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An examination of the metabolic processes underpinning critical swimming in Atlantic cod (*Gadus morhua* L.) using *in vivo* ^{31}P-NMR spectroscopy

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Summary

Traditionally, critical swimming speed has been defined as the speed when a fish can no longer propel itself forward, and is exhausted. To gain a better understanding of the metabolic processes at work during a U_{crit} swim test, and that lead to fatigue, we developed a method using *in vivo* ^{31}P-NMR spectroscopy in combination with a Brett-type swim tunnel. Our data showed that a metabolic transition point is reached when the fish change from using steady state aerobic metabolism to non-steady state anaerobic metabolism, as indicated by a significant increase in inorganic phosphate levels from 0.3±0.3 to 9.5±3.4 μmol g^{-1}, and a drop in intracellular pH from 7.48±0.03 to 6.81±0.05 in muscle. This coincides with the point when the fish change gait from subcarangiform swimming to kick-and-glide bursts. As the number of kicks increased, so too did the Pi concentration, and the pH_i dropped. Both changes were maximal at U_{crit}. A significant drop in Gibbs free energy change of ATP hydrolysis from –55.6±1.4 to –49.8±0.7 kJ mol^{-1} is argued to have been involved in fatigue. This confirms earlier findings that the traditional definition of U_{crit}, unlike other critical points that are typically marked by a transition from aerobic to anaerobic metabolism, is the point of complete exhaustion of both aerobic and anaerobic resources.

Key words: Atlantic cod, *Gadus morhua*, critical swimming speed, *in vivo* ^{31}P-NMR spectroscopy, high-energy phosphates, Gibb's free energy, intracellular pH.

Introduction

The critical swimming speed (U_{crit}) test was originally introduced in a study looking at the fitness of salmon (*Oncorhynchus* sp.) in relation to temperature (Brett, 1964). Brett defined the U_{crit} as the swimming velocity when the fish could no longer propel itself forward off the downstream grid due to exhaustion. The U_{crit} test provides an easy way of directly assessing fitness in fish, and by proxy, the sum of both the aerobic and anaerobic metabolic scopes. More specific tests, such as the burst swimming test and the endurance swimming test, have been developed to separately examine anaerobic and aerobic scope, respectively. Since its inception, however, many experiments have used the critical swimming speed test to assess the fitness of various fishes under different conditions, e.g. after feeding, during hypoxia, in polluted waters (for a complete review, see Hammer, 1995).

A great body of literature exists on the different swimming modes in fish (Schultz and Webb, 2002; Webb, 2002; Jayne and Lauder, 1994; Videler, 1993; Videler, 1981; Beamish, 1978; Brett, 1964) and the metabolic processes fuelling muscle contraction (Jones, 1982). It has been clearly demonstrated that red muscle is oxidative (Johnston, 1977) and produces the slow contractions during subcarangiform swimming (Jayne and Lauder, 1994), while white muscle is glycolytic and responsible for fast twitch contractions (Johnston, 1977), which produce tail kicks during burst swimming (Jayne and Lauder, 1994). A handful of studies have also found white muscle to be recruited during subcarangiform swimming (Rome et al., 1984; Jones, 1982; Greer-Walker and Pull, 1973). Yet to date, few studies have looked at the metabolic processes underpinning the continuum, i.e. from subcarangiform swimming through the gait transition to kick-and-glide bursts to exhaustion.

Although an increase in inorganic phosphate and an acidification of the intracellular milieu is known to be involved in muscular fatigue, the exact processes leading to muscular fatigue are ill-defined. Allen and Westerblad (Allen and Westerblad, 2001) contend that it stems from excess inorganic phosphate altering intracellular concentration of Ca^{2+} and/or the Ca^{2+} sensitivity of the myofilaments. Building on previous work (Hibberd et al., 1985), Debold et al. add that inorganic phosphate and H^+ also reduce the force generated by cross-bridge cycling (Debold et al., 2004). Others have argued that a drop in the Gibbs free energy of ATP hydrolysis ($dG/d\xi_{ATP}$) below a certain threshold results in fatigue (Hardewig et al., 1998).

Nevertheless, the anaerobic products of kick-and-glide bursts leave a 'fingerprint' on the fish's metabolic state, and a small number of studies have examined the relationship between swimming gait and metabolic processes. For example, it was concluded that elevated post-exercise oxygen consumption (EPOC) measured in salmon was a product of the anaerobiosis that fuels swimming shortly before U_{crit} (Lee et al., 2003). Furthermore, a clear relationship was demonstrated between

anaerobic markers (plasma and tissue lactate levels, tissue glycogen and EPOC), and kick-and-glide swimming in smallmouth bass (*Micropterus dolomieu* Lacepedé) (Peake and Farrell, 2004). Thus, Brett's traditional definition of U_{crit} is the speed that causes complete fatigue, i.e. when energy demand has exceeded both the aerobic and anaerobic supply (Brett, 1964).

Traditionally, metabolic critical points such as critical temperatures and critical oxygen tensions, are points marked by a transition from steady state aerobic metabolism to non-steady state anaerobic metabolism (Pörtner, 2002; Pörtner et al., 1985; Claireaux and Dutil, 1992). The traditional definition of U_{crit} does not correspond with these 'critical' parameters. This was initially noted (Brett, 1964) and further discussed (Beamish, 1978) in relation to swimming duration. Subsequent research has expanded upon this; for example, the metabolic cost of swimming in common brief squid (*Loliguncula brevis* Blainville) was investigated using the equivalent of a U_{crit} test, and it was found that anaerobiosis had commenced long before the squid could no longer swim (Finke et al., 1996). Pilot studies in our laboratory have found similar results using Atlantic cod (Pörtner et al., 2002). While Brett's U_{crit} test gives information about the swimming performance, little is known about the underlying metabolic processes, and in particular, what causes fatigue during exhaustive exercise.

The use of *in vivo* ^{31}P-NMR spectroscopy has proved to be an exceptional method for non-invasively monitoring the metabolic status of high-energy phosphates like PCr (phosphocreatine) and ATP, as well as the formation of metabolic products such as intracellular phosphate (Pi), and intracellular changes in pH in muscle (Gadian, 1982). Furthermore, it has been extensively used for mammalian muscle (Cozzone and Bendaham, 1994). A number of pioneering studies used ^{31}P-MRS in freshwater fishes (for reviews, see van den Thillart and van Waarde, 1996; van der Linden et al., 2004), and these methods have been further developed for marine fishes (Bock et al., 2001; Bock et al., 2002b; Sartoris et al., 2003). Early studies were carried out exclusively on restrained or resting animals. However, a technique has recently been developed for non-invasive studies of unrestrained fish under resting conditions (for reviews, see Bock et al., 2002b; Pörtner et al., 2004). Furthermore, recent preliminary trials within our laboratory have successfully investigated the use of *in vivo* ^{31}P-NMR in Atlantic cod during swimming (Bock et al., 2002a; Pörtner et al., 2002).

In this study we report on an online analysis of metabolic processes by *in vivo* ^{31}P-NMR spectroscopy during U_{crit} tests. The resulting data were used to examine three questions: (1) at what point in the U_{crit} test do cod go from using steady state aerobic metabolism to time-limited, non-steady state anaerobic metabolism to fuel swimming and how does this tie in with the different swimming gaits; (2) how does the traditional definition of U_{crit} compare with these underlying metabolic processes; (3) what are the metabolic processes that potentially cause fatigue in swimming Atlantic cod?

Materials and methods

Animals

First generation cultured North Eastern Arctic cod *Gadus morhua* L. (a generous gift from Dr M. Delghandi at the Institute of Marine Research, Tromsø, Norway) were air freighted over a 24 h period to the Alfred Wegener Institut (AWI) in September 2004. Upon arrival, fish were held at 4°C for a minimum of 2 weeks before being moved to the 10°C aquarium.

All fish were fed to satiation twice a week with a mixture of mussels (*Mytilus edulis* L.) and live common shrimp (*Crangon crangon* L.). Feeding was stopped a minimum of 5 days, maximum 7, before experimentation.

All experiments were carried out within German animal care legislature. Mortality during the entire holding period, i.e. from August 2004 to February 2006, was approximately 25%. No fish died during the course of the experiments; however, one died a week after experimentation.

Experimental setup

The swim tunnel consisted of three major parts (Fig. 1). A Perspex^{TM} pipe was fed through a 40 cm inner diameter Bruker Biospec 47/40 (Ettlingen, Germany) operating at 4.7 T. This pipe was attached to a circulation system. When 'closed,' a 256 l volume of seawater was hermetically sealed to measure oxygen consumption. When 'open,' a supplemental 444 l volume with a reservoir of constantly aerated seawater was used to flush the system. Switching between the two circulations was accomplished *via* three large taps. The gas-tightness of the closed system was checked periodically by bubbling the seawater with nitrogen gas overnight, then monitoring the seawater oxygen content over a 12 h period. On no occasion during this test did the oxygen content increase. A digital motion camera system connected to a computer was used for observing the fish. Both circulations were temperature controlled to within ±0.3°C. All swimming speeds were corrected for solid blocking effects using the procedure outlined by Nelson et al. (Nelson et al., 1994).

Surgical procedure

Fish were initially anesthetised with 0.05 mg l^{-1} MS222 in seawater for approximately 5 min, then weighed and the standard length (*SL*) was measured. Fish were then transferred to an operating table where the gills were irrigated with 0.02 mg l^{-1} MS222 in seawater. While anesthetised, a seawater-tight inductive ^{31}P-NMR coil (Bock et al., 2002a), measuring 3 cm×3 cm×0.2 cm, was sewn to the left side of the body, 15 cm distal to the end of the caudal fin with two sutures on the leading edge. The trailing edge was left free to move. This took less than 15 min. Fish were then placed in a tubular shaped cage (15 cm diameter × 60 cm length) within the chamber. Here, the receive coil was positioned to allow optimal signal transduction, while at the same time the fish was observed as it recovered from anaesthesia. Complete recovery usually took approximately 15 min. The cage was then pulled by means of a cord into the centre of the magnet and the fish allowed to recover for at least another 4 h.

Exercise protocol

As part of a larger project looking at the effects of thermal acclimation and acute temperature change on swimming performance (G.J.L., C.H.B. and H.-O.P., manuscript in preparation), all fish were swum twice, once at the acclimation temperature 10°C and once at the non-acclimation temperature of 4°C; however, data for this study

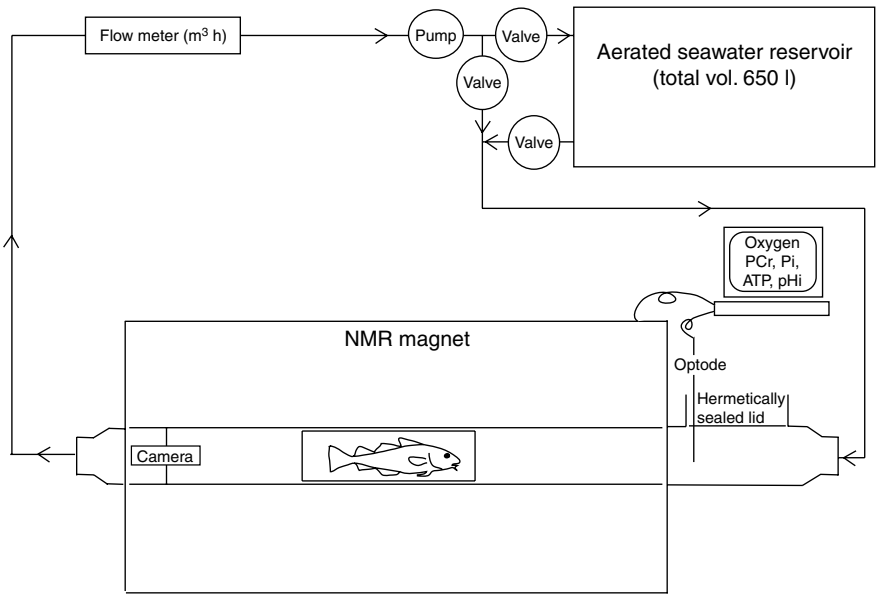

Fig. 1. The NMR/swim tunnel apparatus. Arrows indicate direction of water flow.

were only used from the 4°C swim. Fish were allowed to recover from surgery for a minimum of 4 h before they were cooled in a stepwise manner (2× 3°C steps per 2 h) to 4°C and left at minimal flow, i.e. 1 $m^3 h^{-1}$ (typically 0.15–0.19 *BL* s^{-1}) overnight. The routine metabolic rate was determined in fish swimming with minimal flow. The flow was then increased in approximately 1 $m^3 h^{-1}$ (approx. 0.05 *BL* s^{-1}) stepwise manner with each step lasting 30 min so that sufficient ^{31}P-NMR spectra could be recorded (see below). At sufficiently high water flows, fish would rest on the rear grid of the cage. When two of these successive pauses lasted more than 20 s, a short 9 V electric current was manually applied to a grid downstream. The traditional U_{crit} was defined as the time when the fish was no longer able to move from the grid (as per Nelson et al., 1994), and calculated according to the formula (Brett, 1964):

$$U_{crit} = u_i + \left(\frac{t_i}{t_{ii} \times u_{ii}}\right), \quad (1)$$

where u_i is the highest velocity in *BL* s^{-1}, u_{ii} is the velocity increment, t_i is the time in min that the fish swam at the fatiguing velocity, and t_{ii} is the prescribed swimming period, i.e. 30 min. Following that, the water flow was reduced to a minimum of 3.3 $m^3 h^{-1}$ and the fish were allowed 4 h to recover. They were then warmed to their acclimation temperature in the same stepwise manner outlined above and left overnight before being swum again in the same manner, before they were taken out, the inductive coil removed and placed back in the aquarium. Water ammonium and nitrite content within the swim tunnel were checked every 12 h and water changed when necessary.

Tail-beat frequency measurement

Tail-beat frequency was measured manually by counting the number of tail beats in a 30 s period using the digital camera system. This was repeated eight times at each of the 30 min swimming stages. The mean of these eight was then taken as the tail-beat frequency. Eight 30 s sampling periods were not always possible at U_{crit}, so the mean was taken of as many sampling periods as were permitted, minimum three. The time of the first kick was also recorded.

Oxygen measurement

Oxygen was measured constantly at a sampling rate of 0.5 Hz using Fibox optodes (Presens, Regensburg, Germany) with the temperature compensation entered manually. Optodes were zeroed chemically with sodium dithionite in seawater, and 100% was calibrated by placing the optode in the open swim-tunnel circulation. This was checked periodically against a MultiLine P4 CellOx 325 oxymeter (WTW, Weilheim, Germany) calibrated to fully air saturated seawater.

Oxygen consumption was calculated from the slope of the drop in water oxygen content, which was monitored over a 20 min measurement period at each speed. At no point did the seawater oxygen content drop below 80%. After the initial 20 min measurement period the circulation was opened for a 10 min flush/re-oxygenation. At the end of each experiment when the fish had been removed, a 'blank' oxygen consumption run was performed to quantify any background microbial oxygen consumption. This was then subtracted from the fish's respiration rate. Oxygen consumption rates were corrected for any allometric size effects using the mass exponent of 0.8 (Saunders, 1963):

$$\dot{M}_{O_2} = \left(\frac{1}{M}\right)^{0.8} \times \dot{M}_{O_2 m}, \quad (2)$$

where $\dot{M}_{O_2}$ is the standardised oxygen consumption rate in mg O_2 kg^{-1} h^{-1}, M is the mass of the fish in kg, and $\dot{M}_{O_2 m}$ is the measured oxygen consumption.

^{31}P-NMR spectroscopy

In vivo ^{31}P-NMR spectra included primarily white muscle with a minor contribution from red muscle. Spectra were collected using a 200 μs bp32 pulse with a flip angle of 45°, sweep width was 5000 Hz at 4 k, and repetition time was 0.8 s. 256 scans were collected resulting in a total acquisition time of around 3 min. *In vivo* ^{31}P-NMR spectra were recorded over the whole 30 min time period for each swimming speed.

Statistical analysis, data processing and modeling

^{31}P-NMR spectra were acquired using Paravision 3.0 (Bruker, Ettlingen, Germany). Spectra were processed in Topspin 1.5 (Bruker) first by fast Fourier transformation, then filtered with line broadening in the range of the half width of the PCr signal. Phase and baseline were corrected using a

specially adapted automatic correction routine (R.-M. Wittig, AWI, Germany). Typically, 6–7 spectra were collected per swimming speed, i.e. per 30 min period. The best six spectra were then added for each swimming speed. Metabolite concentrations were determined by operator defined integration limits using the standard integration routine in Topspin 1.5 (Bruker).

The control PCr integral was converted into μmol g^{-1} using the intracellular concentration of 27.3 μmol g^{-1} for resting Atlantic cod (Sartoris et al., 2003). All other concentrations, i.e. subsequent PCr measurements, Pi and ATP, were then calculated relative to this. The intracellular pH was calculated from the Pi chemical shift using the temperature compensated formula given elsewhere (Bock et al., 2001). Gibbs free energy change of ATP hydrolysis ($\Delta G/d\xi_{ATP}$) was estimated for NMR visible metabolites as described earlier (Pörtner et al., 1996; Satoris et al., 2003; van Dijk et al., 1999), except that creatine concentration was estimated using the following equation:

$$\mathrm{Cr} = \left(\frac{\mathrm{PCr}}{0.65}\right) - \mathrm{PCr}, \quad (3)$$

where Cr is the estimated creatine concentration in μmol g^{-1}, PCr is phosphocreatine concentration in μmol g^{-1} and 0.65 is the ratio of PCr:Cr measured in resting fish white muscle (Hardewig et al., 1998).

All statistical analysis and modelling was performed using Graphpad Instat 3.0 and Prism 4.0 software (Graphpad Software, San Diego, CA, USA). For comparative purposes, significant differences were tested using ANOVA with Tukey's post-tests. Differences were considered significant when $P \leqslant 0.05$. Data are presented throughout as mean ± standard deviation (s.d.).

Results

The mean critical swimming speed for all fish was 0.71±0.06 *BL* s^{-1}. Neither the effects of the inductive coil nor the receive coil on swimming performance were directly quantified; however, a decrease of approximately 25–30% in the U_{crit} was seen compared to most values in the literature (Table 1). Because each fish swam at different speeds and had a different critical swimming speed, the relative speeds are given as the % U_{crit}, with the variability shown as horizontal error bars in Figs 2–5.

The oxygen consumption rate increased exponentially and significantly as a function of swimming speed up to the active metabolic rate (208±21.4 mg O_2 h^{-1} kg^{-1}; Fig. 2A). The standard metabolic rate (SMR) was estimated using non-linear regression of the oxygen consumption data at various speeds and extrapolating back to a swimming speed of 0 *BL* s^{-1}. The mean SMR for all fish was 36.3±10.7 mg O_2 h^{-1} kg^{-1}.

The tail-beat frequency increased in a linear fashion up to the point when kicking was initiated and then levelled off (Fig. 2B). Any increase in swimming speed attained thereafter must have been accomplished by an increase in amplitude or the frequency of the kick-and-glide bursts. Kicking started before the traditional U_{crit} was reached (at 89±2.5% U_{crit}). Once initiated, the number of kicks increased in frequency until U_{crit}, when the fish were kicking exclusively for several minutes before they stopped swimming altogether. This paralleled the initial decrease in the PCr signal, an increase in the Pi signal, and a resultant decrease in pH_i (see below).

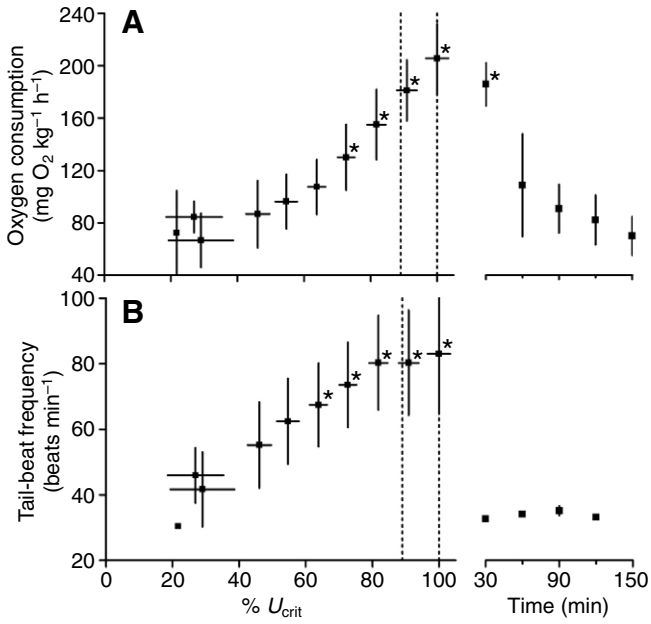

Fig. 2. Oxygen consumption rate (A) and tail-beat frequency (B) during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at 89% represents the mean time when kicking was initiated. Values are means ± s.d., *N*=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

Difficulties were encountered in collecting sufficient ^{31}P-NMR spectra from the moving animals. For optimal signal transduction, both the inductive and receive coils had to be parallel, with a minimal distance between them. Therefore, unless the fish was cooperative and swam near the coil, only a very weak signal could be detected. These difficulties were further exacerbated at higher speeds, in particular at U_{crit}, when fish were kicking and gliding. Thus, it was possible to collect spectra from only three of the six fish at U_{crit}. The loss of signal strength and the broadening of spectral bands due to swimming movements could have been mitigated by restricting the movements of the fish; however, this would have reduced the swimming performance.

Using *in vivo* ^{31}P-NMR we were able to show a significant increase in the relative proportion of the inorganic phosphate signal above resting levels as the fish approached the critical swimming speed (Fig. 3A). Under minimal flow, the Pi signal was often below the detection limit. As the critical swimming speed was approached, the Pi signal began to intensify, until it was maximally and significantly elevated at the traditional U_{crit} (9.5±3.4 μmol g^{-1}). Thereafter it decreased and was again basal at 2 h post exhaustive exercise.

The increase in Pi was coupled with a stoichiometric decrease in the relative proportion of the PCr from 27.3 μmol g^{-1} at

Table 1. *Critical swimming speeds from Atlantic cod (Gadus morhua)*

Study	Origin	U_{crit} (*BL* s^{-1})	*N*	Temperature (°C)	Length (cm)	Mass (kg)
Present study	NEAC	0.71±0.06	6	4	45.5±4.12	0.818±0.190
(Pörtner et al., 2002)	NEAC	0.74±0.03	5	10	46.0±4.45	0.719±0.284
(Reidy et al., 2000)	Scotian Shelf	1.11±0.15	25	5	52.6±6.05	1.42±0.55
(Reidy et al., 1995)	Scotian Shelf	0.99±0.14	8	5	48.5±6.53	0.818–1.68
(Nelson et al., 1994)	Bras d'Or	1.03±0.09	5	2	46.1±1.50	1.00±0.11
	Scotian Shelf	0.95±0.07	6	2	48.8±3.42	1.10±0.19

Values are means ± s.d., *N*=number of animals.

minimal flow, to 16.7±6.2 μmol g^{-1} at U_{crit} (Fig. 3B). A possible slight overshoot in the relative PCr proportion was apparent during recovery. The free ATP at control was 4.5±0.9 μmol g^{-1} and did not change significantly throughout the course of the swimming bout, i.e. 3.8±1.4 μmol g^{-1} at U_{crit}, or recovery, although a slight dip to 2.2±3.0 μmol g^{-1} was seen 90 min post U_{crit}, at approximately the same time as the aforementioned PCr overshoot (Fig. 3C).

The pH_i decreased significantly as the fish approached U_{crit} (Fig. 4). At minimal flow pH_i was 7.48±0.03. The pH_i started to drop at approximately the same time as the Pi concentration began to increase (see below), i.e. between 72±2.6 and 91±3.0% U_{crit}. At 91±3.0% U_{crit} the increase became significant. The pH_i was minimal, 6.81±0.05, at U_{crit} and began to increase back to resting conditions during recovery.

As previously mentioned, under minimal flow conditions the Pi signal was extremely small, sometimes undetectable, as also observed by other authors (Bock et al., 2002b; Sartoris et al., 2003), which reflects the resting condition of the unrestrained fish. However, this made determination of resting intracellular pH difficult. Some of the variation in pH_i at higher speeds, particularly near U_{crit}, where a very clear Pi signal could be discerned, may be a result of the temporal resolution. As the acquisition of each spectrum took approximately 3.0 min, the pH_i may have changed in this time. Furthermore, six of these spectra were then summed for each swimming speed, potentially broadening the Pi signal. In addition, the kicking at U_{crit} would have caused turbulences in the water that may have led to magnetic inhomogeneities, resulting in broadening of all signals. As we predominantly saw a broadening of the Pi signal alone, we believe the former explanation to be correct.

At minimal flow the $\Delta G/d\xi_{ATP}$ was –55.6±1.4 kJ mol^{-1} and had significantly decreased to –49.8±0.7 kJ mol^{-1} at the traditional U_{crit} (Fig. 5). The drop in $\Delta G/d\xi_{ATP}$ was not a linear function of the speed or time before U_{crit}. The largest drops in $\Delta G/d\xi_{ATP}$ of 2.96 kJ mol^{-1} and 4.04 kJ mol^{-1} were seen between 82±2.8% and 91±3.0% U_{crit} and 91±3.0% and U_{crit}, respectively. Non-linear regression and extrapolation to 0% U_{crit}, i.e. resting conditions (Fig. 5), gave a resting $\Delta G/d\xi_{ATP}$ value of –57.3±1.5 kJ mol^{-1}.

Discussion

The primary findings of the present experimental series clearly show that as the fish approached U_{crit} a transition point in metabolism was seen. This transition from steady state aerobic metabolism to anaerobic metabolism occurred at the same time as the fish changed gait from subcarangiform swimming to kick-and-glide. Furthermore, insight was provided into fatigue processes and it was evident that the drop in the $\Delta G/d\xi_{ATP}$ below a threshold appears to be ultimately responsible for fatigue.

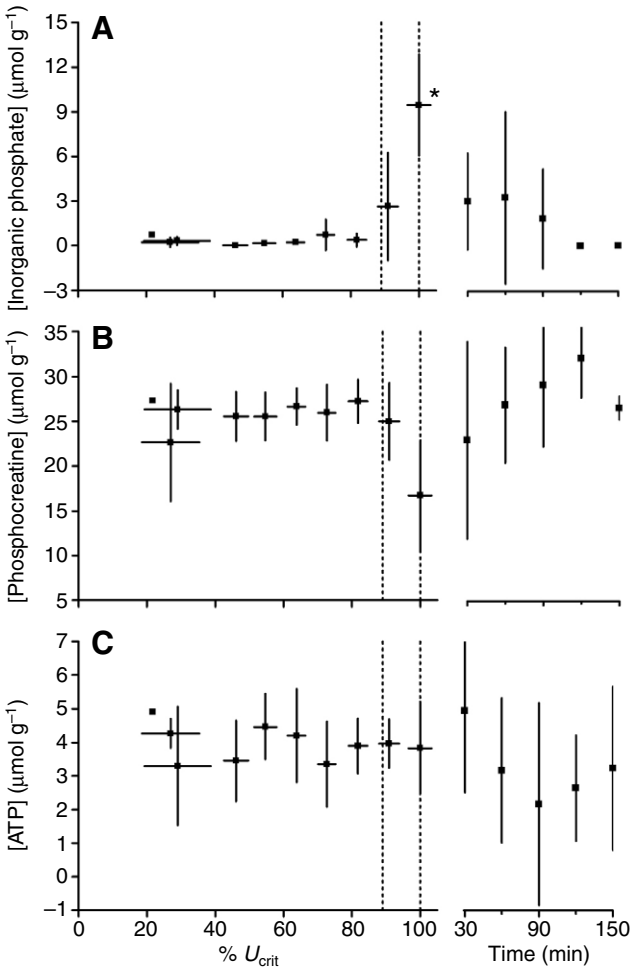

Fig. 3. Inorganic phosphate (A), phosphocreatine (B) and ATP (C) concentrations during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at 89% represents the mean time when kicking was initiated. Values are means ± s.d., *N*=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

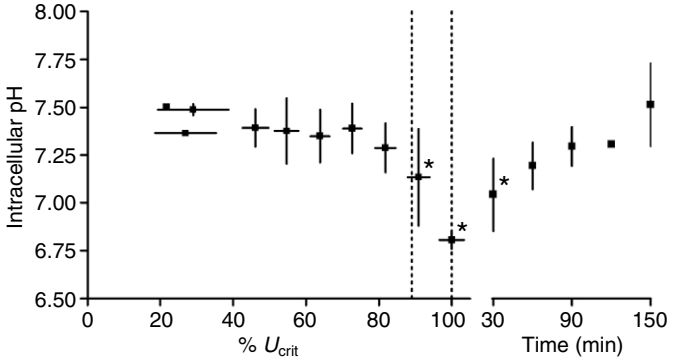

Fig. 4. Intracellular pH values during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at 89% represents the mean time when kicking was initiated. Values are means ± s.d., N=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

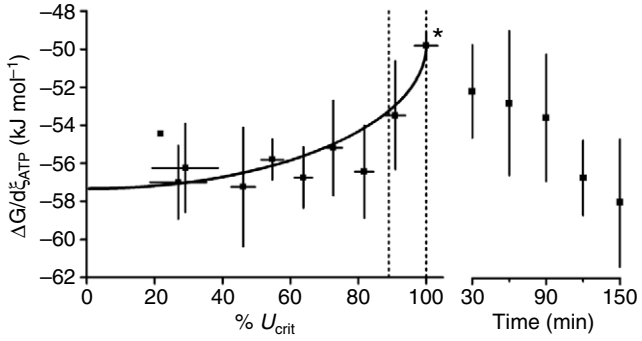

Fig. 5. The time course of the Gibbs free energy change of ATP hydrolysis during the critical swimming speed test (left; speed as % U_{crit}) and the first 150 min of recovery [right; time after U_{crit} (min)]. The right broken vertical line indicates the traditional U_{crit} value; the left broken vertical line at –89% represents the mean time when kicking was initiated. Values are means ± s.d., N=3–6. Horizontal error bars represent s.d. of the mean speed. Asterisks indicated significant difference from control values.

It is worth noting that both the inductive coil and the receive coil produced micro-turbulences. As a result, the flow in the swim tunnel behind the receive coil was turbulent, and thus swimming conditions were not optimal. When compared to literature values of similarly sized cod (Table 1), an approximate decrease of 30% in U_{crit} was observed in the current study, similar to that seen by Pörtner et al. (2002), using a similar set-up. Furthermore, fish in the present study were 10°C acclimated but swum at 4°C. Parallel work found that an acute thermal change from 10°C to 4°C reduced swimming performance by approximately 10–15%, when compared to the performance at the acclimation temperature of 10°C (G.J.L., C.H.B. and H.-O.P., manuscript in preparation).

Energetics of U_{crit}

Several previous studies have used excessive post-exercise oxygen consumption and blood parameters as measures of anaerobic metabolism (Peake and Farrell, 2004; Lee et al., 2003; Nelson et al., 1994; Brett, 1964), assuming that any oxygen debt accumulated during the swimming trial was re-paid during recovery. The online measurement of metabolic processes underpinning the entire swimming trial, i.e. from slow swimming through the transition to kick-and-glide to complete fatigue, confirm previous findings that U_{crit} is indeed the point of complete fatigue, when both the aerobic *and* anaerobic resources have been fully expended.

In the current study, kicking started at 89±2.5% U_{crit}, just as the tail-beat frequency began to plateau (Fig. 2B). At the same time Pi began to increase and the pH_i started to acidify. These kicks avoided any increase in the tail-beat frequency required as speed increased. Although it is possible that the fish were able to compensate by increasing the tail-beat amplitude at the same frequency, we primarily observed that once initiated, the number of kicks increased in frequency until close to exhaustion (i.e. U_{crit}), when the cod were kicking exclusively. This strategy indicated the involvement of white muscle fibres (Jones, 1982) and came at an additional cost fuelled anaerobically on top of enhanced aerobic metabolic rate.

Using electromyography, Rome et al. (Rome et al., 1984) previously found that as swimming velocity increased, mirror carp (*Cyprinus carpio* L.) increasingly recruited more white muscle to increase the power production before kicking was initiated. Similar findings have also been seen with other fish species (Jayne and Lauder, 1994; Rome et al., 1992). Although the precise time when kicking was first initiated was not given, a significant increase in lactate and drop in PCr was seen at 70% U_{crit} in rainbow trout (*Onchorhynchus mykiss* Walbaum) using ^{31}P-NMR (Burgetz et al., 1998). This continued to rise at 80% and was maximal at U_{crit}. A more invasive study (Nelson et al., 1994) looked at, among other parameters, lactate production in Atlantic cod as a proxy for anaerobic metabolism, and found that it had already significantly increased at approximately 80% U_{crit}. The reasons why this switch to anaerobic metabolism occurred later in the present study, i.e. at 89% U_{crit}, are discussed below.

^{31}P-NMR and energetic status

To our knowledge, only our previous study with Atlantic cod (Pörtner et al., 2002) and one other study with rainbow trout (Burgetz et al., 1998) have looked at exercised fish using ^{31}P-NMR spectroscopy. In the latter study, where the trout were swum to 70, 80 or 100% U_{crit} and then transferred to the NMR magnet, the exactitude of the measurements in relation to swimming speeds was limited due to the potential stress incurred during transfer. An artefactual decrease in PCr and an increase in Pi is almost invariably seen in studies after the initial transfer of animals to the NMR magnet (e.g. Sartoris et al., 2003). However, because the trout were restrained, the spectra were of a higher quality.

A previous study (Burgetz et al., 1998) found that PCr had dropped to approximately one half of the 30 μmol g^{-1} control values at 70% U_{crit}. This depletion continued and was maximal at U_{crit} where PCr was approximately 4 μmol g^{-1}. The severe reduction in the PCr concentration, particularly at 70% U_{crit}, is much larger than in our study. This may be due to (i) a stress related artefact, as discussed above, or (ii) species differences between athletic trout and lethargic Atlantic cod.

In the same study (Burgetz et al., 1998), biochemical analysis was used to determine lactate concentrations in the same tissue. A strong correlation (r^2=0.83) was found between the tissue lactate concentration and the pH_i calculated from the Pi shift. At 70% U_{crit}, tissue lactate concentration had already increased significantly (from ~13 μmol g^{-1} tissue to ~21 μmol g^{-1}), and was maximal at U_{crit} (~47 μmol g^{-1}). In the current study pH_i had dropped significantly at 91±3.0% U_{crit}. The disparity between the time of onset and degree of intracellular acidification may be attributable to the differences between the two species. For example, the maximum plasma lactate concentration after exhaustive exercise was found to be ~7 mmol l^{-1} in Scotian Shelf cod and ~10 mmol l^{-1} in Bras d'Or cod (Nelson et al., 1994). In comparison, other authors (Jain and Farrell, 2003) found that plasma lactate levels could get as high as ~20 mmol l^{-1} in rainbow trout post exhaustive exercise. We conclude from the cited evidence that Atlantic cod are physiologically unable to exert themselves to the same degree as trout.

Additionally, the general level of fitness of our fish may have further reduced their swimming capacity as they had been kept in our aquarium for more than 1 year and there was only a slight current against which they could exercise, possibly making them rather lethargic in comparison to freshly caught, wild fish (Soofiani and Preide, 1985; Webb, 1971; Bams, 1967; Bainbridge, 1962; Brett, 1958). The critical swimming speed indicating onset of anaerobic metabolism may not be fixed but be found at variable velocities in relation to U_{crit}. In cod it appears to be tied to the onset of kick-and-glide swimming.

Fatigue

One of the aims of the current study was to observe exactly when the pH_i decreased, i.e. at or before U_{crit}, as this would be an important step in determining the transition to non-steady state anaerobic metabolism. It has previously been hypothesised that the accumulation of physiological concentrations of Pi (e.g. 30 μmol g^{-1}) is responsible for a decrease in the power generated in the muscle as Pi moves into the sarcoplasmic reticulum and precipitates Ca^{2+} (Allen and Westerblad, 2001), and reduces steady state tension by reducing/preventing cross-bridge attachment (Hibberd et al., 1985). The mean Pi concentration at U_{crit} was significantly elevated at 9.5±3.4 μmol g^{-1}, but we expect the effect of Pi on fibre contraction would have been reduced, as levels are 30% of those cited above. Two further points supporting the reduced importance of Pi and pH_i in exercise induced fatigue must be noted. Firstly, increased intracellular Pi in fact increased force re-development during rapid contraction–relaxation cycles (Hibberd et al., 1985), which are typical of kick-and-glide bursts seen here. Secondly, a drop in pH_i led to increased excitability of working skinned muscle fibres from rats (Pedersen et al., 2004).

More important were the changes observed in tissue energetics. The stoichiometric relationship between the Pi increase and the PCr decrease indicates that these cellular energetic stores were being depleted at U_{crit} to buffer cellular ATP concentrations. The consequent drop in pH_i indicated that anaerobic metabolism was also being used to maintain ATP concentrations. Both Pörtner et al. (Pörtner et al., 1996) and Hardewig et al. (Hardewig et al., 1998) have argued that as the cytosol becomes more acidic and, more importantly, as the phosphagen (PCr in case of fish) is lysed to Pi and Cr, there is a drop in $\Delta G/d\xi_{ATP}$. For two species of Zoarcid eelpouts, the free energy of ATP hydrolysis was observed to drop from –60 kJ mol^{-1} to approximately –46 kJ mol^{-1} and for rainbow trout, $\Delta G/d\xi_{ATP}$ dropped from –60 to –47 kJ mol^{-1} after exhaustive exercise (Hardewig et al., 1998).

Various studies have looked at the effects of a drop in free energy values and the detrimental effects on cellular ion transporters (Jansen et al., 2003; Kammermeier et al., 1982). Hardewig et al. (Hardewig et al., 1998) argued that below a threshold of approximately –52 kJ mol^{-1}, cellular processes such as Ca^{2+}-ATPases, essential to muscle function, can no longer derive enough energy to be maintained. We suggest that the muscular fatigue observed in our fish was predominantly due to a drop in $\Delta G/d\xi_{ATP}$ to –49.8±0.7 kJ mol^{-1} at U_{crit}, which was below a certain threshold, potentially –52 kJ mol^{-1} (Hardewig et al., 1998), required by transporters to maintain ion gradients and fuel the muscular machinery.

The control $\Delta G/d\xi_{ATP}$ values in the current study (–55.6±1.4 kJ mol^{-1}) lie below those reported for resting eelpout (Hardewig et al., 1998) and –61 kJ mol^{-1} for resting Atlantic cod (Sartoris et al., 2003). It seems reasonable to conclude that this is because resting values in the two aforementioned studies were obtained from inactive fish, whereas our values were obtained at minimal flow. A relatively linear decrease in $\Delta G/d\xi_{ATP}$ with increasing swimming speed was previously shown at moderate speeds in squid (Pörtner et al., 1996). Consequently, the slow swimming of our fish during control conditions would have led to a shift in steady state energy status, thus reducing $\Delta G/d\xi_{ATP}$ in comparison to values from inactive fish. Extrapolation of our values back to resting (Fig. 5) gave us a base value of –57.3±1.5 kJ mol^{-1} (r^2=0.90).

Conclusions

Through the use of *in vivo* ^{31}P-NMR spectroscopy combined with a Brett-type swim tunnel, we were able to show that as Atlantic cod swimming speed increased, and gait was changed from subcarangiform swimming to kick-and-glide swimming prior to traditional U_{crit}, i.e. at 89±2.5% U_{crit}, a graded decrease in intracellular pH was observed while the oxygen consumption rate continued to increase exponentially. At the same time, phosphocreatine levels fell and this was accompanied by a significant increase in inorganic phosphate. All these changes were maximal at the traditional U_{crit} (i.e. exhaustion), when the fish were kicking exclusively. These changes were subsequently restored during recovery. The Gibbs free energy change of ATP hydrolysis was also minimal at the traditional U_{crit} (i.e. down from –55.6±1.4 at control to –49.8±0.7 kJ mol^{-1}), and this was argued to be the leading cause for muscular fatigue leading to exhaustion of the cod. Thus, a transition from steady state aerobic metabolism to non-steady state anaerobic metabolism led to a complete exhaustion of aerobic and anaerobic resources at the traditional critical swimming speed.

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