Finding the best estimates of metabolic rates in a coral reef fish

D.G. Roche[1 *], S.A. Binning[1], Y. Bosiger[2], J.L. Johansen[2] and J.L. Rummer[2]

[1] *Australian Research Council Centre of Excellence for Coral Reef Studies, Division of Evolution, Ecology and Genetics, Research School of Biology, The Australian National University, Canberra ACT 0200 Australia.*

[2] *Australian Research Council Centre of Excellence for Coral Reef Studies, School of Marine and Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia*

***** Author for correspondence: dominique.roche@anu.edu.au

SUMMARY

Metabolic rates of aquatic organisms are estimated from measurements of oxygen consumption rates ($\dot{M}O_2$) through swimming and resting respirometry. These distinct approaches are increasingly used in eco- and conservation physiology studies; however, few studies have tested whether they yield comparable results. We examined whether two fundamental $\dot{M}O_2$ measures, standard metabolic rate (SMR) and maximum metabolic rate (MMR), vary based on the method employed. Ten bridled monocle bream (*Scolopsis bilineatus*) were exercised using (1) a critical swimming speed (U_{crit}) protocol, (2) a 15 min exhaustive chase protocol and (3) a 3 min exhaustive chase protocol followed by brief (1 min) air exposure. Protocol (1) was performed in a swimming respirometer whereas protocols (2) and (3) were followed by resting respirometry. SMR estimates in swimming respirometry were similar to those in resting respirometry when a three-parameter exponential or power function was used to extrapolate the swimming speed-$\dot{M}O_2$ relationship to zero swimming speed. In contrast, MMR using the U_{crit} protocol was 36% higher than MMR derived from the 15 min chase protocol and 23% higher than MMR using the 3 min chase 1 min air exposure protocol. For strong steady (endurance) swimmers, such as *S. bilineatus*, swimming respirometry can produce more accurate MMR estimates than exhaustive chase protocols because oxygen consumption is measured during exertion. However, when swimming respirometry is impractical, exhaustive chase protocols should be supplemented with brief air exposure to improve measurement accuracy. Caution is warranted when comparing MMR

estimates obtained with different respirometry methods unless they are cross-validated on a species-specific basis.

Short title: Estimating fish metabolic rates

Keywords: eco-physiology, oxygen consumption rate, maximum metabolic rate, standard metabolic rate, critical swimming speed, respirometry

INTRODUCTION

Eco-physiology is the study of how organisms respond physiologically to environmental stressors (Fry, 1947; Fry, 1971; Schurmann and Steffensen, 1997; Claireaux and Lefrançois, 2007). Given the prevalence of anthropogenic stressors in natural systems, conservation physiology is rapidly growing as a discipline that aims to better understand and predict organisms' responses to these environmental changes (Wikelski and Cooke, 2006; Kieffer, 2010; Cooke et al., 2012). Respirometry, in particular, is increasingly used by eco-physiologists as advances in technology and equipment accessibility are facilitating studies (Kieffer, 2010), especially in less studied groups such as tropical fishes (e.g., Donelson et al., 2011; Munday et al., 2012). However, as conservation physiology and respirometry continue to grow in popularity, standardized methods must be used to ensure that physiological data are robust and comparisons among studies are valid.

In aquatic respiratory physiology, two types of respirometry chambers are commonly used to conduct either swimming (Fry and Hart, 1948; Blazka et al., 1960; Brett, 1964; Steffensen et al., 1984) or resting respirometry (Teal and Carey, 1967; Hemmingsen and Douglas, 1970; Fry, 1971). Resting respirometry is sometimes also referred to as static respirometry (e.g., Reidy et al., 2000; Brick and Cech, 2002; Barnes et al., 2011), but this terminology is much less common. Despite differences in their complexity and ease of use, both methods allow measuring oxygen consumption rates ($\dot{M}O_2$) to estimate metabolic rates during or following varying levels of activity (e.g., resting vs. active swimming) (Reidy et al., 1995; Peake and Farrell, 2006; Killen et al., 2007). Different calculations can also be used within each method to compute the same estimates of metabolic rate. This probably introduces variation in metabolic rate estimates but studies have yet to carefully examine

whether data obtained in different ways produce comparable results (but see Reidy et al., 1995; Reidy et al., 2000).

Two key physiological parameters characterize the upper and lower bounds of a fish's capacity to uptake oxygen: standard (resting) metabolic rate (SMR or $\dot{M}O_{2,min}$), and maximum metabolic rate (MMR or $\dot{M}O_{2,max}$). SMR corresponds to the minimum maintenance metabolism of a resting fish in a post absorptive state (Fry, 1971; Brett and Groves, 1979; Schurmann and Steffensen, 1997), whereas MMR corresponds to a fish's maximum rate of oxygen consumption (Fry, 1971; Beamish, 1978; Schurmann and Steffensen, 1997; Korsmeyer and Dewar, 2001; Clark et al., 2011). During exercise, MMR is measured at a fish's maximum swimming speed during prolonged swimming (Bushnell et al., 1994; Schurmann and Steffensen, 1997; Korsmeyer and Dewar, 2001), which requires anaerobic metabolism and typically ends in fatigue within 200 min (Beamish, 1978; Peake and Farrell, 2004). In contrast, active metabolic rate (AMR) is a term describing the oxygen consumption rate of fish at their maximum sustained swimming speed (U_{max}). Unlike prolonged swimming, sustained swimming can be maintained for > 200 min and is powered solely by aerobic metabolism (Beamish, 1978; Peake and Farrell, 2004). Beyond U_{max}, fish generally engage in burst-and-coast swimming and $\dot{M}O_2$ begins to asymptote (Sepulveda and Dickson, 2000; Claireaux et al., 2006). As a result, MMR often slightly exceeds AMR since fish are forced to swim beyond their maximum sustained swimming speed for a limited time (Bushnell et al., 1994; Schurmann and Steffensen, 1997). Once measured, SMR and MMR can be used to calculate a fish's aerobic scope for activity (AS), which determines the range of metabolic energy available for aerobic activities (Fry, 1947; Bushnell et al., 1994; Cutts et al., 2002; Claireaux and Lefrançois, 2007; Clark et al., 2011). SMR and MMR exclude metabolic activities powered anaerobically because anaerobic metabolism cannot be measured directly through oxygen consumption at the time of exercise (Korsmeyer and Dewar, 2001).

In swimming respirometry, the most common means of estimating a fish's metabolic rate is using a critical swimming speed (U_{crit}) protocol such as the one initially developed by Brett (1964) (Reidy et al., 1995; Plaut, 2001; Farrell, 2007). Fish are made to swim against a laminar water flow in a swimming respirometer while water velocity is increased incrementally, at regular intervals, until the fish fatigues. The U_{crit} is the swimming speed at which fish become exhausted and stop swimming. Because oxygen consumption is measured

continuously while fish are exercised to exhaustion, swimming respirometry is thought to provide a very accurate estimate of MMR (Farrell and Steffensen, 1987; Plaut, 2001; Shultz et al., 2011). In contrast, SMR is not directly measured using this method, but can be calculated by extrapolating the non-linear swimming speed-$\dot{M}O_2$ relationship to a swimming speed of zero (Bushnell et al., 1994; Reidy et al., 2000; Korsmeyer and Dewar, 2001; Korsmeyer et al., 2002; Binning et al., 2013). Despite many advantages of this method for measuring MMR, U_{crit} protocols can be time consuming and species that are poor swimmers (e.g., ambush predators) often lack the motivation to swim in a respirometer (Peake and Farrell, 2006).

To circumvent the limitations of swimming respirometers, exhaustive chase protocols have been developed to estimate MMR whereby fish are manually chased to exhaustion (Black, 1958; Milligan, 1996; Kieffer, 2000) and immediately placed into a resting respirometer (Cutts et al., 2002; Jordan and Steffensen, 2007; Norin and Malte, 2011). Variations of this method also exist in which fish are briefly held out of the water after chasing (Ferguson and Tufts, 1992; Donaldson et al., 2010; Clark et al., 2012). Air exposure contributes to increasing metabolic demands and has been argued to simulate exercise stress associated with catch-and-release fisheries, where fish are temporarily held out of the water to allow hook removal (Donaldson et al., 2010; Clark et al., 2012). Because the volume of resting respirometers is generally small relative to the size of the fish, individuals tend to remain immobile in the chamber and MMR is measured during recovery from exercise/chasing (Steffensen, 2005). This method relies on post-exercise oxygen consumption rates and MMR therefore corresponds to the sum of the fish's routine metabolic rate (RMR; $\dot{M}O_2$ during activities that elevate SMR (Schurmann and Steffensen, 1997; Steffensen, 2005)) and excess post-exercise oxygen consumption (EPOC) to repay the oxygen debt incurred from anaerobic metabolism during chasing (Killen et al., 2007). One major advantage of using resting respirometry is that SMR can be measured while fish have remained inactive in the chamber for several hours (typically between 2 to 24 hours, depending on the species), thus allowing both SMR and MMR to be calculated in one trial (Cutts et al., 2001; Brick and Cech, 2002; Cutts et al., 2002; Nilsson and Ostlund-Nilsson, 2004; Nilsson et al., 2009; Nilsson et al., 2010; Donelson et al., 2011; Norin and Malte, 2011; Clark et al., 2012).

Some studies suggest or anecdotally report that similar MMR measurements can be obtained using both exhaustive chase protocols and U_{crit} protocols (e.g., Killen et al., 2007;

Gingerich et al., 2010). However, a comprehensive comparison of key metabolic parameters measured with different respirometry methods has yet to be conducted. Here, we compare data obtained using three common methods of measuring SMR and MMR in fishes: (1) a traditional U_{crit} protocol, (2) an exhaustive chase protocol by manual chasing, and (3) an exhaustive chase protocol by manual chasing followed by brief (1 min) air exposure. $\dot{M}O_2$ measurements for protocol (1) were carried out in a swimming respirometer whereas measurements for protocols (2) and (3) were performed in resting respirometers.

MATERIAL AND METHODS

Study site and species

We chose the coral reef fish *Scolopsis bilineatus* (Nempiteridae) for this study due to its high abundance on the Great Barrier Reef (Boaden and Kingsford, 2012; Roche et al., In press), adequate size relative to the respirometry equipment used, and amenable behaviour in the swimming respirometer (Binning et al., 2013). Adult fish were collected by divers using barrier and hand nets between February and March 2012 from reefs surrounding Lizard Island, on the northern Great Barrier Reef, Australia (14° 40’ S; 145° 28’ E). Fish were transported live in buckets to the aquarium facilities at the Lizard Island Research Station within two hours of capture and held in individual aquaria (40.0W× 29.0L× 18.0H cm) with a flow-through water system directly from the reef. Fish were fed once daily with pieces of raw prawn (mean wet weight approx. 1g) and maintained in aquaria for a minimum of three days before the respirometry trials. Length measurements for individual fish were obtained by holding each fish in a plastic bag half-filled with water and measuring total length (TL), body width and body depth with handheld callipers. Body mass (M) was measured directly on a balance. Fish were fasted for 24h prior to the experimental trials (Johansen and Jones, 2011; Shultz et al., 2011) to evacuate the digestive tract and standardize a post-absorptive state that maximizes energy availability for swimming (Niimi and Beamish, 1974). Ten fish (TL= 17.6 ± 0.4 cm; M = 97.0 ± 7.7 g; mean ± s.d.) were subjected to each of three protocols in a random order: a critical swimming speed (U_{crit}) trial (Brett, 1964; Beamish, 1978; Eliason et al., 2011; Johansen and Jones, 2011), a 15 min exhaustive chase trial (see Cutts et al., 2002; Killen et al., 2007; Fu et al., 2009; Norin and Malte, 2011; Shultz et al., 2011), and a three minute exhaustive chase followed by one minute air exposure trial (Ferguson and Tufts, 1992; Donaldson et al., 2010; Clark et al., 2012). The same fish (n=10) were subjected to each protocol following a repeated measures design (Reidy et al., 1995) to minimize inter-

individual variation in metabolic rates. Fish were fed and allowed a minimum of 48 hours to recover between trials. Prior to trials, individuals were starved for at least 24 h, but never more than 36 h. In all three protocols, oxygen consumption rates were measured using intermittent-flow respirometry (Steffensen et al., 1984; Steffensen, 1989).

Swimming respirometry

Swimming trials were carried out in an 11.9 L Loligo flow tank respirometer (swim chamber dimensions 40.0L× 10.0W × 10.0H cm) filled with well-aerated, filtered and UV-sterilized seawater and maintained at a constant temperature of 28 ± 0.1°C (mean ± actual range). Oxygen levels in the respirometer were recorded using a Fibox 3 fiber optic oxygen meter (PreSens, Regensburg, Germany) online feed into the AutoResp 1 Software (Loligo Systems, Copenhagen, Denmark). Flow in the working section of the respirometer was calibrated using a digital TAD W30 flow-meter (Höntzsch, Waiblingen, Germany). Solid blocking effects of the fish in the working section were corrected by the respirometry software (AutoResp, Loligo Systems, Copenhagen, Denmark) following Bell & Terhune (1970). We used 10 min determination periods with a 240s flush, 60s wait and 300s measurement cycle (Binning et al., 2013). Once an individual's length and mass were inputted into the software, three determinations were run without fish to measure initial background rates of respiration from bacterial load in the test chamber. The fish was then placed in the respirometer and left to habituate to the chamber for six to eight hours at a swimming speed of 0.75 BLs^{-1} until oxygen consumption rates stabilized (Johansen et al., 2010; Binning et al., 2013). This speed corresponded to the lowest water flow necessary to ensure constant swimming and minimize spontaneous activity in this species. To start the trial, the flow speed was slowly increased to 1.25 BL s^{-1} and maintained constant for three $\dot{M}O_2$ determinations (see Brett, 1964). Flow speed was incrementally increased by 0.5 BL s^{-1} every three determinations for the duration of the experiment. Trials were complete when fish could no longer maintain their position in the swim chamber and were forced to rest against the back grid of the chamber (U_{crit}) for > 5s (Johansen and Jones, 2011). The time and speed was recorded and the water flow was reduced to 0.75 BL s^{-1} to ensure the fish's recovery from oxygen debt. We calculated U_{crit} following Brett (1964):

$$U_{crit} = U + U_i * (t/t_i) \quad (1)$$

where U is the penultimate swimming speed before the fish fatigued and stopped swimming; U_i is the swimming speed at which the fish was unable to continue swimming; t is the length of time the fish swam at the final swimming speed where fatigue occurred; t_i is the amount of time fish were swam at each speed interval (i.e., 30 min). The fish was then removed from the test chamber and returned to its holding tank. Three additional determinations were run to measure final background rates of respiration in the chamber. Background oxygen consumption rates at the end of each cycle were determined from the slope of the linear regression between initial and final background rates, and were subtracted from each $\dot{M}O_2$ determination. All slopes aside from background respiration rates had an r^2 greater than 0.97. To reduce bacterial growth and respiration in the chamber, the respirometer was drained and rinsed in freshwater when the background consumption rates exceeded 15% of the resting metabolic rate of the fish.

We calculated oxygen consumption rate at U = 0.75 BL s^{-1} following three different methods commonly used in swimming respirometry studies: (1) by averaging the three lowest $\dot{M}O_2$ measurements (SMR_{swim_low}) before increasing U to 1.25 BL s^{-1} (Schurmann and Steffensen, 1997); (2) by averaging the three last $\dot{M}O_2$ measurements (SMR_{swim_last}) immediately before increasing U to 1.25 BL s^{-1} (Binning et al., 2013); and (3) by generating a frequency distribution of $\dot{M}O_2$ (bin size = 5 mg O_2 kg^{-1} h^{-1}) at U = 0.75 BL s^{-1} and averaging values in the lowest mode (SMR_{swim_hist}) to exclude elevated values resulting from spontaneous activity (Steffensen et al., 1994; Korsmeyer et al., 2002; Jordan and Steffensen, 2007; Svendsen et al., 2012). For this third method, a double normal distribution was fitted to a frequency histogram of the raw $\dot{M}O_2$ data: elevated values of $\dot{M}O_2$ corresponding to the first normal distribution were excluded and the second normal distribution with lower $\dot{M}O_2$ values was used to provide an estimate of $\dot{M}O_2$ at U = 0.75 BL s^{-1}. We averaged three $\dot{M}O_2$ measurements in (1) and (2) for consistency with $\dot{M}O_2$ calculations at higher swimming speeds. Oxygen consumption rate ($\dot{M}O_2$) was then plotted against swimming speed (U) to produce an oxygen consumption curve, including only speeds that resulted exclusively in aerobic activity (i.e., from U = 0.75 to U = 3.25 BL s^{-1}). The onset of anaerobic activity was determined as the swimming speed when fish transitioned gait from steady to unsteady (bursting-and-coasting) swimming (Peake and Farrell, 2004). Bursting and coasting was defined when the fish used caudal fin beats (typically 1, 2 or 3 beats) and a subsequent forward glide motion >5 cm. Standard metabolic rate (SMR) was obtained by extrapolating the curve to U = 0 BL s^{-1} (Steffensen et al., 1994; Schurmann and Steffensen, 1997;

Korsmeyer et al., 2002) using either a traditional exponential function (Brett, 1964; Webb, 1975; Korsmeyer et al., 2002; Binning et al., 2013) with two (equation 2) or three (equation 3) parameters, or the hydrodynamics-based power function with three-parameters (equation 4) (Wu, 1977; Videler, 1993; Korsmeyer et al., 2002; Johansen et al., 2010; Svendsen et al., 2010).

$$\dot{M}O_2 = a10^{bU} \quad (2)$$

$$\dot{M}O_2 = a + b10^{cU} \quad (3)$$

$$\dot{M}O_2 = a + bU^c \quad (4)$$

Therefore, for each of the three different functional forms, three SMR estimates were obtained (SMR_{swim_low}, SMR_{swim_last}, SMR_{swim_hist}) following different calculations of $\dot{M}O_2$ at U = 0.75 BL s^{-1}. Maximum metabolic rate (MMR_{swim}) was measured at the maximum swimming speed where fish completed at least one 10 min $\dot{M}O_2$ determination; we averaged $\dot{M}O_2$ values when fish completed more than one determination (up to three determinations).

Resting respirometry

Resting respirometry differs from swimming respirometry in that resting chambers are simpler and more affordable, allowing the benefit of testing multiple fish simultaneously. Individual chambers are connected to flush pumps that turn on intermittently after each $\dot{M}O_2$ determination to replenish the chamber with oxygenated seawater; a closed-loop recirculation pump also mixes the water inside the chamber during $\dot{M}O_2$ determinations. Our resting respirometry system consisted of four darkened cylindrical chambers 3.48 L in volume, fitted with fiber optic oxygen probes and immersed in a temperature-controlled aquarium (100 x 52 x 49 cm; length x width x height) filled with aerated seawater. The water temperature was maintained at 28 ± 0.5°C (mean ± actual range) and dissolved oxygen concentration was recorded with a four channel FireSting O_2 Optical Oxygen Meter (Pyroscience, Aachen, Germany). We used two different methods to estimate MMR. The first consisted of a 15 min exhaustive chase trial in which individual fish were placed in a 110 cm diameter circular tank and chased continuously with a 20 mm diameter pvc tube until exhaustion (i.e., all fish became unresponsive within 12-15 min) (see Cutts et al., 2002; Fu et al., 2006; Killen et al., 2007; Fu et al., 2009; Norin and Malte, 2011). The experimenter would only touch the tail of the fish if it slowed down or stopped swimming. Fish swam primarily with their caudal fin, occasionally bursting-and-coasting. The second method consisted of a 3 min exhaustive chase

followed by 1 min of air exposure (Ferguson and Tufts, 1992; Donaldson et al., 2010; Clark et al., 2012). Fish were chased in an identical manner and subsequently maintained out of the water in a rubber mesh net for 1 min. Following either procedure, each fish was immediately placed in a resting respirometry chamber and their oxygen consumption measured for 5 min. The measurement period started within 10 s from cessation of chasing for the 15 min chase protocol, and 10 s following the end of air exposure for the 3 min chase 1 min air exposure protocol. This $\dot{M}O_2$ measurement corresponded to the maximum metabolic rate: MMR_{chase} or MMR_{air}, depending on the method employed. Subsequently, $\dot{M}O_2$ was measured continuously following a 10 min measurement and 10 min flush cycle. SMR was obtained by leaving the fish in the chamber overnight between 6 and 12 hours, and calculated in one of three ways: (1) by averaging the three lowest $\dot{M}O_2$ measurements recorded (SMR_{rest_low}); (2) by averaging the three last $\dot{M}O_2$ measurements recorded (SMR_{rest_last}); and (3) by generating a frequency distribution of all $\dot{M}O_2$ measurements recorded (bin size = 5 mg O_2 kg^{-1} h^{-1}) and averaging values in the lowest mode (SMR_{rest_hist}). Previous resting respirometry studies have averaged either three (Cutts et al., 2001; Brick and Cech, 2002; Cutts et al., 2002) or six $\dot{M}O_2$ measurements to obtain SMR estimates (Schurmann and Steffensen, 1997; Gingerich et al., 2010; Norin and Malte, 2011; Shultz et al., 2011). We chose to average three measurements for consistency with $\dot{M}O_2$ calculations in the U_{crit} protocol. Oxygen consumption rate ($\dot{M}O_2$ in mg O_2 kg^{-1} h^{-1}) was calculated with LabChart v. 6.1.3 (ADInstruments, Dunedin, New Zealand) as the slope of the linear regression of oxygen concentration decline over time for each determination cycle using the equation (Bushnell et al., 1994; Schurmann and Steffensen, 1997):

$$\dot{M}O_2 = sV_{resp}\alpha M^{-1} \quad (5)$$

where s is the slope (mmHg h^{-1}), V_{resp} is the volume of the respirometer minus the volume of the fish (L), α is the solubility of oxygen in water (μgO_2 L^{-1} $mmHg^{-1}$) adjusted for temperature and barometric pressure and M is the mass of the fish (kg). Three determinations were run before and after each trial to measure background rates of bacterial respiration in individual chambers, which were subtracted from $\dot{M}O_2$ values upon calculation. The system was rinsed in freshwater every third day to insure that background oxygen consumption rates remained below 15% of the resting metabolic rate of fish.

Statistical analysis

We used a linear mixed effects model (LMM; lme function in R) to compare values of maximum metabolic rate (MMR_{swim}, MMR_{chase}, MMR_{air}). We used a second LMM to compare SMR estimates obtained in resting respirometry (SMR_{rest_low}, SMR_{rest_last}, SMR_{rest_hist}) with those obtained in swimming respirometry (SMR_{swim_low}, SMR_{swim_last}, SMR_{swim_hist}) using three different functional forms to describe the relationship between swimming speed and $\dot{M}O_2$ (i.e., a two-parameter exponential function, a three-parameter exponential function and a three-parameter power function). Linear mixed models can be used to reduce inter-individual variation in metabolic rates and control for the non-independence of data points obtained on the same individuals (Bolker et al., 2009). Diagnostic plots and Shapiro-Wilk's test were used to ensure that the data met the assumptions of the models. We compared the fit of non-linear relationships by computing the proportion of variance explained. All analyses were performed in R v2.11.1 (R Development Core Team, 2010).

RESULTS

The mean (± s.e.m.) critical swimming speed for all fish was 3.76 ± 0.10 BL s^{-1}, whereas the mean (± s.e.m.) maximum swimming speed at which fish completed at least one 10 min $\dot{M}O_2$ determination was 3.85 ± 0.10 BL s^{-1}. At 4.25 BL s^{-1}, only three out of ten fish completed one $\dot{M}O_2$ determination. MMR differed according to the respirometry method employed (LMM; $F_{2,18} = 19.2$, $p < 0.001$; Fig. 1A,B): MMR_{swim} was 36% higher than MMR_{chase} (estimate = -167.69, 95% CI = -221.35 to -114.03, t = -6.13, $p < 0.001$) and 23% higher than MMR_{air} (estimate = -107.15, 95% CI = -160.81 to -53.49, t = -3.91, $p = 0.001$). MMR_{air} was significantly higher than MMR_{chase} (estimate = 60.54, 95% CI = 6.88 to 114.20, t = 2.21, $p = 0.04$; Figs 2B, 3A).

SMR estimates obtained in resting respirometry differed based on the calculation method used: SMR_{rest_low} was significantly lower than SMR_{rest_last} (estimate = 23.75, 95% CI = 6.71 to 40.78, t = 2.73, $p < 0.01$) but not different from SMR_{rest_hist} (estimate = 7.84, 95% CI = -9.19 to 24.87, t = 0.90, $p > 0.3$); there was no significant difference between SMR_{rest_last} and SMR_{rest_hist} (estimate = -15.91, 95% CI = -32.94 to 1.12, t = -1.83, $p = 0.07$) (Fig. 2A,B). In contrast, when calculated for each of the three functional forms, SMR estimates obtained in swimming respirometry did not differ significantly, irrespective of the calculation method employed (LMM; all p values > 0.05; Fig. 2B).

Fitting a two-parameter exponential function produced SMR estimates 25% lower, on average, than the lowest SMR estimate obtained in resting respirometry (LMM; contrast group = SMR_{rest_low}; SMR_{swim_low} estimate = -27.49, 95% CI = -44.59 to -10.39, t = -3.15, p = 0.004; SMR_{swim_last} estimate = -24.29, 95% CI = -41.40 to -7.19, t = -2.78, p = 0.01; SMR_{swim_hist} estimate = -22.37, 95% CI = -39.47 to -5.27, t = -2.56, p = 0.016). Alternatively, SMR_{rest_low} did not differ from SMR_{swim_low} when we fit a three-parameter exponential function or a three-parameter power function to the swimming speed-$\dot{M}O_2$ relationship (LMM; all p values > 0.05; Table 1; Fig. 2B). When using either a three-parameter exponential or power function, most differences between SMR obtained in resting versus swimming respirometry occurred between SMR_{rest_last} and SMR_{swim_last} (Table 1; Fig. 2B); there were very few significant differences between SMR_{rest_hist} and SMR_{swim_hist} (Table 1; Fig. 2B). Including $\dot{M}O_2$ measurements at speeds that induced bursting-and-coasting (U = 3.75 and 4.25 BL s^{-1}) into the swimming speed-$\dot{M}O_2$ relationship did not change these results qualitatively.

DISCUSSION

We found notable differences in MMR, a key metabolic rate parameter, measured using different respirometry methods (Fig. 1). Previous studies have suggested that resting and swimming respirometry produce similar MMR estimates (Gingerich et al., 2010), with some support from data on the lumpfish *Cyclopterus lumpus* (Killen et al., 2007). Although there was overlap in SMR estimates obtained with different methods, MMR estimated using a U_{crit} protocol was significantly higher than MMR obtained using two different exhaustive chase protocols combined with resting respirometry.

Using swimming respirometry, SMR is indirectly measured by extrapolating the swimming speed-$\dot{M}O_2$ relationship to U = 0 BL s^{-1} (Brett, 1964; Bushnell et al., 1994; Schurmann and Steffensen, 1997). Following this approach, we used three common calculations to estimate SMR, either by averaging 1) the lowest three $\dot{M}O_2$ measurements at U = 0.75 BL s^{-1} (SMR_{swim_low}), 2) the last three $\dot{M}O_2$ measurements at U = 0.75 BL s^{-1} (SMR_{swim_last}), or 3) all values in the lowest mode of an $\dot{M}O_2$ frequency distribution at U = 0.75 BL s^{-1} (SMR_{swim_hist}). Despite different calculations, the three SMR estimates did not significantly differ from each other (Fig. 2) and considerably overlapped SMR_{rest_low} and SMR_{rest_hist} estimates (Table 1) when extrapolated based on a three-parameter exponential or power function (Fig. 3B,C). SMR_{rest_last} differed from the two other SMR estimates in resting

respirometry because spontaneous activity elevated $\dot{M}O_2$ values in the early morning, towards the end of the trials. This was not the case in swimming respirometry as U_{crit} trials began shortly before sunrise.

In a study on the Atlantic cod, *Gadus morhua*, Schurmann and Steffensen (1997) found similar results when comparing SMR estimated using both swimming and resting respirometry. Our findings also suggest that SMR can accurately be estimated by extrapolating the swimming speed-$\dot{M}O_2$ relationship obtained from U_{crit} protocols. Importantly however, when we fit a more simple, two-parameter exponential function to this data, SMR values estimated with the U_{crit} protocol were ~25% lower than SMR_{rest_low}, irrespective of the calculation employed (Table 1, Fig. 3C). This finding is in stark contrast with those of Korsmeyer et al. (2002), who recommend using the traditional two-parameter exponential function. While this simpler, function requires deriving only two constants (Korsmeyer et al., 2002), it may not be the most reliable functional form to extrapolate $\dot{M}O_2$ beyond the range of swimming speed values measured. In contrast, the hydrodynamics-based power function is believed to overestimate SMR since it places more weight on higher swimming speed values (Videler and Nolet, 1990; Korsmeyer et al., 2002). Our SMR estimates from the hydrodynamics-based power function were higher than estimates from the three-parameter exponential function, but this difference was not significant (Fig. 2).

We obtained higher MMR estimates in the swimming respirometry protocol compared to either exhaustive chase protocols. Several factors may explain this difference. Chasing by an experimenter may not have induced complete exhaustion in *S. bilineatus* even if fish became unresponsive towards the end of the chase. However, the duration of our 15 min protocol greatly exceeded that of typical 1-5 min chases in the published literature (Cutts et al., 2002; Fu et al., 2009; Gingerich et al., 2010; Norin and Malte, 2011; Shultz et al., 2011; Clark et al., 2012). *S. bilineatus* uses its pectoral and caudal fins for swimming and has intermediate to high sustained swimming abilities (Fulton, 2007; Binning et al., 2013). In contrast, many of the fishes that have been subjected to exhaustive chase protocols thus far are body-caudal fin (BCF) swimmers with high unsteady (burst) swimming performance, such as trout (Ferguson and Tufts, 1992; Norin and Malte, 2011), Pacific salmon (Donaldson et al., 2010; Clark et al., 2012), bonefish (Shultz et al., 2011), and bass (Gingerich et al., 2010). Studies suggest that 1-2 min chases are sufficient to achieve complete fatigue in these species (Gingerich et al., 2010; Norin and Malte, 2011; Clark et al., 2012). Rapid exhaustion

most likely occurs because manual chasing induces repetitive burst swimming (Clark et al., 2012), which is powered by white muscle fibres and anaerobic metabolism (Milligan, 1996; Kieffer, 2000). The use of fast, glycolytic muscles for escape swimming explains why these fishes fatigue rapidly and incur large oxygen debts, which can be measured as post-exercise metabolism in the resting respirometry chamber. Alternatively, some BCF swimmers, such as Atlantic salmon (Cutts et al., 2002), carp and catfishes (Fu et al., 2009) can sustain unsteady swimming for longer periods and require chases up to 5 minutes to reach full exhaustion. Pectoral (e.g., Labridae, Scaridae, Pomacentridae, Cichlidae, Embiotocidae) and pectoral-caudal (e.g., Chaetodontidae, Nemipteridae) swimmers may require even longer exhaustive chases, however, as we observed in the case of *S. bilineatus*. Fishes that use their median-paired fins (MPF) for swimming may burst less frequently during chases (e.g., Gotanda et al., 2009) and utilize both red (aerobic) and white (anaerobic) muscle fibres to power their escape. As such, increased use of red muscle could lead to lower oxygen debts and reduced EPOC required to clear metabolites resulting from anaerobic activity. Since the magnitude of EPOC directly influences measurements of MMR in resting respirometry (Reidy et al., 1995), fishes that escape using a combination of white and red muscles will likely display lower $\dot{M}O_2$ values than fishes relying predominantly on white muscle and anaerobic metabolic pathways. Although swimming respirometry appears to be a better method for measuring MMR in fish that are good steady swimmers, the opposite may be true of fish with better unsteady swimming performance (see Peake and Farrell, 2006). For example, in a study of post-exercise metabolic rates in Atlantic cod, Reidy et al. (1995) found that MMR during recovery after exhaustive chasing significantly exceeded $\dot{M}O_2$ measurements at U_{crit}, which is contrary to our results.

Lastly, the short 3 min exhaustive chase and 1 min air exposure protocol yielded higher estimates of MMR than the prolonged 15 min chase. Brief periods of air exposure have been used in a number of studies to simulate fisheries encounters (Ferguson and Tufts, 1992; Donaldson et al., 2010; Clark et al., 2012), and likely push fish beyond their anaerobic threshold, leading to increased EPOC. Given the considerable variability in the duration of fish responses to chase protocols and the likelihood of additional variation from using different chasing techniques and intensity (e.g., tail pinching vs. manual or stick chasing), air exposure may provide a very effective method of standardizing exhaustive chase protocols and improving the accuracy of MMR estimates across species.

Conclusions and recommendations

Our experiment demonstrated that, for *S. bilineatus*, the U_{crit} swimming protocol provided a more accurate estimate of MMR than chase protocols combined with resting respirometry. However, because swimming respirometry is impractical for some species (Reidy et al., 1995; Jordan and Steffensen, 2007), chasing followed by air exposure likely provides the best alternative. Furthermore, we found that SMR can accurately be estimated from data obtained using swimming respirometry. However, extrapolating the oxygen consumption curve depends on the functional form used to describe the swimming speed-$\dot{M}O_2$ relationship. As such, resting respirometers provide a reliable measure of SMR with which to compare estimates from U_{crit} protocols and should be used whenever possible. Additional studies are required to test how data produced with various respirometry methods compare across fish species with different life histories (e.g., demersal vs. pelagic, predatory vs. herbivorous) and swimming behaviours (e.g., pectoral, pectoral-caudal and caudal swimming). However, caution is warranted when comparing results obtained with different approaches, particularly in the case of MMR, unless cross-validation has been performed on a species-specific basis. Bearing in mind these results and the limitations of the methods used, researchers should carefully choose the apparatus and method most appropriate for their species and specific research questions before conducting respirometry studies.

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Table 1. *P* values from LMM contrasts between SMR obtained in resting respirometry (SMR_{rest}) and swimming respirometry (SMR_{swim}). SMR_{swim} was calculated using three different functional forms: a two-parameter exponential function (exp 2), a three-parameter exponential function (exp 3), and a three-parameter power function (power 3). Three different calculation methods were used for all SMR estimates: by averaging 1) the three lowest $\dot{M}O_2$ determinations (low), 2) the three last $\dot{M}O_2$ determinations (last), and 3) $\dot{M}O_2$ values in the lowest mode of an $\dot{M}O_2$ frequency distribution (hist). Significance is denoted by * with α = 0.05.

	SMR_{swim} exp 2			SMR_{swim} exp 3			SMR_{swim} power 3		
SMR_{rest}	**low**	**last**	**hist**	**low**	**last**	**hist**	**low**	**last**	**hist**
low	<0.01*	<0.01*	0.01*	0.24	0.89	0.5	0.78	0.13	0.052
last	<0.001*	<0.001*	<0.001*	<0.001*	0.01*	0.04*	0.02*	0.22	0.44
hist	<0.001*	<0.001*	<0.001*	0.04*	0.44	0.82	0.54	0.55	0.29

667 **FIGURE LEGENDS**

668

669 **Fig. 1** A) Maximum metabolic rate (MMR) for 10 *S. bilineatus* obtained with three different
670 methods: 1) in a swim respirometer (MMR_{swim}), 2) in a resting respirometer after a 15 min
671 exhaustive chase trial (MMR_{chase}), and 3) in a resting respirometer after a three min
672 exhaustive chase trial followed by one min air exposure (MMR_{air}); B) Paired difference
673 between MMR_{swim} and MMR_{chase} as well as MMR_{air}, controlling for the non-independence of
674 measurements on the same fish. Error bars are s.e.m.

675

676 **Fig. 2** A) Standard metabolic rate (SMR) for 10 *S. bilineatus* obtained with two different
677 respirometry methods and three distinct calculations: first, in a resting respirometer (resting)
678 by averaging 1) the three lowest $\dot{M}O_2$ determinations (low, light grey bar), 2) the three last
679 $\dot{M}O_2$ determinations (last, grey bar), and 3) $\dot{M}O_2$ values in the lowest mode of a $\dot{M}O_2$
680 frequency distribution (hist, dark grey bar); second, in a swimming respirometer (swim) by
681 extrapolating the swimming speed-$\dot{M}O_2$ relationship to a speed of zero after averaging 1) the
682 three lowest $\dot{M}O_2$ determinations at U=0.75 BL s^{-1} (low, light grey bar), 2) the three last $\dot{M}O_2$
683 determinations at U=0.75 BL s^{-1} (last, grey bar), and 3) $\dot{M}O_2$ values in the lowest mode of a
684 $\dot{M}O_2$ frequency distribution at U=0.75 BL s^{-1} (hist, dark grey bar). Three different functional
685 forms were fitted to the swimming speed-$\dot{M}O_2$ relationship in swimming respirometry: a two-
686 parameter exponential function (swim exp 2), a three-parameter exponential function (swim
687 exp 3) and a three-parameter power function (swim power 3). B) Paired differences
688 controlling for the non-independence of measurements on the same fish using SMR_{rest_low} as a
689 contrast. Error bars are s.e.m.

690

691 **Fig. 3** The relationship between the rate of oxygen consumption ($\dot{M}O_2$) and swimming speed
692 (U) for the coral reef fish, *S. bilineatus* (n=10). SMR estimates were calculated by
693 extrapolation of A) a two-parameter exponential function (turquoise line; $\dot{M}O_2$ = 62.9 (± 6.45
694 s.e.m.) * $10^{0.21(\pm0.02)\,U}$, r^2 = 0.77); B) a three-parameter exponential function (blue line; $\dot{M}O_2$
695 = 77.72 (± 25.70 s.e.m.) + 14.12 (± 11.50 s.e.m.) * $10^{0.38(\pm0.10)\,U}$, r^2 = 0.78); and C) a three-
696 parameter power function (red line; $\dot{M}O_2$ = 104.13 (± 11.99 s.e.m.) + 7.26 (± 4.75 s.e.m.) * U
697 $^{2.86(\pm0.54)}$, r^2 = 0.78). Intercepts are slightly different from those displayed in Fig. 2, which are
698 mean intercepts for ten relationships. Solid lines indicate relationships based on aerobic
699 swimming only ($0.75 \geq U \leq 3.25$ BL s^{-1}); broken lines indicate extrapolations to U = 0 BL s^{-1}
700 and beyond U = 3.25 BL s^{-1}, at speeds that resulted in bursting-and-coasting (anaerobic

701 metabolism). Symbols are described in the legend and error bars are s.e.m. Values of SMR
702 are offset at U = 0 BL s^{-1} to avoid overlap. Mean U_{crit} is shown as a vertical solid line with
703 broken grey lines indicating s.e.m. Sample sizes are n = 10 at $U \leq 3.25$ BL s^{-1}, n = 9 at U =
704 3.75 BL s^{-1} and n = 3 at U = 4.25 BL s^{-1}.
705