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Influence of photoperiod and protocol length on metabolic rate traits in ballan wrasse *Labrus bergylta*

Malthe Hvas 🄳

Animal Welfare Research Group, Institute of
Marine Research, Matre, Norway

Correspondence
Malthe Hvas, Animal Welfare Research Group,
Institute of Marine Research, 5984 Matre,
Norway.
Email: malthe.hvas@imr.no

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Abstract

In this study, ballan wrasse *Labrus bergylta* were subjected to either a conventional
1-day or an extended 5-day respirometry protocol. Additionally, in the 5-day protocol
the fish were subjected to a 12 h light–dark cycle to assess the effects of photope-
riods on metabolic rates ($\dot{M}O_2$). Diurnal patterns in routine and resting $\dot{M}O_2$ were not
observed, suggesting that circadian rhythms in metabolism largely are driven by activ-
ity patterns rather than being of endogenous origin. Moreover, lack of a detectable
circadian $\dot{M}O_2$ may be an adaptation to lower costs of living in ballan wrasse. Proto-
col length influenced standard metabolic rates (SMR) where estimates decreased by
13% and 17% when using 48 h and 5 days, respectively, compared to 24 h. The maxi-
mum metabolic rate (MMR) and the derived absolute aerobic scope (MMR-SMR)
were unaffected by protocol length. However, factorial scopes (MMR/SMR) were
reduced from 8.5 to 6.4 in the 5-day protocol, showing that factorial scopes are more
sensitive to how SMR are obtained. The critical oxygen tension (P_{crit}) was reduced
from 15% PO_2 in the 1-day group to 11% PO_2 in the 5-day group. However, $\dot{M}O_2$ in
response to decreasing PO_2 was similar, which together with a similar oxygen extrac-
tion coefficient, α ($\dot{M}O_2/PO_2$), suggested that the higher P_{crit} in the 1-day group was
an artefact of overestimating SMR. Finally, α was 12% lower at MMR compared to at
P_{crit}, which either means that MMR was underestimated in proportion to this differ-
ence or that α is not constant in the entire PO_2 range. In summary, this study found
that a conventional 1-day respirometry protocol may overestimate SMR and thereby
alter the derived P_{crit} and aerobic scope, while α is unaffected by protocol length.
Moreover, alternating light conditions in the absence of other stressors did not influ-
ence $\dot{M}O_2$ in ballan wrasse.

KEYWORDS
aerobic scope, circadian rhythm, hypoxia, oxygen extraction coefficient, P_{crit}, respirometry

1 | INTRODUCTION

Many animals display a circadian rhythm in numerous biochemical and
physiological traits that is influenced by natural photoperiods that
allow them to anticipate temporal changes in their environment
(Albrecht, 2012; Hastings *et al.*, 2007). For instance, in fish, melatonin
release rates from the photoreceptive pineal organ are largest in the
dark (Falcón *et al.*, 2010; Iigo *et al.*, 2007), while other hormones such

as cortisol, ghrelin and leptin also tend to follow a circadian rhythm (Isorna *et al.*, 2017; Pickering & Pottinger, 1983). These and other endogenous oscillators may synchronize with daily variation in foraging and resting behaviours, and thereby help govern diurnal patterns in anabolic and catabolic processes (Nisembaum *et al.*, 2014; Paredes *et al.*, 2014; Vatine *et al.*, 2011). Integrating metabolism to the whole-animal level should therefore reveal predictable patterns in energy usage on a 24 h cycle.

Evidence for such metabolic patterns can be seen in the heart rates of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) and Atlantic salmon *Salmo salar* (L. 1758), which follow a pronounced circadian rhythm with lower heart rates at night (Brijs *et al.*, 2018; Hvas *et al.*, 2020a), suggesting lower energy expenditure when it is dark in these species. Similarly, in Nile tilapia *Oreochromis niloticus* (L.) and the surface form of the Mexican tetra *Astyanax mexicanus* (De Filippi 1853) metabolic rates are highest during light periods (Moran *et al.*, 2014; Ross & McKinney, 1988), while in lake sturgeon *Acipenser fulvescens* (Rafinesque 1817) metabolic rates peak at dawn (Svendsen *et al.*, 2014). However, the eyeless cave form of the Mexican tetra lacks a circadian metabolism, which may be an adaptation for constant dark environments (Moran *et al.*, 2014).

Diurnal changes in activity levels could perhaps be the main driver for circadian whole-animal metabolic rates. For instance, Atlantic salmon are known to swim more slowly at night (Hansen *et al.*, 2017; Oppedal *et al.*, 2001), coinciding with the above-mentioned lower heart rates. However, measurements of metabolic rates over several days while accounting for activity and feeding have revealed circadian rhythms of strictly endogenous origin in other species (Kim *et al.*, 1997; Moran *et al.*, 2014; Ross & McKinney, 1988). Furthermore, such rhythmicity tends to be larger when using alternating light–dark periods but is still present at a depressed rate in constant darkness (Ross & McKinney, 1988). Hence, circadian rhythms in metabolic rates appear to be caused by both endogenous (*e.g.*, hormones) and exogenous (*e.g.*, photoperiod) factors, while daily fluctuations in energy usage should be further enhanced when fish engage in their natural diurnal feeding and swimming behaviours.

Metabolic rates in fish are usually estimated indirectly with respirometry by measuring oxygen uptake rates ($\dot{M}O_2$) (Clark *et al.*, 2013; Steffensen, 1989). Provided that the experimental settings are adequate, various $\dot{M}O_2$ traits of interest can be measured, such as the standard metabolic rate (SMR), the maximum metabolic rate (MMR), the aerobic scope (AS) and the critical oxygen tension (P_{crit}). The SMR is the minimum energetic requirement to maintain basal homeostasis in inactive and non-digestive fish at their acclimation temperature (Chabot *et al.*, 2016). The MMR is the highest rate of aerobic metabolism achieved during strenuous activities or intensive stress (Norin & Clark, 2016). The AS is the difference between MMR and SMR, and signifies the capacity to perform fitness-related aerobic activities (Clark *et al.*, 2013; Fry, 1971). The P_{crit} is the point where $\dot{M}O_2$ becomes dependent on ambient oxygen levels and is commonly used as an indicator of hypoxia tolerance (Rogers *et al.*, 2016; Speers-Roesch *et al.*, 2013).

Owing to the popularity of respirometry in diverse areas of fish biology, significant attention has been given to methodological

approaches where several studies have investigated impacts of respirometry designs, protocols, calculations and fish husbandry practices when measuring $\dot{M}O_2$ traits (Clark *et al.*, 2013; Reidy *et al.*, 1995; Rummer *et al.*, 2016; Svendsen *et al.*, 2016; Zhang *et al.*, 2020). Ideal methodology will also depend on species and the overall purpose of the experiment (Hvas & Oppedal, 2019a; Killen *et al.*, 2017). As such, the best approaches are still being debated, and any two unrelated respirometry studies are likely to differ in methods regardless of them reporting the same parameters.

The SMR can be particularly difficult to measure owing to its requirements of the fish being in an inactive and nondigestive state, since such experimental conditions can be hard to satisfy in practice (reviewed by Chabot *et al.*, 2016). For instance, introduction into a confined resprimeter is stressful and several hours are usually needed for the fish to calm down and acclimate to the experimental setup, meaning that accurate SMR measurements are time-consuming and typically are completed over a 24 h period. Moreover, to satisfy the requirement of being in a nondigestive state, fish are typically fasted for 1–2 days prior to experimental trials, which generally should be sufficient time to avoid overestimating SMR owing to confounding specific dynamic action effects.

Recently, interpretations and usefulness of the P_{crit} as an indicator of hypoxia tolerance have been under scrutiny owing to seemingly arbitrary methods of calculation and an apparent disconnect between naturally encountered hypoxia of fish species and their P_{crit} (Reemeyer & Rees, 2019; Regan *et al.*, 2019; Seibel *et al.*, 2021; Wood, 2018). Its definition may also vary in nuances between the point where $\dot{M}O_2$ becomes dependent on ambient oxygen levels (*e.g.*, Speers-Roesch *et al.*, 2013) and the point where $\dot{M}O_2$ falls below SMR as measured in normoxia (*e.g.*, Ern *et al.*, 2016). Here, the former defines P_{crit} for a range of activity levels where a specific $\dot{M}O_2$ can be maintained, while the latter is a stricter definition that only pertains to SMR conditions. Using SMR to define P_{crit} means that P_{crit} will be influenced by any methodological flaws when estimating SMR, while using any routine $\dot{M}O_2$ to define P_{crit} arguably is too dependent on arbitrary contexts.

A proposed way to circumvent issues with methodology and interpretations of the P_{crit} is the introduction of the oxygen extraction coefficient, α ($\dot{M}O_2/PO_2$) (Seibel *et al.*, 2021; Seibel & Deutsch, 2020). Rather than being a measure of hypoxia tolerance, the P_{crit} instead becomes a measure of oxygen supply capacity at a given oxygen partial pressure where α is maximized. Moreover, since α theoretically should be a constant at a given temperature, P_{crit} can be determined for any $\dot{M}O_2$ or PO_2, and in normoxia P_{crit} therefore becomes the MMR. This approach may relax some of the methodological constraints in respirometry as the state of the fish becomes less of a concern when studying physiological responses to hypoxia (Seibel *et al.*, 2021).

The purpose of the present study was twofold; first to investigate the effect of alternating light–dark periods on metabolic rates in the absence of other stimuli, and second, to assess the influence of protocol length in respirometry trials for obtaining commonly reported metabolic rate traits in fish. The study animal used for these experiments

was the ballan wrasse *Labrus bergylta* (Ascanius 1767), a temperate labriform species native to the north-eastern Atlantic Ocean that inhabits shallow rocky, reef and kelp habitats. Ballan wrasse were either subjected to a conventional 1-day or an extended 5-day respirometry protocol, and in the 5-day protocol they were maintained in a 12 h light–dark cycle. Additionally, the response to progressive hypoxia was measured at the end of all trials.

It was hypothesized that $\dot{M}O_2$ would be elevated during light periods relative to dark periods owing to a photostimulated circadian rhythm in metabolism. Furthermore, increased protocol length was hypothesized to decrease SMR owing to additional time for acclimation to the experimental setup as well as a longer fasting period to further reduce specific dynamic action effects. Since SMR here is used to calculate both AS and P_{crit}, any differences in SMR estimates would then also affect these parameters. Finally, whether measured α values remain constant between different P_{crit} states and the MMR, as theorized by Seibel *et al*. (2021), was also assessed along with its general usefulness in respirometry studies.

2 | MATERIALS AND METHODS

2.1 | Fish husbandry

Hatchery-reared ballan wrasse were obtained from a commercial supplier and transported to the Matre Research Station, Institute of Marine Research, Norway. Here, they were maintained in circular indoor holding tanks with a diameter of 1.05 m and a water volume of 0.93 m^3. Each holding tank contained approximately 90 fish that were fed in excess *via* automated feeding devices each day between 13:00 and 17:00 (Atlantic Gold, 2.0 mm pellet size; PTAqua, Dublin, Ireland). The temperature was maintained at 9°C, the salinity was full-strength seawater of 33 ppt and holding tanks were subjected to 12 h light–dark photoperiods. The water supply was aerated, filtered and sterilized with UVC light, and a constant inflow of ∼30 l min^{-1} without water recycling ensured constant good water quality in the holding tanks. The fish had been acclimating in these conditions for over a month prior to the experimental trials.

The experimental trials were performed in June and July 2021 and were approved by the Norwegian Food Safety Authorities under permit number 27406, following ethical and legal obligations to vertebrate animals in scientific research.

2.2 | Respirometry setup

To measure $\dot{M}O_2$ in the ballan wrasse, a four-chamber automated static intermittent-flow respirometry system was used, allowing for four individual fish to be tested simultaneously (Loligo Systems, Viborg, Denmark). The size of the cylindrical-shaped acrylic chambers was 30 cm in length and 8 cm in internal diameter. Each chamber was connected to an internal loop with gas-tight PVC tubes running through a circulation pump and a flow-through oxygen sensor cell

connected to an optic fibre cable that measured oxygen concentrations at 1 Hz. All oxygen sensors were carefully calibrated prior to the experimental trials following the manufacturer's instructions. The estimated volume of this closed system, including tubes, was 1.584 l. To allow for intermittent flushing the respirometry chambers also had an open loop connected to a flush pump (5 l min^{-1}) with the upstream PVC tube reaching above the surface layer. Each respirometry chamber together with flush pump, circulation pump and oxygen sensor was submerged in its own rectangular tank with a water volume of 0.14 m^3.

The water supply into the tanks containing the respirometers was from the same source and of the same quality as that used for the holding tanks. A constant in- and outflow through all four tanks maintained constant water temperature and continuously replaced the respriometer water to minimize waste accumulation and bacterial proliferation within each setup.

Oxygen sensors and flush pumps were connected to computer software (AutoResp; Loligo Systems), allowing for automatic flushing of the respirometers periodically and to log changes in oxygen concentration during closed periods.

The entire setup, including water supply and electronics, was located in a secluded room to which only the experimenter was allowed access. This ensured that unwanted disturbance from other activities was avoided and lights could be turned on and off, and remain so, in the desired time intervals during the experimental trials.

2.3 | Experimental protocols

Prior to being introduced into a respirometer, all fish were randomly netted from a holding tank and individually subjected to an exhaustive chase protocol to provide an estimate of the MMR at the onset of the measurement protocol (Norin & Clark, 2016). In this chase protocol, the fish was momentarily moved to a new tank containing ≈100 l of water where it was forced into repeated burst swimming by tactile stimulation for 3 min. At the end of the chase period, the fish had limited responsiveness and could be handled almost as if sedated, which suggested physiological exhaustion. The fish was then subjected to 30 s of air exposure before being transferred into the respirometer. The respirometer was then sealed off as quickly as possible so that $\dot{M}O2$ measurements could begin. The delay from the end of the chase protocol to the start of the first $\dot{M}O_2$ measurement period was about 1 min.

An automated intermittent-closed measurement protocol was then repeated in 10 min cycles consisting of a 6 min closed $\dot{M}O_2$ measurement period, followed by a 3 min open flush period to reestablish oxygen levels and a 1 min closed wait period to stabilize flow conditions before the next closed $\dot{M}O_2$ measurement period.

All respirometry trials were started before the daily feeding schedule at 13:00 so that the fish would have been fasting overnight for a minimum of 18 h. This was done to reduce the confounding metabolic effects associated with digestion when attempting to estimate the SMR in fish (Chabot *et al*., 2016).

Two different treatment protocols were performed, which both started with chasing of the fish, as described above. In the first

treatment, fish were kept in the respirometers overnight for 24 h. Then the measurement cycle was changed to omit the flush and wait periods, which caused progressive hypoxia within the respirometer chamber as the fish gradually used up the remaining oxygen. In this way, $\dot{M}O_2$ measurements in response to decreasing ambient oxygen levels were obtained. Prior to loss of equilibrium, the fish were removed from their chambers and euthanized with a blow to the head whereafter weight and length were recorded.

In the second treatment, fish were kept in the respirometers for 5 days (120 h). During this time the light was turned on and off in 12 h intervals. At the onset of these trials, the first light period lasted 8 h while the final light period lasted 4 h, allowing for five dark and four light periods of 12 h in between. After 120 h, intermittent flushing was stopped to measure $\dot{M}O_2$ during progressive hypoxia followed by euthanizing the fish, as described in the first treatment protocol. The progressive hypoxia part of the trial lasted for 3.02 ± 0.23 (mean ± s.e.m.) h across both treatments.

After the fish had been removed, the respirometers were resealed to measure background respiration rates. Four measurement cycles were completed in the empty chambers and the mean values of these measurements were subtracted from all prior measurements where fish were present to correct for background respiration. The respirometer chambers, tubes and pumps were then disassembled and thoroughly cleaned in preparation for the next trial. Twelve fish were tested in both treatment groups.

2.4 | Calculations and statistics

The $\dot{M}O_2$ was calculated in all measurement periods from the linear decrease in dissolved oxygen over time as:

$$\dot{M}O_2 = \frac{\frac{\Delta O_2}{\Delta t}(V_{sys} - V_b)}{M_b}$$

In this formula $\Delta O_2 / \Delta t$ is the slope of the linear decrease (mg O_2 h^{-1}), V_{sys} is the volume of the respirometer, and V_b and M_b are the volume (L) and mass of the fish (kg), respectively, assuming a fish density of 1 kg l^{-1}. The R^2 of the linear regressions used to calculate $\dot{M}O_2$ were most often >0.97. If R^2 fell below 0.9, those data points were omitted from further analyses.

The SMR was estimated from the average of the 10% lowest $\dot{M}O_2$ values obtained during the entire measurement period until the onset of progressive hypoxia. If any outliers (±2 standard deviations from the mean) were found, they were removed and a new average was calculated as the reported SMR based on the remaining data points (Clark et al., 2013). In addition, for the 5-day protocol, the SMR was calculated with this approach using different measurement periods (e.g., 1, 2, 3, 4 or 5 days).

The MMR was defined as the highest $\dot{M}O_2$ measured, which in the majority of cases was in the beginning of the trial after the fish had been subjected to an exhaustive chase protocol. However, in a few cases some of the fish were able to approach and exceed this

initial peak $\dot{M}O_2$ at seemingly random times in the latter part of the trials, presumably as an expression of spontaneous escape behaviour. The absolute and factorial AS were then calculated as MMR minus SMR and MMR divided by SMR, respectively.

To assess the effect of photoperiod on $\dot{M}O_2$ in the 5-day protocol, two mean values were calculated in each consecutive light–dark period either based on all data points or the 10% lowest data points after having omitted outliers (exceeding ±2 standard deviations from the mean). The mean of all data points was used to represent routine metabolic rates and the mean of the lowest values was used to approximate resting metabolic rates in each light interval.

The P_{crit} was defined as the ambient oxygen level where $\dot{M}O_2$ decreased below the SMR estimated beforehand (Ern et al., 2016; Reemeyer & Rees, 2019). The oxygen extraction coefficient, α, was calculated as $\dot{M}O_2/PO_2$ at the point of P_{crit} and at the point of MMR, where the median PO_2 in a given measurement interval was used as the denominator. In addition, α_0 values were calculated in the entire PO_2 range as $\dot{M}O_2/PO_2$ to further explore the response to progressive hypoxia (Seibel et al., 2021).

Finally, the condition factor of each fish was calculated as 100 x weight / length^3)$^{-1}$ as a simple morphometric measure (Fulton, 1904; Nash et al., 2006).

Statistical differences between the two treatment protocols in the various measured categories were assessed with a t-test. Statistical differences in $\dot{M}O_2$ during consecutive alternating photoperiods and when using increasing interval length for SMR estimations in the 5-day protocol were assessed with a repeated measures ANOVA followed by Tukey's post hoc test to identify which data points differed. A two-way ANOVA was used to compare α values at P_{crit} and MMR, and between treatment groups. Prior to performing these tests, equal variance and normal distribution were confirmed with Levene's mean test and Shapiro–Wilks tests, respectively. In the case of α values at P_{crit} and MMR, it was necessary to use a log transformation to adhere to test assumptions. A P value below 0.05 was considered significant. To further compare data between the two treatment protocols, scatter plots with linear regression lines are reported for $\dot{M}O_2$ versus PO_2. Data are reported as mean ± s.e.m. unless specified otherwise.

3 | RESULTS

The weight, length and condition factor of ballan wrasse from the two treatment groups were statistically similar (t-test, d.f. = 22, P > 0.05 for all size parameters) (Table 1). The mean sizes across groups were 77.3 ± 5.3 g weight, 16.5 ± 0.4 cm length and 1.68 ± 0.04 condition factor.

The $\dot{M}O_2$ of all consecutive data points (six per hour) during the 5-day trial of alternating photoperiods are shown in Figure 1a, and included five 12 h dark periods and four 12 h light periods with an 8 h light period at the start and a 4 h light period at the end. The initial period of elevated $\dot{M}O_2$ is here caused by the exhaustive chase protocol prior to starting the measurements. The mean of the 10% lowest

data points (minus outliers), used as an approximation of resting metabolic rates in each photoperiod, did not show a diurnal pattern (Figure 1b). However, significant differences were found between the first light period and all the following photoperiods, while the final dark period was statistically lower than the first dark and the final light periods (repeated measures ANOVA, d.f. = 127, $P < 0.001$). The routine metabolic rate expressed as the mean of all data points (minus outliers) in each photoperiod also did not show a diurnal pattern (Figure 1c). Here, statistical differences were only found between the first light period and all the following photoperiods (repeated measures ANOVA, d.f. = 127, $P < 0.001$).

TABLE 1 Size parameters of ballan wrasse in the two treatment groups, $N = 12$

|	Weight (g)	Length (cm)	Condition factor
1-day trial	76.6 ± 6.6	16.3 ± 0.5	1.73 ± 0.06
5-day trial	78.0 ± 8.7	16.6 ± 0.5	1.63 ± 0.04

Note: All parameters were statistically similar between treatments (*t*-test, $P > 0.05$). Data are mean ± s.e.m.

Estimations of SMR were influenced by protocol length (Figure 2). Specifically, the 1-day treatment group and data from the first 24 h in the 5-day treatment group yielded similar estimations (*t*-test, d.f. = 22, $P = 0.529$). However, SMR estimations decreased significantly when increasing the measurement period to 48 h, whereafter they decreased slightly but not significantly over the remaining days of the 5-day protocol (repeated measures ANOVA, d.f. = 57, $P < 0.001$). Here, the first 24 h resulted in an SMR estimation of 43.9 ± 2.11 mg O_2 kg^{-1} h^{-1}, while expanding to 48 h reduced SMR to 38.4 ± 2.05 mg O_2 kg^{-1} h^{-1}, corresponding to a reduction of 12.7%. Moreover, this reduction increased to 16.6% when comparing the first 24 h to the entire 5-day period.

The SMR, MMR, absolute AS, factorial AS and P_{crit} in the two protocols are shown in Figure 3. While the SMR was significantly lower in the 5-day protocol (*t*-test, d.f. = 22, $P = 0.013$), MMR was similar between the two protocols (*t*-test, d.f. = 22, $P = 0.217$), with a mean across treatments of 294.9 ± 7.3 mg O_2 kg^{-1} h^{-1}. The difference in SMR was not sufficiently big to cause a significant difference in the absolute AS (*t*-test, d.f. = 22, $P = 0.057$), with a mean across treatment of 252.6 ± 7.4 mg O_2 kg^{-1} h^{-1}. However, the differences in
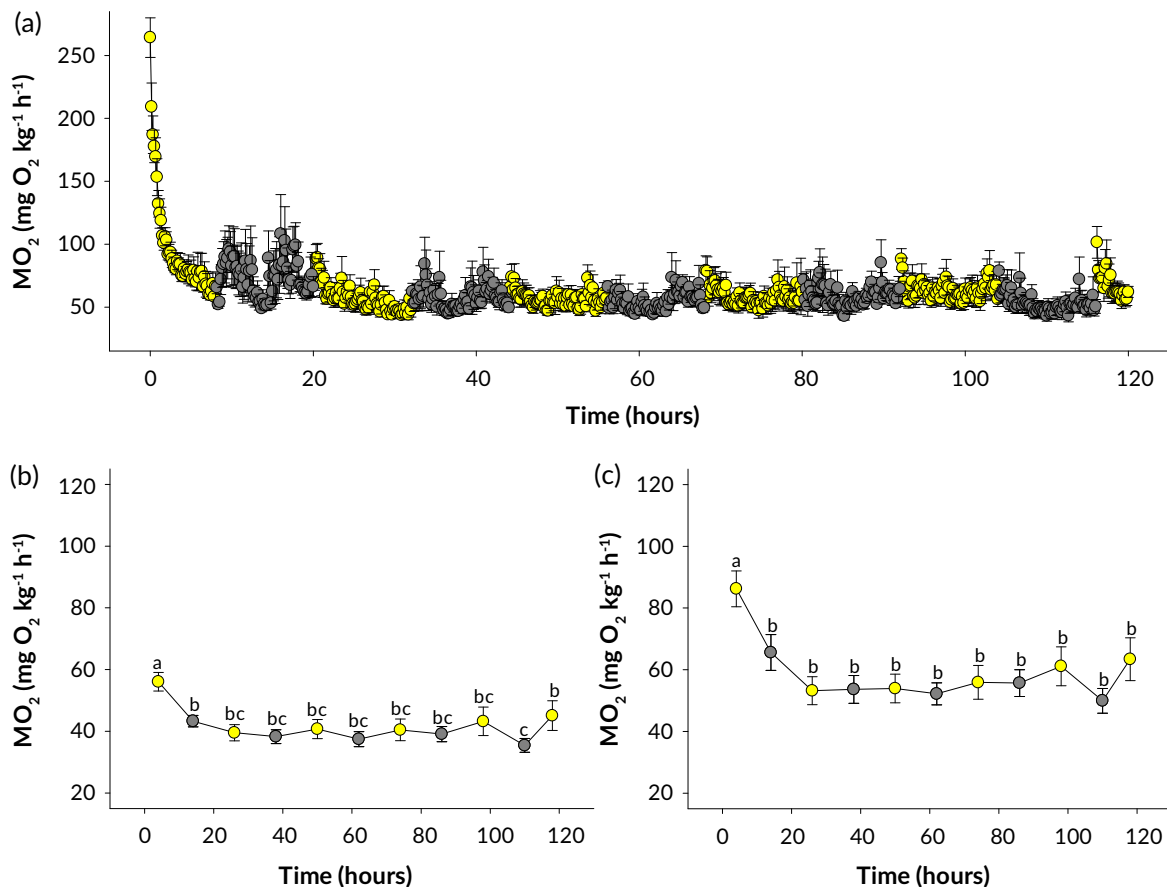
FIGURE 1 The effect of a 12 h light–dark photoperiod on oxygen uptake rates (ṀO$_2$) over a 5-day period. (a) Mean ± s.e.m. values of all consecutive measurement points (six per hour). (b) Mean ± s.e.m. values of the 10% lowest data points (minus outliers) as an approximation of resting metabolic rates in each consecutive light–dark period. (c) Mean ± s.e.m. values (minus outliers) as a representation of routine metabolic rates in each consecutive light–dark period. Significant differences in (b) and (c) are indicated with different letters (repeated measures ANOVA, $P < 0.05$). $N = 12$. (—○—) light; (—○—) dark
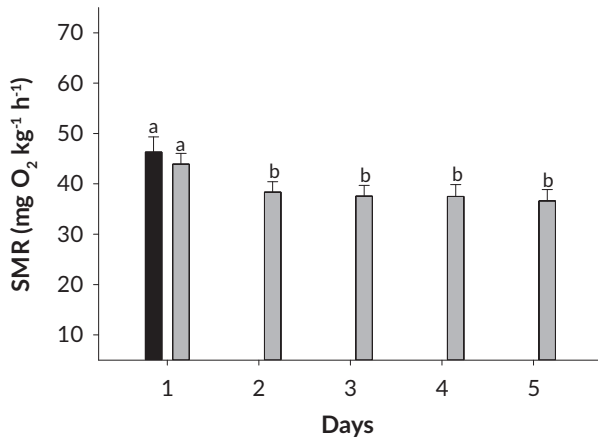
FIGURE 2 Standard metabolic rate (SMR) estimations when using different time periods for calculation. Different letters indicate significant differences (repeated measures ANOVA within the 5-day group and *t*-test between the two groups at the 1-day period, *P* < 0.05). *N* = 12 and data are mean ± s.e.m. (■) 1-day; (▨) 5-day

SMR did cause a significant difference in the factorial AS (*t*-test, d.f. = 22, *P* < 0.001), showing that the latter is more sensitive to how SMR is estimated. Here, the factorial AS was 6.4 ± 0.4 and 8.5 ± 0.4 in the 1-day and 5-day groups, respectively. The P_{crit} was 14.6 ± 1.4 %PO₂ in the 1-day group and 10.6 ± 0.8 %PO₂ in the 5-day group, and these differences are statistically significant (*t*-test, d.f. = 22, *P* = 0.023).

Scatter plots for all $\dot{M}O_2$ data points versus PO_2 during the progressive hypoxia part of the experiment together with MMR data points from both treatment groups are shown in Figure 4a. In the final phase of the hypoxia trial, where the relationship between $\dot{M}O_2$ and PO_2 becomes linear, data points between the two groups overlap substantially and the slopes of the linear regression lines are similar (Figure 4b), suggesting that the response to hypoxia is the same despite differences in the estimated SMR and P_{crit} in the two treatment groups.

A two-way ANOVA (total d.f. = 45) showed that α was unaffected by protocol length (*P* = 0.162) and that protocol length did not
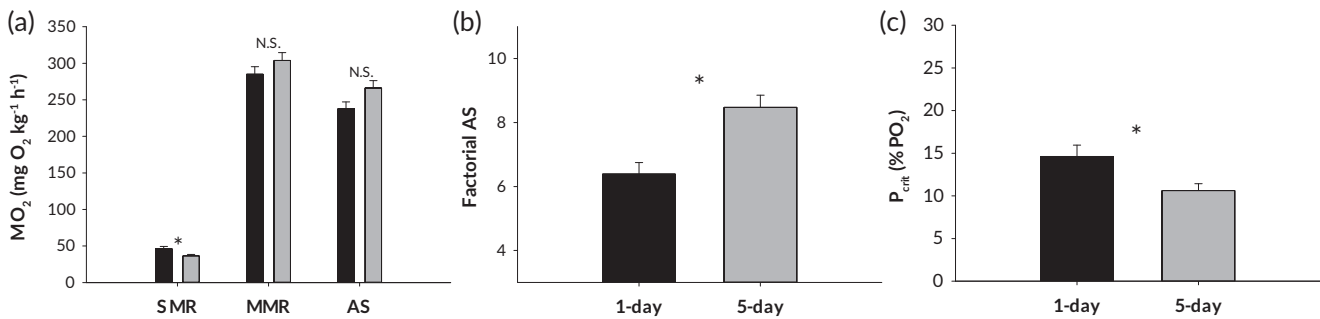

FIGURE 3 The standard and maximum metabolic rates (SMR and MMR), aerobic scope (AS) (a), factorial AS (b) and critical oxygen tension (P_{crit}) (c) in the two treatment groups. Asterisks mark a significant difference. N.S., nonsignificant difference (*t*-test, *P* < 0.05). *N* = 12 and data are mean ± s.e.m. (■) 1-day; (▨) 5-day
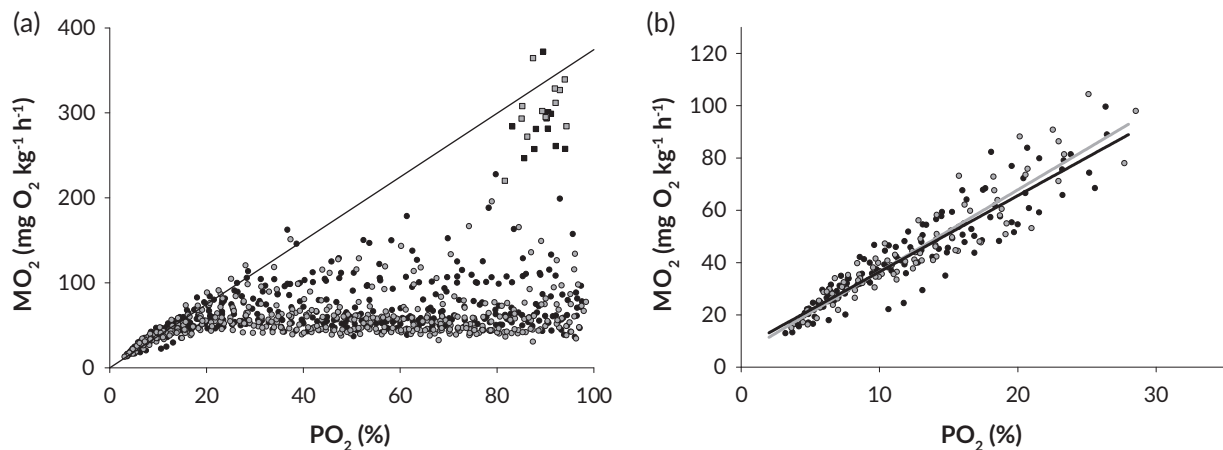
FIGURE 4 The oxygen uptake rate ($\dot{M}O_2$) versus ambient oxygen levels (PO_2) during progressive hypoxia in the two treatment groups. (a) Merged scatter plot of all data points from all the fish in the entire PO_2 range following the onset of progressive hypoxia as well as the beforehand measured MMR of individual fish in normoxia together with the α-line derived from α at P_{crit}. (b) Scattered data points of the final phase of the trial where $\dot{M}O_2$ decreases linearly with PO_2 along with global regression lines and their equations from each treatment group. (●) 1-day hypoxia; (◉) 5-day hypoxia; (■) 1-day MMR; (▨) 5-day MMR; (——) α-line; (●) 1-day, $y = 2.916x + 7.297$, $R^2 = 0.858$; (◉) 5-day, $y = 3.134x + 5.149$, $R^2 = 0.874$
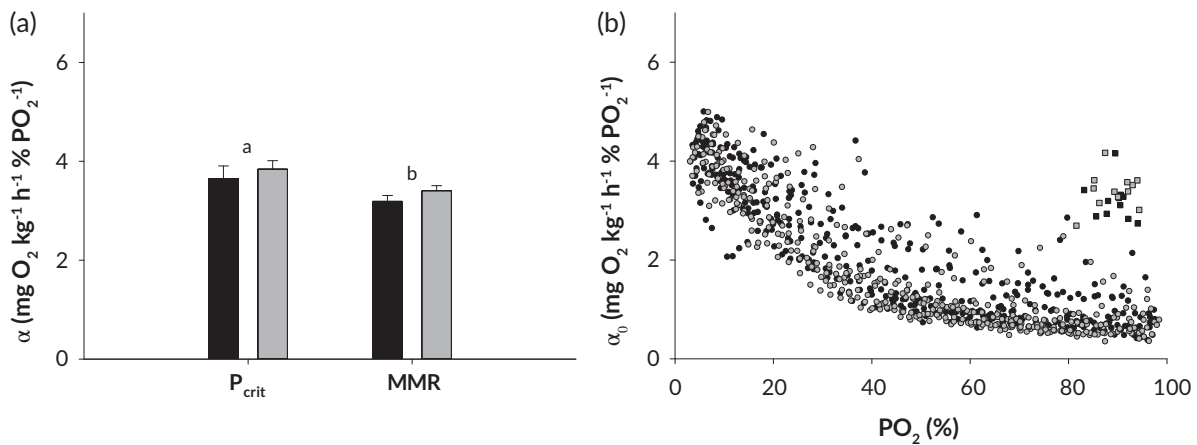
FIGURE 5 The oxygen extraction coefficient (α) (mean ± s.e.m.) at the critical oxygen tension (P_{crit}) and the maximum metabolic rate (MMR) in the two treatment groups (a). Different letters in (a) indicate a significant difference between P_{crit} and MMR (two-way ANOVA, $P < 0.05$). $N = 12$. (b) Scatter plot of α_0 (MO_2/PO_2) during progressive hypoxia together with α values of the MMR from individual fish in the two treatment groups. (■) 1-day; (▨) 5-day; (●) 1-day hypoxia; (○) 5-day hypoxia; (■) 1-day MMR; (▧) 5-day MMR

interact with α values for P_{crit} and MMR ($P = 0.997$), but was significantly different between P_{crit} and MMR ($P = 0.013$) (Figure 5a). Specifically, α was $3.75 ± 0.15$ mg O_2 kg^{-1} h^{-1} %O_2^{-1} at P_{crit} and $3.30 ± 0.08$ mg O_2 kg^{-1} h^{-1} %O_2^{-1} at MMR across treatments, corresponding to a 11.8% lower α at MMR. A scatter plot of α_0 data points in the assessed PO_2 range during progressive hypoxia together with α at MMR is shown in Figure 5b, and shows that α_0 is low initially in normoxia when undisturbed, but increases towards α as hypoxia becomes more severe.

The background respiration rates were $1.28 ± 0.43$ and $2.94 ± 0.62$ mg O_2 kg^{-1} h^{-1} in the 1-day and 5-day trials, respectively, and these values were significantly different (t-test, d.f. = 22, $P = 0.0396$). This suggests some minor increase in bacterial proliferation over the 5-day trial.

4 | DISCUSSION

4.1 | Lack of diurnal rhythm in metabolic rates in ballan wrasse

Contrary to the hypothesis, ballan wrasse in the present study did not display a diurnal rhythm in metabolic rates when maintained in respirometers on a 12 h light–dark cycle for 5 days. A 24 h rhythmicity in metabolism can originate from a combination of endogenous, exogenous and activity-related factors (Kim *et al*., 1997; Ross & McKinney, 1988; Vatine *et al*., 2011). As the fish were confined to small static resprimeters and not fed during the experiment, natural diurnal variation in swimming activity, feeding behaviours and digestive processes that normally would influence $\dot{M}O_2$ were either restricted or eliminated. Any observed circadian rhythm in metabolism should therefore primarily be caused by endogenous oscillators and the alternating photoperiod. The $\dot{M}O_2$ in each consecutive light interval was calculated as both resting and routine metabolic rates to

provide measures for minimum and random spontaneous activity, respectively. Since resting levels did not vary significantly between consecutive 12 h light–dark periods, it can be concluded that endogenous oscillators and photoperiod on their own did not affect the metabolism at the whole-animal level in ballan wrasse. In addition, similar routine metabolic rates suggest that spontaneous activity also was unaffected by diurnal cycles, but this may have been an artefact of the confined respirometer chamber, which limited swimming behaviours.

The absence of endogenous circadian metabolic rates in the ballan wrasse is perhaps a surprising finding when considering that other fish species studied with similar approaches generally have shown an oscillating rhythmicity in $\dot{M}O_2$, even in the absence of alternating photoperiods (*e.g*., Kim *et al*., 1997; Moran *et al*., 2014; Ross & McKinney, 1988). In the study by Moran *et al*. (2014), circadian physiology of the eyed surface form was compared to the eyeless cave form of the Mexican tetra, and here it was found that the cave variant did not have a circadian rhythm in metabolic rates. These authors suggested that this could be an adaptation to cave environments and be a common trait among species living in perpetual darkness, such as in caves or deep water. Furthermore, the absence of circadian metabolic rates in the cave form resulted in substantial energy savings compared to the surface form, which provides an advantage in food-limited habitats such as caves (Moran *et al*., 2014).

Ballan wrasse are nonmigratory and found in various shallow marine environments where they feed on various hard-shelled crustaceans among seaweeds and rocks. They are therefore naturally exposed to alternating photoperiods and their lack of circadian metabolic rates is not an adaptation to dark habitats. However, ballan wrasse and other temperate wrasse species have slow growth rates and low metabolic rates compared to other fish species maintained in similar conditions (Corkum & Gamperl, 2009; Hvas & Oppedal, 2019b; Yuen *et al*., 2019). Species-specific adaptations in energetics and life-history strategies could therefore be a plausible explanation for the

lack of a detectably circadian metabolic rate in ballan wrasse, as it may provide valuable energy savings, as suggested for the cave variant of the Mexican tetra (Moran *et al.*, 2014).

4.2 | Influence of protocol length on metabolic rate traits

As hypothesized, the length of the respirometry protocol influenced estimates of the SMR, which consequently altered the derived AS and P_{crit}. Since the MMR was estimated at the onset of each trial, how long the fish remained in the respirometer afterwards was irrelevant for this parameter and it therefore did not differ between treatment groups.

The reduction in SMR estimations obtained by extending the protocol length beyond the conventional 24 h caused a significant difference between the two treatment groups in the factorial AS but not in the absolute AS. A recent methodological study also found that the absolute AS is less sensitive to variation in SMR than the factorial AS when MMR is fixed (Halsey *et al.*, 2018). Hence, if any unrelated studies report contrasting factorial AS this may be explained by differences in how the SMR was obtained. Furthermore, the absolute and factorial AS often have different responses to changes in important environmental and biological factors such as temperature and body size (*e.g.*, Killen *et al.*, 2007; Yuen *et al.*, 2019). Whether one is more ecologically relevant to report than the other has therefore been debated as they may provide opposing interpretations. However, both can provide valuable insights into energetic capacities and limits when contexts are considered, so best practice is to always report both (Clark *et al.*, 2013; Halsey *et al.*, 2018).

The reduction in SMR estimates was most notable when increasing protocol length from 24 to 48 h, causing a 12.7% reduction, while a greater reduction of 16.6% was achieved over the entire 5-day period. Protocol length may influence SMR estimations owing to a combination of digestive status, stress levels, restlessness, acclimation and increasing background $\dot{M}O_2$ from bacterial proliferation within the respirometer (Chabot *et al.*, 2016).

While background $\dot{M}O_2$ generally remained low in the present study, it was significantly higher in the 5-day protocol. However, using only data from the first 24 h in the 5-day protocol resulted in similar SMR estimates as in the 1-day protocol despite having subtracted a slightly higher background $\dot{M}O_2$ from the data, as measured at the end of the trials. This suggests that bacterial proliferation did not become a significant issue in the extended protocol. Moreover, the experiment was performed at a relatively low temperature of 9°C, which together with thorough cleaning of the setup between trials helped to reduce background $\dot{M}O_2$. At higher water temperatures bacterial proliferation can be a major concern in respirometry studies, especially in setups with high surface-to-volume ratios designed for smaller fish. In those cases it may therefore not be feasible to run trials for several days, unless elaborate efforts are made to more accurately account for the dynamics of background $\dot{M}O_2$ over time (*e.g.*, Norin *et al.*, 2014).

The gradual decline in SMR estimates with increasing protocol length could perhaps reflect decreasing specific dynamic action effects owing to the fish not having fasted for sufficiently long prior to the respirometry trials, meaning that the first estimates did not adhere to the requirements of a true SMR. However, the digestive physiology of ballan wrasse is unusual in that they lack a stomach and therefore are unable to eat and retain large meals, while they instead eat smaller meals in frequent intervals and have a relatively quick gut evacuation rate of 12–14 h (Le *et al.*, 2019). Specific dynamic action effects in this species should therefore inherently be low and completed by the onset of the respirometry trials, as the fish had been fasting for at least 18 h prior to this point.

Prolonged fasting may gradually reduce metabolic rates in fish owing to adaptive physiological changes to preserve energy during periods of food shortages (Fu *et al.*, 2005; Mehner & Wieser, 1994). For instance, Atlantic salmon fasted for 1 week had a 15% reduced SMR and 3–4 weeks of fasting reduced SMR by 22% (Hvas *et al.*, 2020b). Even though the fasting period used in the present study was shorter, it may still have been a contributing factor for the observed reduction in SMR with increasing protocol length.

Full acclimation to the experimental setup and complete recovery from the initial acute stress at the onset of the trial may also explain the effect on the SMR estimates, especially between using 24 or 48 h protocols, which is where the largest difference was found. Furthermore, increasing protocol length also improves the chance of capturing true resting behaviours, particularly in fish with pronounced restless behaviours, although ballan wrasse are calm fish and generally remained inactive within the respirometers.

Evidently, SMR estimates are sensitive to protocol length, which emphasizes that it is a highly context-dependent parameter even when adhering to its strict definition of true resting states in fish. In practice, this means that direct comparisons between studies are difficult to make as numerous differences in methodology likely will exist before even considering the impacts of biological and environmental variations. Nevertheless, within the context of the same experiment comparisons can obviously be made, as any overestimations of the true SMR owing to insufficient protocol length or other methodological flaws will be unbiased as all compared groups otherwise should have been subjected to the same respirometry protocol.

4.3 | P_{crit} and the usefulness of α

Extending the respirometry protocol from 1 to 5 days reduced the P_{crit} from 15% to 11% PO_2 when defined as the point where $\dot{M}O_2$ fell below the SMR measured beforehand in normoxia. The higher P_{crit} in the 1-day group was presumably an artefact of having overestimated the SMR, as discussed above. This is corroborated by the fact that $\dot{M}O_2$ during progressive hypoxia was indistinguishable between the two treatment groups, which suggests that hypoxia responses in fact were similar. Alternatively, a true difference in hypoxia tolerance should have been revealed by different slopes of the linear decline in $\dot{M}O_2$ with decreasing PO_2 once $\dot{M}O_2$ becomes dependent on PO_2.

Regardless of protocols used, the reported P_{crit} values were low compared to other species from similar marine environments and

support previous notions of high hypoxia tolerance in temperate wrasse species (Corkum & Gamperl, 2009; Hvas & Oppedal, 2019b). However, whether ballan wrasse actually encountered severe hypoxic conditions in their natural environment is more doubtful. The low P_{crit} values may instead simply reflect low metabolic rates in this species and not an evolutionary adaptation to survive in hypoxia *per se*. As such, it is presumably not an ecologically relevant marker of hypoxia tolerance as discussed by others when scrutinizing the utility of the P_{crit} (*e.g.*, Wood, 2018). Interpreting the P_{crit} as a measure of oxygen supply capacity at a given PO_2 where α is maximized rather than as a marker of hypoxia tolerance, as suggested by Seibel *et al.* (2021), could therefore seem more appropriate in the case of ballan wrasse. Furthermore, α values at P_{crit} were similar between the two treatment groups, which confirms similar hypoxia responses and that the differences in P_{crit} were an artefact of having overestimated SMR in the 1-day protocol. Similar α values regardless of protocol in the present study also show that this parameter is more flexible to variations in methodological approach and the state of the fish when studying hypoxia effects with respirometry (Seibel *et al.*, 2021).

In theory, α is constant throughout the PO_2 range, and in normoxia the MMR should therefore provide the same α values as in P_{crit} conditions (Seibel *et al.*, 2021). However, in the present study α was significantly lower at MMR, with a mean difference of 12%. Different α values between P_{crit} and MMR could either mean that MMR was underestimated in proportion to this difference, or that α in fact is not constant between normoxia and hypoxia. The constant nature of α was recently challenged (Farrell *et al.*, 2021) and if α indeed increases in hypoxia, this implies that some respiratory mechanisms are involved with improving the oxygen extraction capacity in ballan wrasse. Considering the numerous ways fish species can modify oxygen uptake and transport in response to environmental challenges (*e.g.*, Crans *et al.*, 2015; Matey *et al.*, 2008; Wells *et al.*, 1989), this may seem plausible.

However, a simpler explanation is that the MMR was underestimated. In the present study, MMR was obtained *via* an exhaustive chase protocol prior to introducing the fish to the respirometer. Previous studies have shown that chase protocols can underestimate the MMR in various fish species when compared to other methods such as swimming until exhaustion or confinement stress (Andersson *et al.*, 2020; Hvas & Oppedal, 2019a; Rummer *et al.*, 2016). Provided that α is constant, robust and easily obtained measurements of α in severe hypoxia could then be used to assess the quality of MMR estimates in normoxia, as capturing the true maximum oxygen uptake rate of fish in experiments can be technically difficult (*e.g.*, Norin & Clark, 2016). As such, calculation of α values provide an additional tool to analyse respirometry data, and the fact it is more robust to methodological approach than the P_{crit}, if nothing else, may facilitate a way to compare different studies more fairly.

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ORCID

Malthe Hvas https://orcid.org/0000-0002-2967-5525

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