

## METHODS &amp; TECHNIQUES

# Finding the peak of dynamic oxygen uptake during fatiguing exercise in fish

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## ABSTRACT

As fish approach fatigue at high water velocities in a critical swimming speed ( $U_{crit}$ ) test, their swimming mode and oxygen cascade typically move to an unsteady state because they adopt an unsteady, burst-and-glide swimming mode despite a constant, imposed workload. However, conventional rate of oxygen uptake ( $\dot{M}_{O_2}$ ) sampling intervals (5–20 min) tend to smooth any dynamic fluctuations in active  $\dot{M}_{O_2}$  ( $\dot{M}_{O_2,active}$ ) and thus likely underestimate the peak  $\dot{M}_{O_2,active}$ . Here, we used rainbow trout (*Oncorhynchus mykiss*) to explore the dynamic nature of  $\dot{M}_{O_2,active}$  near  $U_{crit}$  using various sampling windows and an iterative algorithm. Compared with a conventional interval regression analysis of  $\dot{M}_{O_2,active}$  over a 10-min period, our new analytical approach generated a 23% higher peak  $\dot{M}_{O_2,active}$ . Therefore, we suggest that accounting for such dynamics in  $\dot{M}_{O_2,active}$  with this new analytical approach may lead to more accurate estimates of maximum  $\dot{M}_{O_2}$  in fishes.

**KEY WORDS:** Active oxygen uptake, Iterative algorithm, Swimming, Respirometry, Maximal exercise, Maximum rate of oxygen uptake

## INTRODUCTION

Many disciplines in biology use the maximum rate of oxygen uptake ( $\dot{M}_{O_2,max}$ ) attained by an animal both as an index of cardiorespiratory fitness and to better understand aerobic capabilities. Human physiologists typically assess  $\dot{M}_{O_2,max}$  using a graded exercise test to fatigue (Albouaini et al., 2007; Dlugosz et al., 2013), with the rate of oxygen uptake ( $\dot{M}_{O_2}$ ) peaking despite a further increase in the imposed workload (Taylor et al., 1955). Although testing protocols used with humans are still debated (Robergs et al., 2010; Beltz et al., 2016; Poole and Jones, 2017; Van Breda et al., 2017; Green and Askew, 2018), they all benefit from some average of breath-to-breath  $\dot{M}_{O_2}$  measurements. Fish physiologists also use graded exercise tests to estimate  $\dot{M}_{O_2,max}$ , e.g. a critical swimming speed ( $U_{crit}$ ) test, which remains a valid testing protocol despite dating back over 70 years (Fry and Hart, 1948; Beamish, 1964; Brett, 1964), as recently discussed along with the exhaustive chasing testing protocol (Norin and Clark, 2016; Zhang and Gilbert, 2017). In fact, nearly all  $U_{crit}$  tests that measure  $\dot{M}_{O_2}$  have assigned  $\dot{M}_{O_2,max}$  to the peak value for active  $\dot{M}_{O_2}$  ( $\dot{M}_{O_2,active}$ ) during swimming. However, when fish approach fatigue in  $U_{crit}$  tests, they typically supplement their sustainable steady swimming gait with a burst-and-glide swimming gait (Peake and Farrell, 2004, 2005, 2006). Consequently,  $\dot{M}_{O_2,active}$  may become

dynamic as swimming gait changes typically during the penultimate and/or final water velocity increment ( $U_{final}$ ). Unfortunately, because breath-to-breath  $\dot{M}_{O_2}$  measurements are nearly impossible with a swim tunnel, a typical sampling period to estimate  $\dot{M}_{O_2,active}$  (e.g. 5–20 min) is too long to measure such gait dynamics, and peak  $\dot{M}_{O_2,active}$  could be smoothed out, potentially underestimating  $\dot{M}_{O_2,max}$ .

Therefore, we reasoned that minimizing the duration of the sampling window would improve the temporal resolution for  $\dot{M}_{O_2,active}$  and  $\dot{M}_{O_2,max}$  determinations, as well as help with the application of more consistent criteria. To date, sampling windows have varied considerably for the two most common testing protocols used with fishes: from 5 to 60 min for  $U_{crit}$  tests (e.g. Fry and Hart, 1948; Brett, 1964; Bushnell et al., 1994; Nelson et al., 1996; McKenzie et al., 2001; Gallagher et al., 2001; Lee et al., 2003a,b; Claireaux et al., 2005, 2006; Marras et al., 2010; Svendsen et al., 2010; Ejbye-Ernst et al., 2016; Di Santo et al., 2017) and from 0.5 to 60 min for chase-to-exhaustion tests (Lucas and Priede, 1992; Reidy et al., 1995; Norin and Malte, 2011; Clark et al., 2012, 2013; Norin and Clark, 2016; Norin et al., 2014; Gräns et al., 2014; Auer et al., 2016; Zhang et al., 2017). Fortunately, fibre optic oxygen sensors greatly facilitate exploration of the dynamic nature of  $\dot{M}_{O_2,active}$  in fishes by having a fast response time (<15 s; pyroscience.com/index.html), unlike early swim tunnel respirometry that had to use the Winkler method (Winkler, 1888) to follow the decline in dissolved oxygen (DO) over lengthy periods (30–60 min) to estimate  $\dot{M}_{O_2,active}$ .

Also, we examined an alternative to applying a high coefficient of determination (e.g.  $R^2 > 0.95$ ) for linear regressions of decline in water DO concentration for  $\dot{M}_{O_2,active}$  determinations, which is a recommended criterion when determining  $\dot{M}_{O_2}$  with closed respirometry (Svendsen et al., 2016). Although a minimum  $R^2$  is certainly satisfactory for estimating standard metabolic rate (Chabot et al., 2016a,b) and a steady  $\dot{M}_{O_2,active}$ , capturing rapid changes in  $\dot{M}_{O_2,active}$  by minimizing the sampling window unfortunately reduces the number of measurements in the regression, which can decrease  $R^2$  (Cornell and Berger, 1987). Therefore, as a new analytical approach with swim-tunnel respirometer to reduce the reliance on  $R^2$ , we determined the conservative minimum sampling window needed to reliably measure the background  $\dot{M}_{O_2}$  of the respirometer, i.e. a stable, but very low  $\dot{M}_{O_2}$ , and to reliably measure a stable  $\dot{M}_{O_2,active}$  for a fish swimming steadily (~50%  $U_{crit}$ ). Using this minimum sampling window in conjunction with an iterative mathematical algorithm, we then explored the dynamics of  $\dot{M}_{O_2,active}$  while rainbow trout (*Oncorhynchus mykiss*) transitioned from steady to burst-and-glide swimming modes as they approached fatigue at  $U_{crit}$ . Specifically, we used these methods to test the null hypothesis that an  $\dot{M}_{O_2}$  estimate is independent of the sampling window duration and whether it is analysed by an iterative algorithm. If rejected, we predicted that dynamics in  $\dot{M}_{O_2,active}$  would be related to dynamic swimming behaviour, which we assessed semi-quantitatively.

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**List of symbols and abbreviations**

DO	dissolved oxygen
$\dot{M}_{O_2}$	rate of oxygen uptake
$\dot{M}_{O_2\text{active}}$	$\dot{M}_{O_2}$ as measured when an animal exercises at any workload above rest.
$\dot{M}_{O_2\text{max}}$	maximum $\dot{M}_{O_2}$
$U$	water velocity in the swim tunnel respirometer
$U_{\text{crit}}$	critical swimming speed
$U_{\text{final}}$	final water velocity increment in which a fish fatigues

**MATERIALS AND METHODS****Experimental animals**

Animal holding and experimental procedures were approved by the University of British Columbia Animal Care Committee (A16-0049, A15-0035 and A15-0266). A hatchery-reared, river-dwelling strain (Blackwater) of rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] was obtained from Fraser Valley Trout Hatchery (Abbotsford, British Columbia, Canada; Freshwater Fisheries Society of BC) and held at 14°C in 200-litre circular tanks containing dechlorinated Vancouver tap water in the Zoology Aquatic Facility. Water temperature was monitored daily and fish were fed a daily maintenance ration of commercial trout pellets (Skretting Canada Inc., Vancouver, BC, Canada).

**Experimental protocol**

Swimming performance trials were conducted with 14 fish (body mass: 69.1±3.2 g) that were fasted for 48 h. Individual fish were placed in a 10-litre swim tunnel (Loligo systems, Tjele, Denmark; water volume to fish mass ratio=144) in a thermally regulated laboratory (14°C) where the fish habituated for 1 h to the swimming section (40×10×10 cm) at a water velocity ( $U$ ) of ~0.5 body lengths (BL) s<sup>-1</sup>. The swim tunnel was shaded with an opaque curtain to minimize visual disturbances through the transparent swim section of the respirometer. A ramp- $U_{\text{crit}}$  protocol (Jain et al., 1997) increased  $U$  up to ~40% of  $U_{\text{crit}}$  (4 cm s<sup>-1</sup> every 1 min for 5 min), after which  $U$  was increased in increments of 9 cm s<sup>-1</sup> (i.e. ~10% of  $U_{\text{crit}}$ ) every 20 min until the fish reached a fatigue velocity (Fig. S1), as previously recommended (Farlinger and Beamish, 1977; Hammer, 1995). Fish were deemed fatigued when they remained on the rear grid of the swimming section for >5 s. A ramp- $U_{\text{crit}}$  protocol assumes that salmonids can swim aerobically for sustained periods at <50%  $U_{\text{crit}}$  (Beamish, 1978; Hammer, 1995). Following the ramp, the present study had five to seven  $U$  increments of 20 min duration before fish became fatigued; total test duration was <130 min. Validation of the ramp- $U_{\text{crit}}$  protocol (and its earlier versions, Randall et al., 1987; Brauner et al., 1994) has been performed for wild and hatchery-reared salmonids in a variety of field and laboratory conditions (Farrell et al., 1998; Jain et al., 1998; Lee et al., 2003a,b).

DO in the water (expressed as % air saturation) was measured (~1 s interval) with a fibre optic probe (Robust Oxygen Probe OXROB10, PyroScience GmbH, Aachen, Germany) mounted in the centre of the flow straightening vanes ahead of the swimming section to allow proper water mixing. A flush pump (Universal 2400, EHEIM GmbH & Co KG, Deizisau, Germany), attached via a one-way outflow valve to the swim tunnel, was manually controlled to maintain DO above 80% every time the respirometer was closed to measure  $\dot{M}_{O_2}$ . We deliberately avoided flushing the swim tunnel during the transition to  $U_{\text{final}}$  so we could follow the dynamics of  $\dot{M}_{O_2\text{active}}$  immediately after the speed transition.

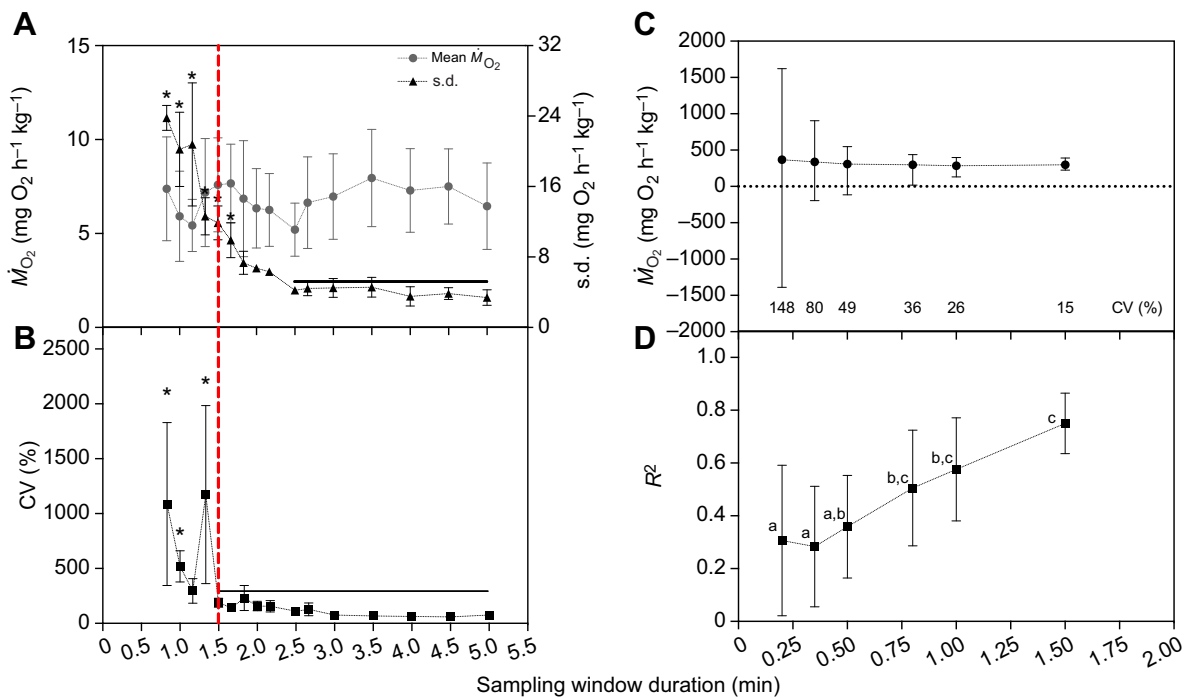
Swim behaviour was recorded with an infrared camera (1000TVL, Zosi Technology, Hong Kong, China) mounted ~1 m above the swim tunnel and was semi-quantitatively analysed using a behavioural event logging software (BORIS; Friard and Gamba, 2016). We scored four mutually exclusive swimming modes that extended beyond an earlier semi-quantitative assessment (Farrell and Clutterham, 2003). These four modes accounted for all observed behaviours and were: (1) maintaining station (M) using a steady swimming mode and with only minor drift; (2) burst-and-glide swimming (B) with repeated cycles of forward progress followed by backwards drift; (3) intense burst swimming (I), where a fish reached the front of the swim section and sustained its swimming effort against the grid without gaining any forward progress; and (4) resting (R), when a fish ceased swimming activity and drifted back against the rear grid (<5 s). The total time spent in each of these four swimming modes is reported as a proportion of a 20-min swimming period.

**Data analysis****Obtaining the minimum duration of a sampling window for a reliable  $\dot{M}_{O_2}$  measurement**

The background  $\dot{M}_{O_2}$  of a respirometer, which is routinely recorded in such studies, has a very low signal-to-noise ratio but is stable over a 20-min period. In contrast,  $\dot{M}_{O_2\text{active}}$  when a fish is swimming steadily at an intermediate water velocity (e.g. 50%  $U_{\text{crit}}$ ) provides a much larger signal. Therefore, both signals were used to determine the minimum duration of a sampling window for a reliable  $\dot{M}_{O_2}$  measurement, i.e. determine the shortest sampling window while still minimizing the variance around the  $\dot{M}_{O_2}$  estimate. We used five 20-min DO traces of background  $\dot{M}_{O_2}$  over 20 min ( $N=5$ ) (see Eqn 1) using different durations for the sampling windows (i.e. 0.8, 1.0, 1.2, 1.3, 1.5, 1.7, 1.8, 2, 2.2, 2.5, 2.7, 3, 3.5, 4, 4.5 and 5.0 min):

$$\dot{M}_{O_2} = \left[ \frac{dDO[i, (i+a)]}{dt[i, (i+a)]} \times (V_r - V_f) \times S_O \right] / (t \times M_f), \quad (1)$$

where  $\dot{M}_{O_2}$  is calculated in mg O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>, dDO/dt is the slope of DO saturation over time,  $V_r$  is the volume of the respirometer,  $V_f$  is the volume of the fish,  $S_O$  is oxygen solubility at 14°C freshwater at 1 atm,  $t$  is the time constant (3600 s),  $M_f$  is the fish mass,  $a$  is measurement duration (s),  $i$  is 1 s forward from the end of previous measurement interval until the last sampling window, where 1 s is the sampling frequency. This generated a minimum of four and a maximum of 25 estimates of the same  $\dot{M}_{O_2}$ , each having the same inherent noise level, from which we determined the mean, standard deviation (s.d.) and coefficient of variation (CV) as a function of the duration of the sampling window (Fig. 1). Given that the background  $\dot{M}_{O_2}$  represents a worst-case scenario in terms of signal-to-noise ratio (peak  $\dot{M}_{O_2\text{active}}$  was at least 70 times larger),  $\dot{M}_{O_2\text{active}}$  at ~50%  $U_{\text{crit}}$  (see Fig. 1C,D) typically provided a less steady but much larger signal to explore even shorter sampling window durations (0.20, 0.35, 0.50, 0.80, 1.0 and 1.5 min). This generated a minimum of 10 and a maximum of 76 estimates of  $\dot{M}_{O_2\text{active}}$  that were used to calculate the mean, s.d., CV and  $R^2$  for each sampling window. Data were log transformed to achieve normality and homogeneity of residuals. One-way ANOVA with Tukey's *post hoc* tests were conducted to compare the differences among the pooled data for the mean, s.d., CV and  $R^2$  as a function of sampling duration.



**Fig. 1. An analysis of variation in sampling window duration for an individual estimate of rate of oxygen uptake ( $\dot{M}_{O_2}$ ;  $n=5$ ).** (A,B) A 20-min background in the respirometer; (C,D) a 15-min measurement of  $\dot{M}_{O_2\text{active}}$  of a rainbow trout (*Oncorhynchus mykiss*) swimming steadily at  $\sim 50\%$  of critical swimming speed ( $U_{crit}$ ). (A) Means and standard deviations (s.d.) for the individual  $\dot{M}_{O_2}$  determinations (mean  $\pm$  s.e.m.). (B) Coefficient of variation (CV) for the individual  $\dot{M}_{O_2}$  determinations (mean  $\pm$  s.e.m.). The suggested 1.5-min minimum duration of sampling window is indicated by a vertical dashed line (red). (C) Mean and CV for the individual  $\dot{M}_{O_2\text{active}}$  determinations (mean  $\pm$  range). (D) Coefficient of determination ( $R^2$ ) for the individual  $\dot{M}_{O_2\text{active}}$  determinations (mean  $\pm$  s.d.). Statistical differences ( $P < 0.05$ ; one-way ANOVA) are indicated by either an asterisk or different letters, while the horizontal black lines indicate stable minimum values for s.d. and CV.

### Dynamic $\dot{M}_{O_2}$ and $\dot{M}_{O_2\text{max}}$ determinations

Even though  $\dot{M}_{O_2\text{active}}$  varied among individuals at a given water velocity,  $\dot{M}_{O_2}$  always increased for  $U_{final}$  (Fig. S1). Therefore, the detailed analysis ( $N=14$  fish) of the dynamic nature of  $\dot{M}_{O_2}$  was limited to  $U_{final}$  except when  $U_{final}$  was too short to analyse with the full range of sampling windows, in which case the penultimate step was included (see Fig. 2G). Changes in swimming gait were always observed. Also, a detailed analysis is presented for two representative fish that were swimming steadily at an intermediate  $U$  (mostly M mode). Each  $\dot{M}_{O_2}$  analysis, which was timed to start at the onset of the new  $U$ , examined whether a longer sampling window duration dampened the dynamics of  $\dot{M}_{O_2\text{active}}$ .  $\dot{M}_{O_2\text{active}}$  was calculated using a conventional sequential interval regression analysis (Eqn 1) using consecutive 1.5, 2.0, 2.5, 3.0, 5 or 10-min durations for the sampling window (except when interrupted by a respirometer flush period). The mean  $R^2$  for these determinations was  $>0.93$  regardless of the sampling window duration. Peak  $\dot{M}_{O_2\text{active}}$  was assigned to the greatest  $\dot{M}_{O_2\text{active}}$  for a given sampling window duration and all the 1.5-min estimates of  $\dot{M}_{O_2\text{active}}$  were averaged to generate the  $\sim 20$ -min estimate. However, the conventional sequential interval regression analysis cannot fully account for the dynamics of  $\dot{M}_{O_2\text{active}}$ . Therefore, we also used an iterative process to estimate peak  $\dot{M}_{O_2\text{active}}$  by conducting a rolling regression (termed an iterative algorithm; Eqn 2):

$$\text{Peak } \dot{M}_{O_2} = \max \left\{ \left[ \frac{dDO[n, (n+90)]}{dt[n, (n+90)]} \times (V_r - V_f) \times S_O \right] / (t \times M_f) \right\}, \quad (2)$$

where  $n$  is a 1 s increment from the first DO measurement until the last (90th) DO measurement.

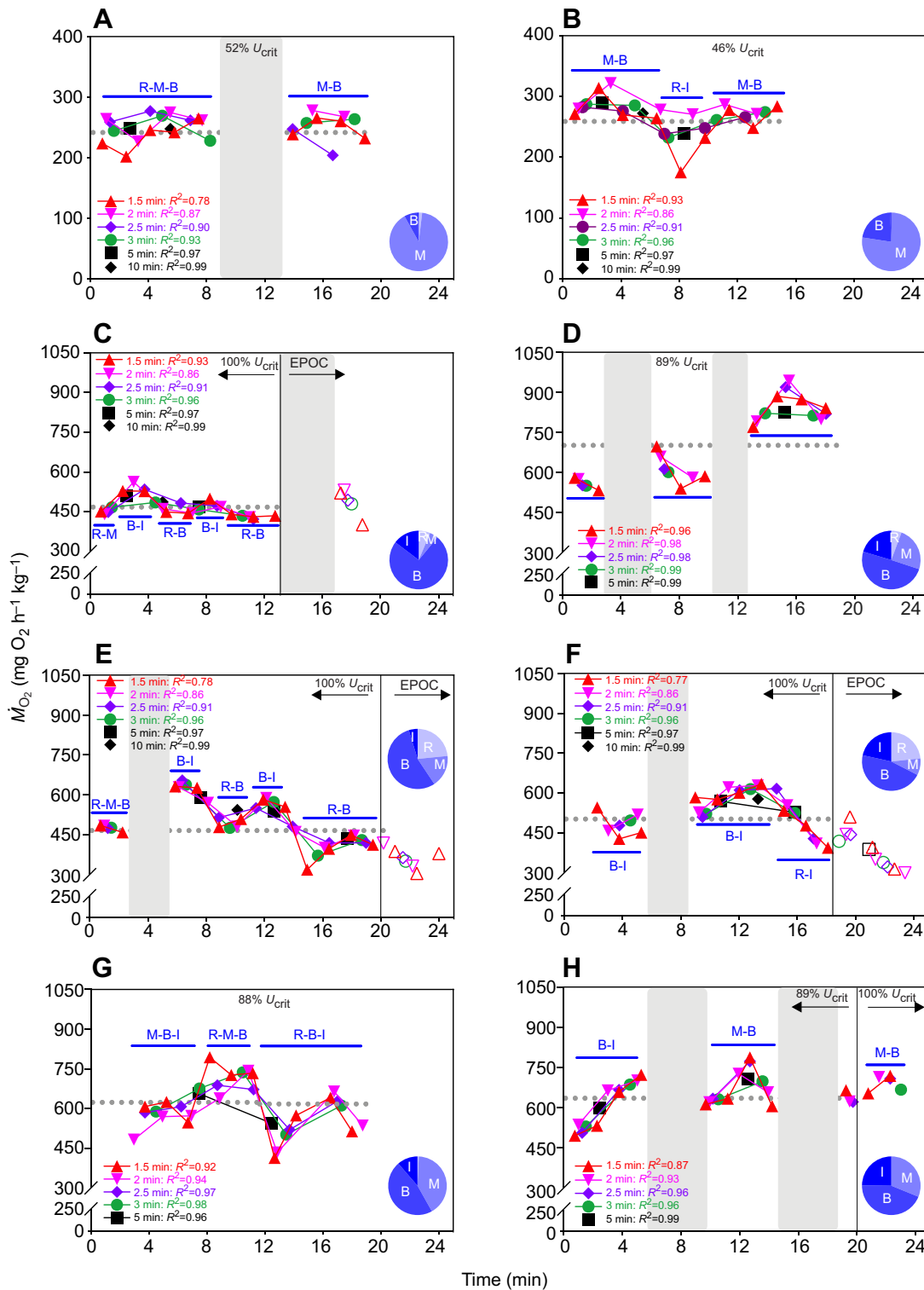
This iterative algorithm repeatedly advanced the shortest reliable sampling window (1.5 min) by a single DO measurement through the DO data rather than using sequential intervals. Mean values for the various estimates of peak  $\dot{M}_{O_2\text{active}}$  were compared with a repeated-measures (mixed-effects model) one-way ANOVA. If necessary, data were log transformed to meet the assumptions of normality of residuals. We predicted that the highest estimate of peak  $\dot{M}_{O_2\text{active}}$  would be that generated by the iterative algorithm used with a 1.5-min sampling window. In this case, we would reject our null hypothesis that the estimate of  $\dot{M}_{O_2\text{max}}$  was independent of the duration of the sampling window and the mathematical analysis of it.

Data analysis and presentation were completed in GraphPad 8.0 (GraphPad Software, San Diego, CA, USA), Labchart 8.0 (ADInstruments, Colorado Springs, CO, USA) and Excel 16.2 (Redmond, WA, USA). Statistical significance was assigned when  $\alpha < 0.05$ .

### RESULTS AND DISCUSSION

As expected, the mean value of the constant background  $\dot{M}_{O_2}$  of the respirometer was independent of the sampling window duration (Fig. 1). However, reliability in the background  $\dot{M}_{O_2}$  estimate was dependent on the duration of the sampling window because s.d. and CV both decreased with the sampling window duration, with CV stabilizing for a sampling window duration  $\geq 1.5$  min, and s.d. stabilizing after 2.5 min (Fig. 1A,B). Thus, depending on the criterion (CV or s.d.), the minimum sampling window duration for this very low  $\dot{M}_{O_2}$  signal was between 1.5 and 2.5 min.

Despite anticipating an even shorter duration of sampling window for a fish swimming steadily (Fig. 1C,D), the larger  $\dot{M}_{O_2\text{active}}$  signal had a greater noise level given that s.d. and CV increased with a sampling window duration  $< 1.5$  min. In fact,



**Fig. 2. Examples of oxygen uptake ( $\dot{M}_{O_2}$ ) dynamics (closed symbols) for individual rainbow trout during a  $U_{crit}$  test with  $U$  expressed as a percentage of  $U_{crit}$ .** (A,B) Steady swimming at an intermediate  $U$ . (C–H) The final or penultimate  $U$  before fatigue. Some analyses continued into recovery (EPOC: excess post-exercise oxygen consumption; open symbols). Grey block indicates when the respirometer was being flushed. Different sampling window durations (1.5 to 10 min) used in a sequential interval regression analysis have different coloured lines (see key, which also shows  $R^2$  for each measurement method). The line of identity (grey dotted) is for the average of all 1.5-min  $\dot{M}_{O_2}$  values for the entire swimming step (as shown in Fig. S1). The pie diagram represents the proportion of time the fish spent using different swimming behaviours: rest (R), maintain stationary (M), burst (B) and intense burst (I). The horizontal blue bars and associated letters indicate when these different swimming behaviours predominated.

spurious negative  $\dot{M}_{O_{2active}}$  values occurred with a 0.5-min sampling window. Also,  $R^2$  decreased significantly ( $P \leq 0.015$ ) with a sampling window duration  $< 1.5$  min (Fig. 1), so we adopted

1.5 min as a conservative minimum sampling window duration for peak  $\dot{M}_{O_{2active}}$  analysis. Because a universal requirement for fish respirometry is a background  $\dot{M}_{O_2}$  check, the variance of this signal



could be applied broadly with future respirometry studies as the primary criterion to set the sampling window duration. Analysis of a stable  $\dot{M}_{O_2\text{active}}$  of sufficient duration could be an additional quality control.

By not relying on the  $R^2$  value as the sole quality criterion for regression analysis of DO (Svendsen et al., 2016; Chabot et al., 2016a,b), we did not restrict our analysis of  $\dot{M}_{O_2}$  dynamics as rainbow trout approach fatigue. Nevertheless, we included  $R^2$  as a secondary reference criterion. Regardless of the sampling window duration, the lowest  $R^2$  among all our dynamic  $\dot{M}_{O_2\text{active}}$  estimates was 0.77 and 80% of the values were  $\geq 0.90$ , as is commonly prescribed. Importantly, the average  $R^2$  value for all 1.5-min sampling window was 0.95 (lowest=0.91) using a sequential interval regression and 0.94 (lowest=0.83) using an iterative algorithm. Thus, our approach did not markedly sacrifice quality as measured by  $R^2$  alone. The important message is that rather than unnecessarily lengthening the duration of the sampling window to improve  $R^2$  (and possibly underestimate  $\dot{M}_{O_2\text{active}}$ ), an iterative algorithm can better detect dynamics in  $\dot{M}_{O_2\text{active}}$  without rejecting data because of an unsatisfactory  $R^2$ .

A sampling window lasting around 1.5-min may have technical and biological merits. Both the water mixing time in the swim tunnel (e.g. Rodgers et al., 2016) and the blood transit time around the circulatory system of a fish have a similar duration (1–2 min: Cameron and Polhemus, 1974; Maren and Swenson, 1980; Hughes et al., 1981; Harter et al., 2018). Whether 1.5-min is an appropriate minimum sampling window for  $\dot{M}_{O_2}$  measurements in other fish species and test apparatuses can be checked using the methodology applied here, but we recognize that sampling window duration could easily change whenever the signal-to-noise ratio changes with different testing apparatuses.

Despite the constant imposed workload, the  $\dot{M}_{O_2\text{active}}$  of rainbow trout swimming at  $U_{\text{final}}$  (Fig. 2) was dynamic, which contrasted with a steady swimming mode at an intermediate  $U$  (Fig. 2A–C). Indeed, whenever swimming gait varied during  $U_{\text{final}}$ ,  $\dot{M}_{O_2\text{active}}$  usually changed (Fig. 2). Moreover, the dynamic nature of  $\dot{M}_{O_2\text{active}}$  was best revealed using a sampling window duration of 1.5 min in combination with the iterative algorithm. Thus, we reject our null hypothesis that peak  $\dot{M}_{O_2\text{active}}$  is independent of sampling window duration and the mathematical analysis method. Importantly, peak  $\dot{M}_{O_2\text{active}}$  estimated using a 1.5-min iterative algorithm was significantly higher (6–23%;  $P \leq 0.022$ ) than the other analytical approaches used to estimate peak  $\dot{M}_{O_2\text{active}}$  (Table 1). Consequently, we recommend that future studies of  $\dot{M}_{O_2\text{max}}$  in fishes adopt an iterative algorithm using a minimum sampling window duration; one that minimizes variance of background  $\dot{M}_{O_2}$ .

We semi-quantitatively assessed swimming behaviour to examine associations between swimming modes and  $\dot{M}_{O_2\text{active}}$  dynamics. Beyond individual variations (Fig. 2), we discovered clear spikes in  $\dot{M}_{O_2\text{active}}$  that coincided with the swimming modes that produced (or should produce) a ground speed, i.e. combining

burst (B) and intense (I) burst swimming, or transitioning to burst swimming (B). Conversely,  $\dot{M}_{O_2}$  decreased when a fish frequently rested on the rear grid (R), as expected. Thus, our analytical approach opens up the possibility of refining quantitative assessments of oxygen costs associated with swimming behaviour, as opposed to traditional determinations for the oxygen cost at an imposed  $U$ , which was  $616.8 \text{ mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$  over the entire period for  $U_{\text{final}}$  in the present experiment, i.e. approximately 16% lower than the peak  $\dot{M}_{O_2\text{active}}$ . Given that fish likely show a variable willingness of to engage in burst swimming (e.g. Peake and Farrell, 2005), being able to resolve  $\dot{M}_{O_2\text{active}}$  dynamics may be important for studying behaviours that otherwise cannot be easily controlled.

Anaerobic metabolism largely powers burst swimming with fast-twitch, white muscles (Rome et al., 1984, 1985; Rome, 1990; Peake and Farrell, 2004), as evidenced by the accumulation of tissue lactate (Milligan, 1996). Moreover,  $\dot{M}_{O_2}$  appears to peak and rapidly decline shortly after exercise to both exhaustion (e.g. Scarabello et al., 1992; Zhang et al., 2018a,b) and fatigue (Farrell et al., 1998; Wagner et al., 2005; Farrell, 2007; Eliason et al., 2013). Thus, the present finding where  $\dot{M}_{O_2\text{active}}$  peaks in association with periods of burst-and-glide swimming suggests a more rapid and partial repayment of this deferred oxygen cost (excess post-exercise oxygen consumption; EPOC). Unfortunately, our 1.5-min sampling window prevented precise pinpointing of the temporal association of transient peaks in  $\dot{M}_{O_2\text{active}}$  with the semi-quantitative analysis of swimming modes. Regardless, what we know is that venous oxygen content is temporarily depleted in association with burst swimming (Farrell and Clutterham, 2003). Therefore, when this oxygen-depleted blood reaches the gills, it would temporarily increase  $\dot{M}_{O_2\text{active}}$  provided cardiac output and arterial oxygen content are unchanged. Alternatively, white muscle recruitment could consume additional oxygen during burst-and-glide exercise (Moyes et al., 1992). Clearly, a more quantitative analysis of swimming behaviours in conjunction with dynamic  $\dot{M}_{O_2\text{active}}$  could provide a better insight into the mechanistic basis of these peaks in  $\dot{M}_{O_2\text{active}}$  associated with burst swimming during a  $U_{\text{crit}}$  test.

We believe that adopting an iterative algorithm and a minimum duration for the sampling window based on criteria other than  $R^2$  may aid in the on-going deliberations over how best to induce and estimate  $\dot{M}_{O_2\text{max}}$  in fishes (Reidy et al., 1995; Sylvestre et al., 2007; Clark et al., 2013).  $\dot{M}_{O_2\text{max}}$  is also commonly estimated immediately following exhaustive exercise (Norin and Clark, 2016). Yet, as revealed here, the traditional 5–20 min sampling window to estimate  $\dot{M}_{O_2\text{active}}$  during a  $U_{\text{crit}}$  test cannot properly capture  $\dot{M}_{O_2}$  dynamics, and thereby underestimated peak  $\dot{M}_{O_2}$  by 6–23%. Thus, given the rapidity with which  $\dot{M}_{O_2\text{max}}$  decays following exhaustive exercise (Scarabello et al., 1992; Zhang et al., 2018a,b), it seems reasonable to expect that post-exhaustion estimates of  $\dot{M}_{O_2\text{max}}$  would benefit in a similar way from using the analytical rigour of an iterative algorithm and a short sampling window.

**Table 1. Comparison of peak active oxygen uptake (peak  $\dot{M}_{O_2\text{active}}$ ) determined with different sampling window durations (1.5 to 10 min) and two types of mathematical algorithms, an iterative algorithm and a sequential interval regression analysis**

	Iterative algorithm		Sequential interval regression analysis				
	1.5 min	1.5 min	2 min	2.5 min	3 min	5 min	10 min
Peak $\dot{M}_{O_2\text{active}}$ ( $\text{mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$ )	731.5±29.9 <sup>a</sup>	689.9±28.6 <sup>b</sup>	674.3±31.2 <sup>b,c</sup>	668.0±33.9 <sup>b,c</sup>	638.7±28.1 <sup>c</sup>	620.8±28.9 <sup>c,d</sup>	502.7±49.7 <sup>e</sup>
Difference from 1.5-min iterative algorithm (%)		−5.6±1.0	−7.9±1.1	−9.0±1.7	−12.7±1.5	−15.2±1.6	−23.3±1.6

Different superscripted letters denote a statistically significant difference ( $P < 0.05$ ) among the absolute values [repeated-measures (mixed-effects model) one-way ANOVA]. Values are presented as means±s.e.m. for both the absolute value of peak  $\dot{M}_{O_2\text{active}}$  and the percent difference from the peak  $\dot{M}_{O_2\text{active}}$  using the 1.5-min iterative algorithm, where  $n=14$  except for the 10 min estimate of  $\dot{M}_{O_2\text{max}}$ , where  $n=11$ .

Indeed, applying uniform analytical approaches may help reconcile previously reported differences between  $\dot{M}_{O_2\max}$  estimates based on chasing and swimming protocols. For instance,  $\dot{M}_{O_2\max}$  estimated using the  $U_{crit}$  test was 29% lower than the  $\dot{M}_{O_2\max}$  estimate obtained after chasing Atlantic cod (*Gadus morhua*) to exhaustion (Reidy et al., 1995). However, both estimates used a 20-min sampling window, which we suggest is far too long for either protocol for determining  $\dot{M}_{O_2\max}$  given the present results. Conversely, Sylvestre et al. (2007) used an even longer sampling window (20–30 min for both methods) but found no difference in  $\dot{M}_{O_2\max}$  for Atlantic cod with the same testing protocols. Applying new analytical rigour in determining  $\dot{M}_{O_2\max}$  would match the rigour now applied when analysing standard  $\dot{M}_{O_2}$  in fishes (Steffensen, 2002; Chabot et al., 2016a,b; Zhang et al., 2016), which may pave the way for a more accurate estimates of  $\dot{M}_{O_2\max}$  and of aerobic metabolic capacity, a fundamental and influential trait in fish respiratory physiology.

In conclusion, beyond optimizing the respirometer water volume relative to fish mass so that the decline in DO can be easily followed when the respirometer is closed (Clark et al., 2013; Rodgers et al., 2016), we recommend fish respirometry studies use rigorous analysis procedure by examining the variance of background  $\dot{M}_{O_2}$  in a respirometer as a reliable criterion to set minimum sampling window duration for measuring  $\dot{M}_{O_2}$  and applying an iterative algorithm to identify peak  $\dot{M}_{O_2\text{active}}$ . Although we demonstrate this analytical approach is particularly useful for estimating the dynamics of  $\dot{M}_{O_2\text{active}}$  in athletic fishes, we suggest that these procedures and principles likely have a much wider applicability in aquatic respirometry.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: Y.Z., A.P.F.; Methodology: Y.Z.; Software: Y.Z.; Validation: Y.Z., M.J.H.G., A.P.F.; Formal analysis: Y.Z.; Investigation: Y.Z., M.J.H.G.; Resources: A.P.F.; Data curation: Y.Z.; Writing - original draft: Y.Z.; Writing - review & editing: Y.Z., A.P.F.; Visualization: Y.Z.; Supervision: A.P.F.; Project administration: Y.Z., A.P.F.; Funding acquisition: A.P.F.

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#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.196568.supplemental>

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