, a new cardiac sampling point was chosen as close to the target sampling time as possible. Cardiac output (Q) (ml min^{-1} kg^{-1}) was calculated as

$$Q = \left(\frac{Q_m}{M}\right) \quad (3)$$

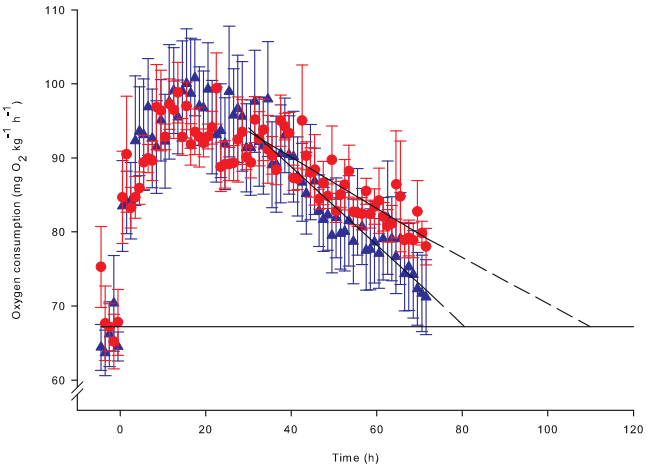

Fig. 3. Oxygen consumption over time for Atlantic cod fed a meal equivalent to 5% body weight and acclimated to 800 (blue triangles, $N = 7$) and 9200 (red circles, $N = 6$) μatm CO_2. Data is presented as mean ± s.e.m. The horizontal line represents the average pre-feeding $\dot{M}O_2$ for both treatments and the dashed lines is least square regressions calculated from time 30–72 h and extrapolated to pre-feeding $\dot{M}O_2$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

where Q_m (ml min^{-1}) is the measured cardiac and M is fish mass (kg). The heart rate (f_H) was calculated as the number of systolic peaks min^{-1} and the stroke volume (Vs) as Q/f_H.

2.7. Statistical analysis

The SMR, SDA, MMR and cardiac performance (Q, f_H, and Vs) variables were calculated per individual along with treatment means ± s.e.m. Differences between treatment means were investigated via t-tests (Sigmaplot 11, Systat Software, Inc.), while differences in pre- and post-feeding values of Q, f_H, and Vs were evaluated using one-way repeated measures ANOVA tests. A significance level of $p < 0.05$ was used for all tests.

3. Results

3.1. Oxygen consumption

There was no significant difference in the standard metabolic rate (SMR) of Atlantic cod exposed to 800 versus 9200 μatm CO_2 (Table 2), suggesting that hypercapnia did not affect metabolic maintenance costs at these concentrations. In contrast, the maximum metabolic rate (MMR) was significantly lower (14%, t-test, $p = 0.03$) for fish exposed to the elevated CO_2 level. The lower MMR measured in hypercapnia resulted in a reduction of the metabolic scope for activity (MS, MMR − SMR) from 2.9 to 2.4 times the SMR, corresponding to an 18% decrease (Table 2).

Mean post-prandial oxygen consumption rate ($\dot{M}O_2$) increased for the first 14 h during the SDA process to a maxima, and decreased in a linear fashion from 30 to 72 h over the subsequent 42 h (Fig. 3). Despite recording oxygen consumption for 72 h post ingestion, the $\dot{M}O_2$ had not returned to pre-feeding levels, therefore, an extrapolation was made to the predicted SDA end time (t_{100}) for both treatments (Fig. 3). SDA_{scope} was similar between the CO_2 treatments (t-test, $p = 0.31$, Table 2). The time to peak post-prandial $\dot{M}O_2$ (t_{peak}) was variable among individuals (range 5–34 h), however, mean t_{peak} was similar for both treatments (between 13 and 14 h post-feeding, Table 2). The t_{50} was significantly longer for Atlantic cod exposed to 9200 μatm CO_2 compared to 800 μatm (66 versus 54 h, t-test $p = 0.04$, Table 2). In contrast, the SDA_{50} or the $SDA_{coefficient}$ did not differ significantly between treatments (Table 2), meaning that the main effect of elevated CO_2 on SDA was to extend the time taken to carry out the SDA process for a given meal size. Neither $SDA_{coefficient}$ nor SDA_{50} account for the effect of a lengthened SDA duration or maintenance costs on the energetic value derived from a meal. When maintenance and SDA energy costs were subtracted to derive the digestive efficiency at t_{50}, 73% of consumed energy was available for assimilation under 800 μatm CO_2, while the extended SDA duration observed under 9200 μatm CO_2 decreased DE_{50} to 67% (Table 2). Extrapolation of the $\dot{M}O_2$ to pre-feeding levels did not substantially alter the interpretation of the effect of CO_2 on SDA. The projected total duration of the SDA process (t_{100}) was markedly longer under hypercapnia

Table 2
Metabolic rate and specific dynamic action of Atlantic cod at 10 °C and acclimated to 800 and 9200 μatm pCO_2.

	800 μatm CO_2	9200 μatm CO_2	t-Test[b]
Maximum metabolic rate			
N	7	8	
Mass (g)	674.1 ± 38.5	576.8 ± 60.9	
MMR[a] (mg O_2 kg^{-1} h^{-1})	176.2 ± 5.71	150.7 ± 7.5	S
Specific dynamic action			
n	7	6	
Mass (g)	648.0 ± 53.5	617.3 ± 39.8	NS
SMR[a] (pre-feeding $\dot{M}O_2$, mg O_2 kg^{-1} h^{-1})	60.7 ± 3.1	63.6 ± 3.2	NS
MS (metabolic scope for activity)	2.9	2.4	
Decrease of MS due to hypercapnia		18.4%	
SDA_{scope} [a] (mg O_2 kg^{-1} h^{-1})	32.6 ± 3.6	27.2 ± 3.0	NS
Decrease of MS due to SDA	28.7 ± 3.7%	30.8 ± 2.7%	NS
t_{peak} (h)	13.7 (5–21)	13.3 (7–34)	NS
SDA duration (t_{50}) (h)	53.6 (45–70)	66.0 (47–76)	S
SDA duration[c] (t_{100}) (h)	80.5	109.9	
SDA_{50} (mg O_2 kg^{-1})	1355.0 ± 157.5	1358.1 ± 185.8	NS
$SDA_{coefficient}$ (%)	8.0 ± 1.2	8.4 ± 1.4	NS
Digestion efficiency t_{50} (DE_{50}) (%)	73 ± 3.2	67 ± 2.3	NS
Digestion efficiency t_{100} [c] (DE_{100}) (%)	61	48	

Values are means ± s.e.m. t_{peak} and t_{50} are shown as mean and range.
[a] $\dot{M}O_2$ values were scaled to an equivalent body mass of 650 g.
[b] S = statistically significant difference ($p < 0.05$), NS = no significant difference ($p \geq 0.05$).
[c] Extrapolated from the hourly mean $\dot{M}O_2$ profile for each treatment given in Fig. 3.

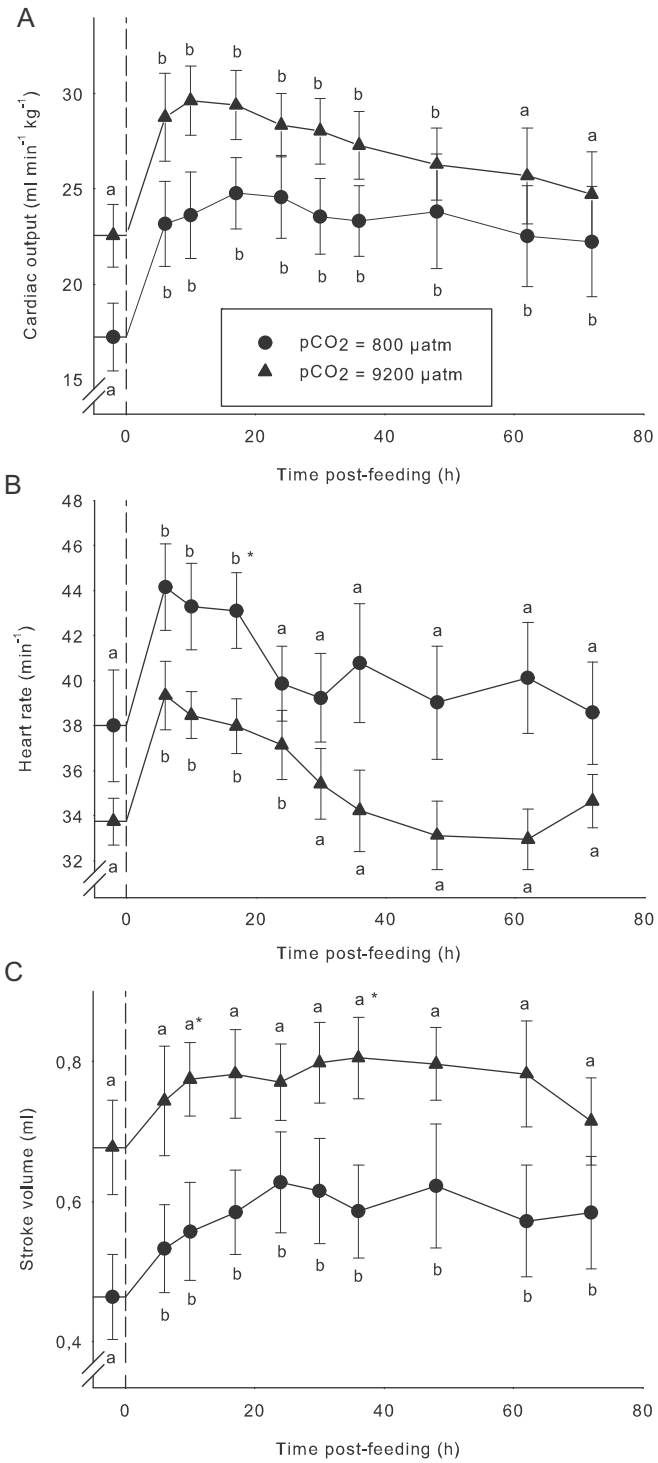

Fig. 4. Cardiac performance over time of Atlantic cod fed a meal equivalent to 5% body weight and acclimated to 800 (circles, $N = 6$) and 9200 (triangles, $N = 5$) µatm CO_2. (A) Cardiac output (Q); (B) heart rate (fH); and (C) stroke volume (Vs). Feeding was conducted at 0 h. Data points represent mean ± s.e.m. Letters indicate statistically significant differences from the pre-feeding value, while * indicates significant differences between CO_2 treatments for each time point.

(110 h versus 81 h), and the longer processing time reduced DE_{100} from 61% to 48% at 800 and 9200 µatm CO_2, respectively (Table 2).

3.2. Cardiac performance

The administration of a meal leads to a significant increase in cardiac output (Q) for both CO_2 treatments and peaked 10–17 h post-feeding (Fig. 4A). The peak increase in Q corresponded to a 44% and 31% increase above pre-feeding levels at 800 and 9200 µatm CO_2, respectively. At the higher test CO_2 level Q returned to pre-feeding levels 60 h post-feeding, whereas fish exposed to 800 µatm maintained significantly elevated cardiac output during the entire SDA process (72 h, Fig. 4A). At all-time points the mean cardiac output at 9200 µatm CO_2 was approximately 30% above that recorded at 800 µatm, however, there was considerable inter-individual variation and all comparisons between treatment means were not significantly different at the $p = 0.05$ level (Fig. 4A). Likewise, mean heart rate (f_H) was consistently lower and stroke volume (Vs) consistently higher at 9200 µatm CO_2, however, the variation was such that the CO_2 treatments were not statistically different at the $p = 0.05$ level (Fig. 4B and C). Stroke volume tended to remain elevated throughout the entire SDA period (Fig. 4C), whereas Vs returned to pre-feeding levels after approximately 24 h (Fig. 4B).

4. Discussion

This study investigated the effect of two pCO_2 levels (800 and 9200 µatm) on the energetics and digestive physiology of Atlantic cod. This section will first discuss the findings regarding minimal and maximal metabolism before turning to the effect of hypercapnia on SDA. Increases in the acid–base load and gill ventilation rates required to cope with exposure to environmental hypercapnia are hypothesized to incur to additional maintenance energy costs, which may have consequences for fish growth, reproduction and survival (Ishimatsu et al., 2008). In the present study we did not observe a significant difference in SMR between treatments despite an order in magnitude increase in water pCO_2, a finding which concurs with a longer term study by Melzner et al. (2009) for the same species and similar test concentration range. The length of time of hypercapnic exposure in the present study (four days) was likely sufficient for acid–base adjustment of blood chemistry (Claiborne and Heisler, 1984; Hayashi et al., 2004; Larsen et al., 1997; Lee et al., 2003), however, it is unclear whether longer term exposure may have resulted in a different pattern in SDA oxygen consumption profile. The agreement in SMR between the current study and the 12 month study by Melzner et al. (2009) suggests that the fish in the current study were not experiencing acute physiological stress. The general pattern that emerges from research to date is that for marine fish environmental hypercapnia does not impose a measurable or consistent increase in maintenance energy costs across a wide range of CO_2 test levels (reviewed in Heuer and Grosell (2014)).

While the present study found no effect of pCO_2 on SMR, the maximum metabolic rate of Atlantic cod exposed to the higher test level of 9200 µatm CO_2 was significantly reduced (by 14%). This finding does not concur with the study of Melzner et al. (2009), who reported no difference in the metabolic rate versus swimming speed at two pCO_2 levels. There are a number of possible explanations for the discrepancy between studies. Melzner et al. (2009) used a longer acclimation period (4 and 12 months) than in the present study (four days), which may infer that this species requires longer than 4 days to fully acclimate. While small changes in the ion regulation capacity of tissues may occur for weeks after hypercapnia exposure (Deigweiher et al., 2008), the acid–base balance is normally reestablished within 24 h and there is an assumption that aerobic capacity is restored (Claiborne and Heisler, 1984; Hayashi et al., 2004; Larsen et al., 1997; Lee et al., 2003). Another explanation could be that CO_2 has an effect threshold on Atlantic cod metabolic scope between 5800 and 9200 µatm (the upper test concentrations of the Melzner et al. (2009) study and our study, respectively). In addition, the two studies differed in the methods used to derive maximum aerobic capacity. In our study fish were exercised to

exhaustion over a short time period (5 min) via chasing and emersion, whereas Melzner et al. (2009) used a critical swimming speed test (U_{crit}). Reidy et al. (1995) compared the two methods and concluded that the chasing method was superior in terms of eliciting maximum oxygen demand in Atlantic cod. The finding that hypercapnia leads to a reduced maximum aerobic capacity and metabolic scope in Atlantic cod agrees with other fish physiology research. Methling et al. (2013) reported that elevated CO_2 levels decreased MMR in the European eel, and Munday et al. (2009) reported consistently decreased metabolic scope in two marine species exposed to environmental hypercapnia. In contrast, studies by Rummer et al. (2013b) show that exposure to predicted year 2100 pCO_2 levels can also lead to increased MMR. Methodological differences in testing has been flagged as a significant issue for ocean acidification studies of fish physiology, and it is worth noting that the present study has used the recommended best practice of intermittent flume respirometry and a chase to exhaustion to establish both SMR and MMR (Heuer and Grosell, 2014; Reidy et al., 1995).

Post-prandial $\dot{M}O_2$ increased similarly for Atlantic cod acclimated to 800 and 9200 μatm CO_2, and peaked at mean of 13–14 h post-feeding (t_{peak}) for both treatments, which is comparable with previous determinations of Atlantic cod SDA (Jordan and Steffensen, 2007; Lyndon et al., 1992). The post-prandial $\dot{M}O_2$ increase (SDA_{scope}) observed in the current study (43–54% above SMR) was less than half the increase reported by Lyndon et al. (1992) and Jordan and Steffensen (2007), but agrees with a value of 64% for a separate study performed in our laboratory using nearly identical diets and analytical approaches (Tirsgaard et al., 2014). Differences between studies in SDA_{scope} for Atlantic cod are therefore likely due to differences in diet digestibility and analytical methods used to determine SDA_{scope}. Exposure of Atlantic cod to the elevated CO_2 treatment caused a prolongation of digestion, with the SDA duration (t_{50}), 23% longer in duration at 9200 μatm CO_2 compared to 800 μatm CO_2. When an extrapolation was made to the time at which digestion was completed (t_{100}), the difference in duration between the treatments became even more pronounced (37%). Other factors such as SDA_{scope}, SDA_{50}, SDA effect on MS (SDA_{scope}/MS) and $SDA_{coefficient}$ did not differ significantly between treatments, supporting the conclusion that the only measurable effect of environmental hypercapnia on SDA is a prolongation of digestion and assimilation processes. Prolonged digestion is likely to result in lower feed intake (due to slower gut clearance) and more energy being used for maintaining basal body functions compared to the energy intake. When accounting for the higher SMR costs incurred by prolonged SDA under hypercapnia, it was calculated that there was 13.2% less of the total meal energy available for growth and activity. This could explain the CO_2 dose effect on growth and condition factor reported by Moran and Støttrup (2011) for juvenile Atlantic cod, and other marine species (Fivelstad et al., 2007; Foss et al., 2003). To the best of our knowledge the only other study of the effect of hypercapnia on SDA in fish was by Methling et al. (2013) on European eel, who likewise reported that elevated pCO_2 and decreased water pH considerably prolonged the SDA process.

The extended SDA process due to hypercapnia might be due to limits on oxygen availability for digestion, as acute respiratory acidosis is known to reduce the blood oxygen carrying capacity by reducing the oxygen binding affinity (Pelster and Decker, 2004). If oxygen delivery capacity to the gut of Atlantic cod was reduced due to environmental hypercapnia, one might expect to observe elevated cardiac output to compensate for the diminished oxygen delivery capacity during the SDA process. There were some indications of this pattern in the current study. As expected, Q was significantly elevated following the administration of a meal. The mean Q of fish at 9200 μatm CO_2 was consistently higher than at 800 μatm, both pre- and post-feeding, suggesting that cardiac performance was generally up regulated due to hypercapnia, however, the inter-individual variation was such that there was no statistically significant difference between treatment means. The lack of statistical significance is perhaps to be expected given that most studies of marine fish report that plasma and intracellular pH are fully, or almost fully, adjusted to normocapnia level within 24 h of exposure to hypercapnia (Claiborne and Heisler, 1984; Hayashi et al., 2004; Larsen et al., 1997; Lee et al., 2003). The loss of maximum respiratory capacity and a consistently elevated Q at 9200 μatm CO_2 suggests that Atlantic cod do experience some loss of oxygen delivery capacity. A loss of maximum respiratory capacity and metabolic scope is hypothesized to have consequences on the burst swimming capacity and specific dynamic action of a fish like Atlantic cod (Clark et al., 2013), but only the latter factor is likely to be relevant in an aquaculture setting, where predation and evasion efficiency are not particularly important. Gilthead seabream (*Sparus aurata*) acclimated from atmospheric CO_2 levels to 5000 μatm CO_2 exhibit an increased reliance on anaerobic metabolism under hypercapnia, presumably due to impaired oxygen delivery to tissues (Michaelidis et al., 2006). Studies at low CO_2 levels, however, indicate that low levels of CO_2 and mild acidosis opposite high levels, might actually increase oxygen offload in the tissue (Rummer et al., 2013a) and increase metabolic scope (Rummer et al., 2013b). Whether a loss of respiratory capacity is the reason underlying the prolongated SDA process is not clear from the results of this study, however, a study by Jordan and Steffensen (2007) on the effects of hypoxia on SDA in Atlantic cod demonstrated that low oxygen availability prolongs the SDA process.

5. Conclusions

The present study provides new insights into the effect of environmental hypercapnia on the metabolic scope and digestive physiology of Atlantic cod. Fish acclimated to 800 and 9200 μatm CO_2 showed no difference in maintenance metabolic rates, which concurs with previous research for this species and other fish species. At 9200 μatm CO_2 Atlantic cod had a significantly diminished (14%) maximum aerobic capacity. Hypercapnia prolonged the SDA by 23%, but did not increase the total oxygen demand for the digestion and assimilation of a meal. The longer SDA process time may offer an explanation for the observation of lower feed intake, growth and condition factor in long-term hypercapnia studies. The mechanism by which CO_2 prolongs SDA may be due to a reduced oxygen delivery capability under hypercapnia, although our results could not definitively demonstrate this effect.

Conflict of interest statement

There are no competing interests in this paper.

Author contributions

Tirsgaard, B. contributed to the conception and design of the experiments, performed the experiments and data analysis and wrote the main part of the manuscript.

Moran, D. contributed to the conception and design of the experiments, and significantly to the writing of the manuscript.

Steffensen, J.F. contributed to the conception and design of the experiments, and edited the manuscript.

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