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Chasing away accurate results: exhaustive chase protocols underestimate maximum metabolic rate estimates in European perch *Perca fluviatilis*

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Abstract

Metabolic rates are one of many measures that are used to explain species' response to environmental change. Static respirometry is used to calculate the standard metabolic rate (SMR) of fish, and when combined with exhaustive chase protocols it can be used to measure maximum metabolic rate (MMR) and aerobic scope (AS) as well. While these methods have been tested in comparison to swim tunnels and chambers with circular currents, they have not been tested in comparison with a no-chase control. We used a repeated-measures design to compare estimates of SMR, MMR and AS in European perch *Perca fluviatilis* following three protocols: (a) a no-chase control; (b) a 3-min exhaustive chase; and (c) a 3-min exhaustive chase followed by 1-min air exposure. We found that, contrary to expectations, exhaustive chase protocols underestimate MMR and AS at 18°C, compared to the no-chase control. This suggests that metabolic rates of other species with similar locomotorty modes or lifestyles could be similarly underestimated using chase protocols. These underestimates have implications for studies examining metabolic performance and responses to climate change scenarios. To prevent underestimates, future experiments measuring metabolic rates should include a pilot with a no-chase control or, when appropriate, an adjusted methodology in which trials end with the exhaustive chase instead of beginning with it.

KEYWORDS

aerobic scope, climate change, exhaustive chase, intermittent-flow respirometry, methods, standard metabolic rate

1 | INTRODUCTION

Global climate change is currently increasing the temperature of water bodies across the world and this trend is likely to continue in the future (IPCC, 2014). As ectotherms, fish are affected by these temperature increases due to the dependence of many of their physiological processes on their thermal environment (Woodward *et al*., 2010). One example is metabolic rate, which scales exponentially with temperature (Clarke & Johnston, 1999; Johnston *et al*., 1991) and can be linked to important life history traits (Auer *et al*., 2018) and impact species distribution and fish community structure (Heibo *et al*., 2005; Ohlberger, 2013; Pörtner & Farrell, 2008). Studies on metabolic rates in fish within the context of warming are increasingly popular and are often used to explain how temperature may impact a species' success

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under various climate change scenarios (Clark *et al*., 2012; Eliason *et al*., 2011; Metcalfe *et al*., 2016).

The oxygen and capacity limited thermal tolerance (OCLTT) hypothesis is the subject of ongoing debate (Jutfelt *et al*., 2018), but within the theory's framework, aerobic scope (AS) is used to measure the full potential capacity of an individual's oxygen transport system above standard metabolic rate (SMR) and has been linked to individual performance and fitness (Pörtner *et al*., 2017). Thus, studies examining how aquatic species will physiologically respond to climate change often measure AS. It is typically quantified as the difference between SMR, which is the rate of oxygen consumption in a resting, post-absorptive individual at a given temperature, and the highest rate of oxygen consumption at that same temperature, maximum metabolic rate (MMR). Depending on the research question, however, AS can also be measured in factorial terms ($AS_{factorial}$ = MMR/SMR) (Halsey *et al*., 2018). In addition to being used to calculate AS, MMR alone has been used to predict future success under climate change scenarios. For example, a thermally fixed MMR may indicate low adaptive capacity and predict limited success under warmer conditions (Sandblom *et al*., 2016) while the MMR of an individual compared with others in the same school can help determine spatial positioning within the school and impact food intake (Killen *et al*., 2012). No matter the framework, for study results to be used to accurately predict the future success of individual species the methods for determining MMR, SMR and subsequently AS must be accurate.

There is a wide range of studies focused on constructing the optimal aquatic respirometer and how to most accurately measure MMR in fishes (Clark *et al*., 2013; Norin & Clark, 2016; Roche *et al*., 2013; Rummer *et al*., 2016; Svendsen *et al*., 2016). Two well-established respirometer designs are the swim tunnel, in which a fish swims against a current generated within the respirometer, and the static respirometer, in which the fish's movement is largely restricted. Both have advantages and disadvantages depending on the focal species and study design. The first study to examine the difference between a swim tunnel and a static respirometer with respect to MMR following an exhaustive chase protocol was performed on cod (Reidy *et al*., 1995). This study showed a higher MMR in cod *Gadus morhua* L. following a chase protocol but hypothesized that this could be due to excessive stress associated with the protocol and that measurements in the swim tunnel were more comparable to natural conditions (Reidy *et al*., 1995). Swim tunnel respirometry has since become a popular method for measuring MMR in species with an active lifestyle that involves continuous swimming and is useful in that it allows measurements both during the period of exercise and immediately following it (Clark *et al*., 2013; Killen *et al*., 2017; Norin & Clark, 2016). For fish that do not naturally swim for prolonged periods, using an exhaustive chase protocol has become a common alternative (Clark *et al*., 2013; Killen *et al*., 2017; Rummer *et al*., 2016). Studies have shown these chase protocols produce values within the same range as those produced *via* burst performance and critical swimming speed protocols in a swim tunnel respirometer (Killen *et al*., 2007, 2017; Sylvestre *et al*., 2007, but see Rummer *et al*., 2016) and that oxygen consumption rate ($\dot{M}O_2$) following exercise is higher than during the exercise itself (Norin & Clark, 2016).

Our focal species European perch *Perca fluviatilis* L. is routinely exercised using an exhaustive chase protocol to determine MMR since it has been cited as unwilling to swim against the current in a swim tunnel (Brijs *et al*., 2015; Jensen *et al*., 2017; Sandblom *et al*., 2016). In past experiments, *P. fluviatilis* has been manually chased till exhaustion (qualified as unresponsive to tactile stimuli) over a period of 1–5 min and then placed in a size-matched intermittent-flow respirometry chamber where the individual is left to recover to its SMR for between 10 and 48 h (Baktoft *et al*., 2016; Brijs *et al*., 2015; Christensen *et al*., 2017; Jensen *et al*., 2017; Sandblom *et al*., 2016). This study aimed to quantify the difference in MMR and AS obtained using exhaustive chase protocols compared to a no-chase control and to establish the most appropriate method to induce maximum oxygen consumption in *P. fluviatilis* at our experimental temperature of 18°C. We compared two of the most common methods used to elicit MMR outside of a swim tunnel, an exhaustive chase and an exhaustive chase followed by 1-min air exposure (Norin & Clark, 2016), with a no-chase control. We compare the MMR, SMR and AS following the 'exercise' protocols with those from a no-chase protocol in which fish were transferred directly from their home tank to a static respirometry chamber. Although there are studies comparing the efficacy of exhaustive chase protocols with a variety of swim tunnel protocols, to the best of our knowledge no prior study has compared the results of MMR following 'exhaustive chase' methods with MMR achieved following a 'no-chase' control.

2 | MATERIALS AND METHODS

All fish collection and experiments were performed under evaluation and permission from the Uppsala Authority for the Ethics of Animal Experimentation (ethics licence #C59/15).

2.1 | Experimental animals

P. fluviatilis were collected *via* angling and beach seining from Lake Erken (59°50′N, 18°33′E) in Sweden during August 2018. After transport to Uppsala University Laboratory (Uppsala, Sweden) fish were anaesthetized using 60 mg L^{-1} benzocaine, individually tagged with coloured elastomer at the base of their caudal fin, and the weight (g) and length (mm) were measured. Individuals were housed with similar sized conspecifics in 105 l (75 × 40 × 35 cm), flow-through aquaria at 16–18°C, with a 16 h light (L):8 h dark (D) cycle and fed to satiation daily [frozen chironomids (Ruto Frozen Fishfood, Netherlands)] for 5 months before experiments began.

One week prior to starting metabolic measurements, *P. fluviatilis* [n = 15, 170 ± 18 mm, 48.9 ± 15 g (means ± S.D.)] were weighed, measured and divided into five groups of three similar-sized individuals which were housed together to simplify size-matching of fish to respirometry chambers and allow easy recapture of individuals tested on the same dates. The water in the tanks in which fish were housed was maintained at 18 ± 0.5°C using thermostat heaters starting

1 week before trials began and for the duration of the experiment. Aside from the stress experienced during chase and chaise + air protocols, stress was minimized for the duration of captivity and over the course of the experiment. Fish were sacrificed at the end of the experiment using an overdose of benzocaine.

2.2 | Respirometry setup

Metabolic rates were measured using intermittent flow respirometry based on the protocols described by Clark *et al.* (2013) and Svendsen *et al.* (2016). The set-up comprised four acrylic respirometry chambers (internal diameter × length 72 × 220 mm or 72 × 185 mm, size-matched to the fish) which were submerged in two aquaria (75 × 30 × 30 cm and 75 × 30 × 20 cm) (two chambers per tank, four total respirometers per trial) containing water and air-stones to maintain oxygen at air-saturation levels. Water within the system was maintained at 18.1 ± 0.03 (mean ± S.D.) using a pump controlled *via* AutoResp software (LoligoSystems, Viborg, Denmark), which pumped water through a metal coil submerged in a heated bath when the system's temperature dropped below 18.0°C. Water was recirculated between the two aquaria using a UV-filter (Eheim ReeflexUV 350, Deizisau, Germany) to reduce background respiration caused by bacterial growth. Each chamber was connected to a flush pump (Eheim 1046, 5 l/min) and a recirculation loop comprised of a pump (Eheim 1046, 5 l min^{-1}), PVC tubing (53.3 ± 0.96 ml, mean ± S.D.) and a flow-through oxygen cell. Oxygen concentration was measured using a Wiltrox4 oxygen meter (LoligoSystems) in conjunction with fibre-optic optodes attached to the flow-through oxygen cells. Each measurement loop lasted for 420 s and consisted of a 180 s flush phase, a 30 s wait phase and a 210 s measurement phase. To prevent the build-up of microbes over the course of the experiment, the system was cleaned between each trial using bleach. After removing the oxygen mini sensors, ~15 ml of bleach was added to each tank and flushed through the system for 5 min. This was followed by thoroughly rinsing the system with fresh water three times. After cleaning, the system was refilled with tap water that had been aerated and maintained at 18 ± 0.5°C for a minimum of 24 h.

2.3 | Protocol description and experimental schedule

Each individual fish was tested using all three protocols following a blocked Latin square experimental design (Figure 1) to counterbalance any possible effects of treatment order. Fish were fasted for 23–25 h prior to the start of each trial, at which point three fish were removed simultaneously from their home tank using a mesh net and placed in a red opaque 9 l bucket with aerated tap water at 18 ± 0.5°C. One of three protocols, A, B, or C, was then performed on each individual before they were placed in the respirometer at the beginning of the 30 s wait phase. The wait phase is necessary to account for a lag in the system which can result in a nonlinear oxygen curve (LoligoSystems, 2020) and fish were placed in the chamber during this phase

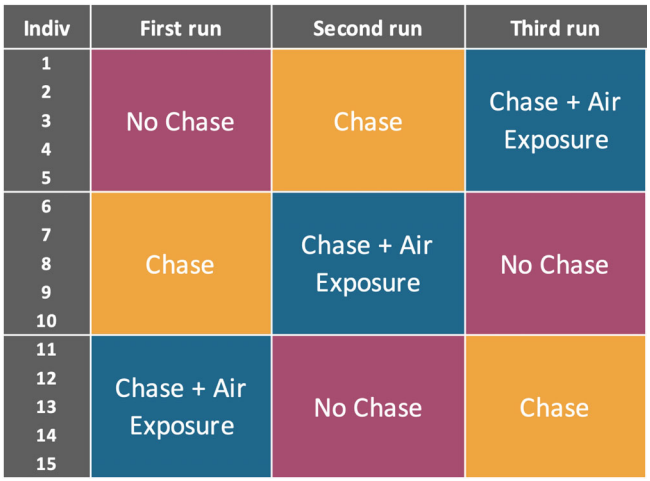

FIGURE 1 Schematic view of the experimental design: maroon, no-chase; yellow, chase; blue, chase + air

so that the first measurement phase would be linear and thereby included in the analysis. Fish remained in the respirometry chambers in a dark room, overnight for a minimum of 17 h and 32 min. The fourth respirometry chamber remained empty for the duration of the trial in order to measure background respiration.

Protocol A, no chase: The individual was transported from its home tank (submerged in water) and placed directly into the respirometer during the wait phase (Chabot *et al.*, 2016). The individual assigned this protocol was always placed in its respirometry chamber first.

Protocol B, chase: The individual was placed in a circular arena (outer diameter = 50 cm with a clear acrylic cylinder in the middle of the arena inaccessible to the fish, diameter = 14 cm, water depth = 12 cm) with aerated tap water at 18 ± 0.5°C and then chased manually with a hand net for 3 min (Brijs *et al.*, 2015; Sandblom *et al.*, 2016; Svendsen *et al.*, 2016). Fish swam away when approached with the hand net at the beginning of the chase and became unresponsive to being tapped on the caudal fin by the end of the chase period. Following the chase, individuals were removed from the arena, transported (submerged in water) and placed in the respirometry chamber during the 30 s wait phase. The first measurement phase commenced approximately 35 s after the end of the chase.

Protocol C, chase + air exposure: The individual was chased following the method described in Protocol B. Following the chase, fish were scooped into a mesh net and held out of the water for 1 min (Clark *et al.*, 2012, 2013; Rummer *et al.*, 2016), after which the individual was placed in the respirometry chamber during the wait phase. The first measurement phase commenced approximately 30 s from the end of air exposure.

2.4 | Determination and calculations of MMR, SMR and AS

Since trials ran for a nonuniform amount of time [18 h and 50 min ± 1 h and 45 min (mean ± S.D.)], raw data was cut to 17 h and

32 min (length of the shortest trial) and measures of $\dot{M}O_2$ were calculated from this reduced data set. Nonmass-specific $\dot{M}O_2$ ($mgO_2\ h^{-1}$) was estimated from the linear decline in dissolved oxygen over each 210 s measure phase using AutoResp software (version 2.2.0, LoligoSystems). To correct for background respiration a linear regression was fit to all $\dot{M}O_2$ measures from the empty chamber in each trial. Fitted values estimating background respiration at each time point were subtracted from measures of $\dot{M}O_2$ for each fish at the corresponding time point. These background-corrected measures of $\dot{M}O_2$ were then divided by individual fish weight to calculate mass-specific $\dot{M}O_2$ ($mgO_2\ kg^{-1}\ h^{-1}$). Estimates of $\dot{M}O_2$ with $R^2 < 0.95$ were removed prior to calculating SMR, MMR and AS.

SMR was calculated as the mean of the lowest 10% of $\dot{M}O_2$ measures (Baktoft *et al*., 2016). MMR was calculated as the global $\dot{M}O_2$ maximum (the highest $\dot{M}O_2$ recorded at any time over the course of the 17 h and 32 min reduced trial period) (Supporting information Figures S1 and S2). AS was calculated as the absolute aerobic scope, MMR – SMR. An additional measure of maximum metabolic rate (MMR_3) was calculated as the highest $\dot{M}O_2$ of the first three measures after placing the fish in the respirometry chamber (Baktoft *et al*., 2016). For individual 3 (yel-oj) from the chase + air treatment there is no MMR value and subsequently no AS value due to failure to start the respirometry software before placing the fish in the respirometry chamber (Supporting information Figure S1). R-code for calculations of all metabolic measures is available on the data repository Zenodo, DOI: 10.5281/zenodo.3873396.

2.5 | Statistical analyses

We used linear mixed-effects models to analyse the data set using package *lme4* (Bates *et al*., 2015) in R. Factors "treatment" and "treatment order" were set as fixed effects with three levels: treatments A, B and C, treatment orders ABC, BCA and CAB. "Fish identity" was set as a random effect to account for individual variation between fish. The interaction term Order:Treatment was nonsignificant for all metabolic measures (Supporting Information Table S1) and was therefore not included in the model. Adjusted repeatabilities (*R*), which control for variance caused by fixed effects Treatment and Order, were calculated for each metabolic measure using rptR (Stoffel *et al*., 2017) with a bootstrap value of 1000. The normality of residuals was verified with Shapiro–Wilk normality tests in package *stats* (R Core Team, 2019) and homogeneity of variance was verified using Levene's test in package *car* (Fox & Weisberg, 2019). A Grubbs test using package *outliers* (Komsta, 2011) was used to test for outliers. An ANOVA using package *car* (Fox & Weisberg, 2019) was used to test the significance of "treatment" and "treatment order" using type II Wald F tests with Kenward-Roger df. Finally *post hoc* pairwise comparisons using Tukey's method within package *emmeans* (Lenth, 2019) were used to test for differences in metabolic responses between treatments. Raw data was imported using package *rMR* (Moulton, 2018) and plots were created using *ggplot2* (Wickham, 2016). All analyses were performed in R version 3.5.3 (R Core Team, 2019).

3 | RESULTS

We found a highly significant effect of treatment on MMR and AS (Table 1 and Figure 2b,c). There was a marginally nonsignificant effect of treatment on SMR (Table 1 and Figure 2a). The order in which individual fish received each treatment was not significant for SMR, MMR or AS (Table 1).

Pairwise comparisons using HSD–Tukey *post hoc* analyses showed that MMR was significantly higher in no-chase compared to both chase and chase + air treatments (Supporting Information - Table S2 and Figure 2b). There was no significant difference in MMR following chase compared to chase + air treatments (Supporting Information Table S2 and Figure 2b). The MMR_3 also show that MMR following no-chase was significantly higher than following chase and chase + air protocols (Supporting Information Table S2), but that there was no significant difference between MMR_3 in chase and chase + air treatments (Supporting Information Table S2).

This significant difference between MMR in different treatments was reflected by the pairwise comparisons of AS, which also show higher estimates in no-chase treatments compared to chase and chase + air treatments (Supporting Information Table S2 and Figure 2c), but no significant difference in fish oxygen consumption between chase and chase + air treatments (Supporting Information - Table S2 and Figure 2c). Using the chase protocol resulted in a 16% underestimate of MMR and a 21% underestimate of AS compared to the no-chase control (Table 2). Using the chase + air protocol also resulted in underestimates of MMR (16%) and AS (24%) compared to the no-chase control (Table 2). There was no significant difference between the SMR values in no-chase and chase treatments (Supporting Information Table S2 and Figure 2a). The chase + air treatment, however, gives higher estimates of SMR compared to both no-chase and chase treatments which are only marginally nonsignificant (Supporting Information Table S2 and Figure 2a).

Individual fish showed high adjusted repeatability of MMR ($R = 0.56$, S.E. = 0.15, $P < 0.001$), MMR_3 ($R = 0.75$, S.E. = 0.11, $P < 0.001$) and AS ($R = 0.55$, S.E. = 0.15, $P < 0.001$) across trials. However, they had low adjusted repeatability of SMR ($R = 0.27$, S.E. = 0.17, $P = 0.086$).

4 | DISCUSSION

Measuring oxygen consumption rate ($\dot{M}O_2$) directly following an exhaustive chase protocol is thought to serve as the best measure of MMR using a static respirometry set-up because during this period the fish will be at peak $\dot{M}O_2$ as it recovers and pays off the oxygen debt created during the chase protocol (Clark *et al*., 2013). However, we found that, under our study conditions, both chase and chase + air treatments underestimated MMR by an average of 16% and resulted in subsequent underestimates of AS (chase 21% and chase + air 24%) compared to the no-chase control group when calculating MMR based on the global maximum during the trial. The pattern of lower estimates of MMR following chase protocols even occurs when MMR

TABLE 1 Output of a linear mixed model with fixed factors Order and Treatment and random factor FishID

Factor	SMR		MMR		AS		MMR_3	
Order	**$F_{(2,12)}$ = 0.36**	**P = 0.70**	**$F_{(2,12)}$ = 0.19**	**P = 0.83**	**$F_{(2,12)}$ = 0.22**	**P = 0.80**	**$F_{(2,12)}$ = 0.41**	**P = 0.67**
Treatment	$F_{(2,28)}$ = 3.10	P = 0.061	$F_{(2,27)}$ = 11.95	P < 0.001	$F_{(2,27)}$ = 14.1	P < 0.001	$F_{(2,27)}$ = 13.3	P < 0.001

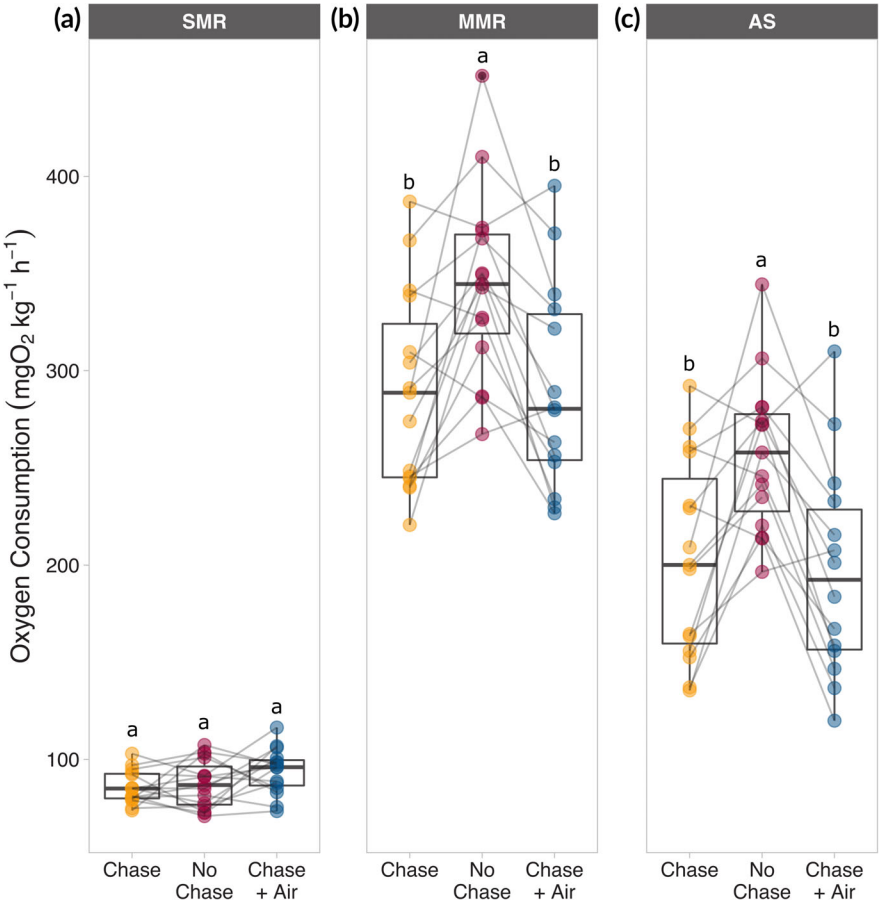

FIGURE 2 Boxplots showing the median and interquartile range of (a) standard metabolic rate (SMR, calculated from the lowest 10% of $\dot{M}O_2$ measures), (b) maximum metabolic rate (MMR, the global maximum $\dot{M}O_2$ measurement) and (c) aerobic scope (AS, calculated as MMR − SMR) measured in each of the three treatments: maroon, no-chase; yellow, chase; blue, chase + air. Different letters indicate significant differences (α = 0.05)

TABLE 2 The least square means estimates and standard errors (S.E., calculated from the raw data) of metabolic measurements

	SMR	MMR	AS	MMR_3
Protocol	Estimate ± S.E.	Estimate ± S.E.	Estimate ± S.E.	Estimate ± S.E.
No chase	87.4 ± 3.19 ^{a}	345 ± 12.9 ^{a}	257 ± 10.5 ^{a}	281 ± 8.34 ^{a}
Chase	85.6 ± 2.21 ^{a}	289 ± 13.7 ^{b}	204 ± 13.8 ^{b}	248 ± 9.08 ^{b}
Chase + air	93.9 ± 3.04 ^{a}	291 ± 14.2 ^{b}	196 ± 14.6 ^{b}	258 ± 9.93 ^{b}

Note: Units for all metabolic measures are (mgO_2 kg^{-1} h^{-1}). Different letters indicate a significant difference (α = 0.05).

is measured based on the first three measures of $\dot{M}O_2$ after placing fish in the respirometry chamber (MMR_3) (chase 12% and chase + air 8%). Thus, our study serves as a call to thoroughly test that methods intended to elicit maximum oxygen consumption are having their desired effect. Our results suggest that a no-chase protocol should be included in pilot studies for any fish species that is tested using an exhaustive chase protocol, as has been recommended for small or nonathletic species (Clark *et al.*, 2013), benthic species and ambush predators (Norin & Clark, 2016), and any other species unwilling to swim in swim-tunnel style respirometers (Brijs *et al.*, 2015; Killen *et al.*, 2007). A potential limitation in our study design is that our chase protocols using a hand-net could be considered mild compared to studies which chase fish by hand and include tail pinching to provoke a strong swimming response (Mochnacz *et al.*, 2017; Roche *et al.*, 2013; Rosewarne *et al.*, 2016).

Oxygen consumption rates following exhaustive chase protocols were less variable than those following the no-chase protocol. Supporting Information Figure S1 shows the difference in oxygen

consumption over time, including the later peaks in $\dot{M}O_2$ in the no-chase treatment (Supporting Information Figure S2), which is assumed to be the result of spontaneous activity. The more limited range of oxygen consumption rates following an exhaustive chase could be the result of stress or a prolonged recovery from anaerobic exercise induced during the chase.

The marginally nonsignificant overestimate of SMR following the chase + air protocol compared to both the no-chase and chase protocols indicates that air exposure may be preventing fish from fully recovering within the timeframe of our experiment. This suggests that chase + air exposure should only be used in cases in which air exposure is an important part of the study question, as opposed to using it to enhance the effect of an exhaustive chase. However, the lack of a significant difference between SMR following the chase compared to no-chase protocols indicates that recovery from an exhaustive chase alone did not prevent fish from reaching their SMR over the duration of the study period, a concern that has been raised previously by Norin and Clark (2016).

We found that when calculating MMR using the global maximum $\dot{M}O_2$, the time at which MMR was reached was spread across the 17 h trial (Supporting Information Figure S2). This indicates that future studies should take advantage of the high temporal resolution of modern respirometry set-ups and include measurements from over the course of the entire trial instead of limiting MMR calculations to the measures immediately following the chase. Jensen *et al*. (2017) found differences in the time MMR was reached between trials at different temperatures. At low water temperatures (5 and 10°C) *P. fluviatilis* MMR always occurred immediately following the chase protocol, while at higher temperatures (15–27°C) MMR could also occur spontaneously over the course of the trial. To account for these differences, we propose future experiments use a method discussed by Norin and Clark (2016) in which the fish is placed directly in the respirometer for a long series of measures and then removed from the respirometer for a chase protocol and returned for a short measurement period following the chase. By taking the global maximum from both portions of the trial, underestimates from both, an unnecessary chase at high temperatures and a lack of chase at low temperatures could be avoided.

Accurate measurements are needed to make accurate predictions of species success under climate change scenarios, especially when making comparisons between species. Measuring MMR and AS are important aspects of conservation physiology, which seeks to understand and predict the ability of different species to survive under a range of current and future thermal regimes (Cooke *et al*., 2013). Many studies have been focused on creating respirometry systems and methods which give accurate estimates of MMR in part so that future predictions within this realm of conservation will be accurate. Our results suggest that future studies using static respirometry should use a no-chase control during pilot studies to determine whether any exhaustive chase protocol is needed. Additionally, studies with multiple temperatures or multiple species could use a chase protocol at the end of the experiment and use the global maximum $\dot{M}O_2$ measure to prevent underestimates.

DATA AVAILABILITY STATEMENT

Data including the AutoResp files (cut to 17 h and 32 min) for each fish and R scripts used for analysis are available on the openly accessible repository Zenodo under DOI: 10.5281/zenodo.3873396.

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CONTRIBUTIONS

All authors contributed to manuscript preparation. M.A.: fish collection, project conception and design, and data analysis; F.S.: data collection, data analysis and interpretation of data; P.E.: interpretation of data and funding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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