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Influence of experimental set-up and methodology for measurements of metabolic rates and critical swimming speed in Atlantic salmon *Salmo salar*

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Abstract

In this study, swim-tunnel respirometry was performed on Atlantic salmon *Salmo salar* post-smolts in a 90 l respirometer on individuals and compared with groups or individuals of similar sizes tested in a 1905 l respirometer, to determine if differences between set-ups and protocols exist. Standard metabolic rate (SMR) derived from the lowest oxygen uptake rate cycles over a 20 h period was statistically similar to SMR derived from back extrapolating to zero swim speed. However, maximum metabolic rate (MMR) estimates varied significantly between swimming at maximum speed, following an exhaustive chase protocol and during confinement stress. Most notably, the mean (±SE) MMR was 511 ± 15 mg O_2 kg^{-1} h^{-1} in the swim test which was 52% higher compared with 337 ± 9 mg O_2 kg^{-1} in the chase protocol, showing that the latter approach causes a substantial underestimation. Performing group respirometry in the larger swim tunnel provided statistically similar estimates of SMR and MMR as for individual fish tested in the smaller tunnel. While we hypothesised a larger swim section and swimming in groups would improve swimming performance, U_{crit} was statistically similar between both set-ups and statistically similar between swimming alone *v.* swimming in groups in the larger set-up, suggesting that this species does not benefit hydrodynamically from swimming in a school in these conditions. Different methods and set-ups have their own respective limitations and advantages depending on the questions being addressed, the time available, the number of replicates required and if supplementary samplings such as blood or gill tissues are needed. Hence, method choice should be carefully considered when planning experiments and when comparing previous studies.

KEYWORDS
aerobic scope, chase protocol, confinement stress, group swimming, *Salmo salar*, salmon, swim-tunnel respirometry

1 | INTRODUCTION

Whole-animal energy expenditure, termed metabolic rate and locomotory ability, are arguably two of the most important physiological traits to investigate when trying to understand the fundamental biology of fish and the range of environments and lifestyles they are adapted to. Respirometry is by far the most commonly used approach to measure metabolic rate (Clark *et al.*, 2013; Fry & Hart, 1948; Steffensen,

1989). Here, oxygen uptake rates ($\dot{M}O_2$), an indirect measure of aerobic metabolism (Nelson, 2016), can be calculated from decreasing oxygen content of water over time in a closed chamber containing a fish. Furthermore, swim-tunnel respirometers have been designed to systematically measure the metabolic rate at different swimming speeds as well as the highest attainable swimming speeds of fish (Blažka *et al.*, 1960; Brett, 1964; Steffensen *et al.*, 1984).

Standard metabolic rate (SMR), maximum metabolic rate (MMR) and the critical swimming speed (U_{crit}) are often reported in respirometry studies. SMR is the $\dot{M}O_2$ of an inactive, non-digestive, unstressed fish at its acclimation temperature and reflects the minimum energetic requirements to maintain basal homeostasis (Chabot *et al.*, 2016; Fry, 1971). MMR is the highest rate of oxygen consumption, which is achieved during high levels of activity and stress (Fry, 1971; Norin & Clark, 2016). U_{crit} is a measure of prolonged swimming capacity and is obtained by incrementally increasing current speeds until the fish being tested fatigues (Brett, 1964; Plaut, 2001). The aerobic scope (AS) can be calculated from the difference between SMR and MMR and is widely used to infer how fish may cope in specific environments, as the AS reflects the available aerobic capacity to perform fitness related activities (Clark *et al.*, 2013; Farrell, 2016; Fry, 1971).

Studies on metabolic rates and swimming performances are used extensively in numerous areas of fish biology. This includes climate change related research to predict consequences of elevated temperatures (Clark *et al.*, 2013; Farrell, 2002, 2016; Lefevre, 2016), mechanistic effects of pollutants and toxicants (Lefevre *et al.*, 2011; Wood *et al.*, 2012), conservation and management of wild populations (Cooke *et al.*, 2012; McKenzie *et al.*, 2016), effect of parasitic infestation (Brauner *et al.*, 2012; Hvas *et al.*, 2017b) and aquaculture in relation to animal welfare and production performance (Anttila *et al.*, 2014; Hvas & Oppedal, 2017).

Owing to broad relevance and growing popularity, several types of experimental set-ups and protocols are commonly used to measure metabolic rates and U_{crit}, which may provide different estimates. Specifically, SMR is typically measured on fasted fish over a 12–48 h period using intermittent-flow respirometry and is derived from the lowest $\dot{M}O_2$ measurements in this period (*e.g.* Chabot *et al.*, 2016; Clark *et al.*, 2013; Svendsen *et al.*, 2016a). However, SMR can also be calculated by extrapolating back to zero activity when $\dot{M}O_2$ has been measured at a range of swimming speeds (Beamish, 1978; Brett, 1964). These two methods have been found to provide both similar and different estimates of SMR (Chabot *et al.*, 2016; Roche *et al.*, 2013).

MMR can be measured directly while the fish is swimming at its maximum capacity in swim tunnels. However, several fish species do not naturally elicit continuous swimming and the U_{crit} test may then not be appropriate or even possible. Moreover, swim tunnels may not be available to researchers as they require more complex logistics and are more expensive than static respirometers. Therefore, MMR is most commonly estimated by chasing fish, sometimes in conjunction with air-exposure, to inflict physiological exhaustion, after which fish are quickly transferred into a static respirometer where $\dot{M}O_2$ is monitored (Norin & Clark, 2016). In species where both methods can be used, a meta-analyses found that swimming and chasing provided similar MMR estimates (Killen *et al.*, 2017), while direct experimental comparisons have both shown a substantial underestimation when using a chase protocol (Rummer *et al.*, 2016) and higher MMR when chasing compared with a U_{crit} test (Reidy *et al.*, 1995), suggesting optimal approach is species dependent.

Swim-tunnel designs vary in shape and size and U_{crit} may be underestimated if the swim section is too small for the fish to fully express natural swimming behaviours such as burst-and-glide (Kern *et al.*, 2017; Peake & Farrell, 2006; Tudorache *et al.*, 2007). Furthermore, some species may gain hydrodynamic advantages from swimming in schools (Herskin & Steffensen, 1998; Marras *et al.*, 2015; Svendsen *et al.*, 2003), while the swim protocol itself in terms of increment duration and magnitude may alter U_{crit} (Downie & Kieffer, 2017; Farlinger & Beamish, 1977). Hence, reported U_{crit} values are dependent on the context in which they were obtained.

Swim-tunnel respirometry is time consuming and laborious and is generally limited to one trial per day, as the fish being tested ideally should be given ample time (typically overnight acclimation) to recover from handling and confinement stress prior to being tested (Kolok, 1999; Norin *et al.*, 2014). Considering that a sufficient number of replicates are needed to infer robust statistical effects and that several treatment groups often are desired, completing a full experiment can be a daunting effort. In cases where individual variations in metabolic rates are less of a focus, swim-tunnel respirometry on groups can be performed, testing a greater number of fish in less time. This allows fish to be tested in larger swim tunnels, where natural swimming behaviours are more likely to be fully expressed (Remen *et al.*, 2016). In this case, fish are subjected to the same experimental treatment and the measured $\dot{M}O_2$ values represent a group average at specific swimming speeds. However, owing to inherent variations in swimming capacities, individual fish may be in different metabolic states during specific time points with regards to becoming exhausted in the swim trials (Hvas *et al.*, 2017a). Hence, whether representative MMR estimates of the group being tested can be obtained is a legitimate methodological concern, especially if the weakest swimmers have been removed due to fatigue and the biomass-to-volume ratio becomes too low for reliable $\dot{M}O_2$ measurements in the remaining best swimmers (Hvas *et al.*, 2017a). To establish if group respirometry is a viable method and to reveal if potential biases in measured variables are present, a direct comparison with traditional single-fish respirometry is therefore currently required.

The purpose of our study was to compare estimates of SMR, MMR and U_{crit} of Atlantic salmon *Salmo salar* (L. 1758) obtained with different protocols and set-ups, to evaluate the best practice approaches for this species, but also to provide some general advice, especially concerning group respirometry. To ensure a robust comparison in methods, all data was obtained from *S. salar* of one genetic strain and of similar sizes that had been reared and maintained under identical conditions, including acclimation temperature. We hypothesised that the U_{crit} would be higher when testing groups of *S. salar* in a larger swim tunnel owing to hydrodynamic advantages from group swimming and owing to the more spacious environment and

that fish swimming continuously until exhaustion would attain higher $\dot{M}O_2$ compared with either confinement stress or following a chase protocol. Minor differences, if any, was expected between methods of SMR estimates.

2 | MATERIALS AND METHODS

This research was approved under the ethics permit 9776 and abided to Norwegian procedures and legislations of animal usage in scientific research.

2.1 | Animal husbandry

All experiments were performed on *S. salar* produced and reared at Matre Research Station, Institute of Marine Research, Norway. After smoltification, fish were kept in large tanks (5.3 m^3) at salinity 34 in an open system with constant inflow of filtered and UVC treated water. This prevented the accumulation of waste products and maintained ambient oxygen levels above 85% saturation at all times. Fish were subjected to a simulated natural photoperiod and fed until satiation with standard commercial feed (Nutra, 3 mm pellet size, Skretting; www.skretting.com) each day through automated feeding devices. All fish were maintained at 13°C for a minimum of 4 weeks prior to experimental trials. Water temperature in the holding tanks were continuously monitored and kept within ±0.1°C by custom software (SD Matre software, Normatic; www.normatic.no) through adequate mixing from ambient (8°C) and heated (20°C) water reservoirs.

2.2 | Swim-tunnel set-ups

2.2.1 | Single-fish system

To measure metabolic rate and swimming performance in individual fish, a submerged 90 l intermittent-flow swim-tunnel respirometer (Loligo Systems; www.loligosystems.com) was used (as described in Hvas *et al.*, 2018a). A constant flow of 13°C seawater ran through the outer tank containing the respirometer to ensure stable temperatures. Within the swim-tunnel respirometer, desired current speeds could be generated with a motor driven propeller, after having determined the relationship between motor output and current speed using a flow meter (Höntzsch Flow Measuring Technology; www.hoentzsch.com). Upstream of the rectangular swim section (66 × 20 × 19.5 cm), a temperature sensor and a dipping probe oxygen mini sensor (Loligo Systems) were attached. A powerful flush pump (57 l min^{-1}) was also placed in the outer tank to allow for rapid flushing of the respirometer chamber *via* an inlet downstream of the swim section. Sensors and the flush pump were connected to computer software (AutoResp, Loligo Systems), which allowed for oxygen concentration and temperature to be logged each second while the flush pump could be turned on and off in desired intervals automatically. To minimise visual disturbance to the fish, the entire set-up was partially covered in black plastic sheets.

2.2.2 | Group-fish system

To measure metabolic rate and swimming performance in groups of fish, as well as swimming performance in a more spacious environment on individual fish, a larger custom build Brett-type swim-tunnel respirometer was used (1905 l; as described in Hvas *et al.*, 2017a; Remen *et al.*, 2016). Here, the swim section was circular, with an internal diameter of 36 cm and a length of 248 cm. Like the smaller set-up, desired water currents were generated with a motor driven propeller after thorough calibration with a flow meter. Water of 13°C was supplied *via* an adjustable inlet opposite of the swim section with a maximum flow capacity of *c.* 280 l min^{-1}. This allowed for rapid flushing of the entire set-up between closed measurement periods. Downstream of the swim section, a camera was deployed to observe fish behaviour. An oxygen sensor (RINKO ARO-FT, JFE Advanced Co.; www.jfe-advantech.co.jp) was placed next to the camera and was connected to computer software and measurements were logged every 2 s. (MiniSoft SD200W, SAIV A/S Environmental Sensors & Systems; www.saiv.no). In contrast to the smaller set-up, the flush system was not controlled by computer software and automated measurement loops were therefore not made during overnight acclimation in the larger swim tunnel. The swim section was covered from visual disturbances, except for at the rear end where the top lid was transparent. This top lid could be removed, allowing us to remove fatigued fish during the swim trials.

2.3 | Experimental groups and protocols

Feed was withheld 1 day prior to the onset of all respirometry trials to reduce the metabolic effects from digestion and allowing for gut evacuation (Handeland *et al.*, 2008; Storebakken *et al.*, 1999). The small swim-tunnel respirometer was used for two experimental protocols, a U_{crit} group and a chase group. In the U_{crit} group, a random fish was netted from a holding tank and quickly transferred to the swim chamber in the afternoon. Measurements of $\dot{M}O_2$ then commenced immediately after the swim chamber had been properly sealed. The computer software was programmed to run loops of 15 min, consisting of a 10 min closed measurement period followed by a 3.5 min flush period and then a 1.5 min wait period. The fish was left in the swim tunnel overnight at a low current speed of 15 cm s^{-1} which did not enforce swimming behaviour. The following morning, current velocity was increased every 30 min in steps of 15 cm s^{-1}, corresponding to *c.* 0.5 body length (L_B) s^{-1}, meaning that two measurement cycles could be completed at each increment speed. Current increments were always made during the flush phase. Some fish were able to hold station against the current at the lowest speeds, but eventually, steady swimming was initiated. At the highest speeds, fish would either fall back on the rear grid or attempt to rest in the bottom corner at the rear end of the swim section. If this occurred, the water current was quickly reduced to allow the fish to recover its swimming position before the desired test speed was resumed. When a fish repeatedly got stuck on the rear grid within 10 s after having resumed a given test speed, hence no longer was able to swim continuously,

the time was recorded and current speed was reduced to a minimum. After swim trials, fish were euthanized with a blow to the head whereafter fork length (L_F), mass (M) and the width and height of the fish were measured.

In the chase group, a random fish was netted from the holding tank and moved to a 90 × 90 × 50 cm tank. It was then manually chased for 3 min. During this period, fish would initially burst swim in circles, but eventually burst swimming became less frequent. At the end of the 3 min period, the fish showed little responsiveness to handling, suggesting physiological exhaustion (Norin & Clark, 2016; Reidy *et al*., 1995). The fish was then moved in to the small swim tunnel set-up and the swim chamber was sealed off as quickly as possible to reduce delays in the subsequent $\dot{M}O_2$ measurements. Time was recorded from the end of the chase protocol until measurements could be started and averaged 55 ± 3 s (mean ± SE). Chased fish were kept in the respirometer for 2 h at 10 cm s^{-1}, with a similar measurement loop used in the U_{crit} group. At the end of the trial, fish were euthanized and their size measurements recorded.

To account for bacterial respiration in the small swim tunnel, a measurement period was completed after each trial when the fish had been removed. The calculated $\dot{M}O_2$ in the empty chamber was then subtracted from each loop where the fish was present. It should be noted that since the set-up was thoroughly cleaned between trials, background respiration was barely detectable and therefore had negligible influences on the reported values. Ten replicates were performed for both treatments in the small swim-tunnel respirometer.

In the big swim-tunnel respirometer, a similar U_{crit} protocol as described for the small tunnel was performed on fish tested individually. Owing to the large volume of this set-up, however, reliable measurements of oxygen uptake rates were difficult to obtain within a reasonable timeframe. Therefore, only swimming performance was measured when testing fish individually in the large set-up. Eight replicate trials were performed in this experimental group.

By testing groups of fish simultaneously, a reliable measurement of $\dot{M}O_2$ is possible in a larger swim-tunnel respirometer due to the increase in biomass relative to the respirometer volume (Hvas *et al*., 2017a; Svendsen *et al*., 2016b). Hence, using groups of *S. salar* in the big swim-tunnel respirometer has been done in several previous studies (Hvas *et al*., 2017a, 2017b, 2018b; Hvas & Oppedal, 2017; Oldham *et al*., 2019). For our analyses here, we have therefore included measured metabolic rates and swimming performances from one of these studies (Hvas *et al*., 2018b), as it contained an experimental group tested at 13°C seawater with similarly sized fish (Table 1). In addition, these fish had been reared in the same environments and all trials were performed by the same researcher at the same location, allowing for a fair direct comparison in set-up and methodology.

The U_{crit} protocol used when testing groups in the larger set-up was generally similar to when testing individuals in the smaller set-up. Groups of 10 fish were moved into the swim tunnel the day before to allow for acclimation to their new environment at a low current velocity of 15 cm s^{-1}. Continuous moderate flushing was used to maintain stable water temperatures and oxygen levels above 90% saturation in the swim tunnel. When the swim trial was initiated the following morning, current velocity was increased by 15 cm s^{-1} every 30 min. The water inflow was closed off in the first 20 min of each increment period and subsequently opened again for the remaining period to re-establish oxygen levels. Oxygen levels were not allowed to fall below 85% saturation in the closed measurement period. As individual fish fatigued, they were removed through the removable lid at the rear end and time was recorded and once the first fish had been removed $\dot{M}O_2$ was no longer monitored. The swim trials continued until all fish had reached fatigue and had been removed from the swim tunnel, following size measurement recordings. Four replicate trials were performed with group respirometry.

TABLE 1 The live mass (M), fork length (L_F) and condition factor (K) for each experimental group of *Salmo salar*

	M (mean ± SE, g)	L_F (mean ± SE, cm)	K (mean ± SE)
Small tunnel, U_{crit}	343 ± 34	31.5 ± 0.8^{ab}	1.06 ± 0.02
Small tunnel, chase	306 ± 14	30.5 ± 0.3^{a}	1.08 ± 0.03
Big tunnel, alone	413 ± 38	33.6 ± 1.4^{b}	1.08 ± 0.03
Big tunnel, group	403 ± 16	33.4 ± 0.4^{b}	1.07 ± 0.01

Different superscript letters indicate significant differences between groups.

2.4 | Calculations

For each closed period and for selected time intervals within a closed period, $\dot{M}O_2$ was calculated from a linear regression fitted to the decrease in oxygen over time within the respirometry chamber as: $\dot{M}O_2 = ((\delta O_2 \delta t^{-1})(V_{sys} - V_b))M_b^{-1}$, where $\delta O_2/\delta t$ is the slope of the regression, representing change in oxygen per unit of time, V_{sys} is the volume of the respirometer, V_b is the volume of the fish and M_b is the mass of the fish. An assumed density of 1 kg l^{-1} was used to calculate V_b.

In the small swim-tunnel respirometer, automated measurement loops of 15 min allowed for several measurements of $\dot{M}O_2$ overnight prior to the swim trial. From this entire period, the average of the lowest 10% $\dot{M}O_2$ measurements was then calculated and any outliers, defined as ±2SD from the mean, were removed; thereafter, the average was recalculated from the remaining data. This final value was defined as the SMR according to Clark *et al*. (2013) and Norin *et al*. (2014).

Another estimate of the SMR in the small swim-tunnel respirometer was obtained by fitting an exponential function to $\dot{M}O_2$ *v*. swimming speed and back-extrapolating to a swimming speed of zero (Beamish, 1978; Brett, 1964). This regression was based on 4 or 5 different speeds where swimming was observed to be steady and continuous, hence, data point from the lowest speeds where fish would stand on the current were omitted from this calculation. In groups of fish tested in the large swim-tunnel respirometer, the SMR was approximated in the same way by back-extrapolating to a swimming speed of zero. However, since the large set-up was not equipped with an automated flushing system, routine $\dot{M}O_2$ measurements during

overnight acclimation were not obtained, meaning that back-extrapolating was the only method available to estimate the SMR here.

In the small swim-tunnel respirometer, the MMR was estimated in three different ways. First, as the highest $\dot{M}O_2$ measured during the swim trials, which coincided with the highest swimming speed attained prior to fatigue. Secondly, as the $\dot{M}O_2$ during the first 5 min after initial transfer into the set-up which represented handling and confinement stress. Third, as the highest $\dot{M}O_2$ measured over either a 3, 4 or 5 min period (whichever was highest and also provided an $R^2 > 0.9$ in the linear regression) immediately after movement into the respirometer following an exhaustive chase protocol.

In groups tested in the large swim-tunnel respirometer, MMR was only obtained using one method; the highest $\dot{M}O_2$ measured during swimming while all fish still remained in the set-up. This MMR represented swimming speeds when or just before the first fish in the group reached its U_{crit}. As all fish typically would become fatigued within one increment speed of each other, this group average MMR estimate was therefore inherently conservative since the achieved $\dot{M}O_2$ of the final remaining best swimmers was not obtained.

The absolute and factorial aerobic scope was expressed as the difference and factorial relationship between MMR and SMR estimates, respectively. The U_{crit} was calculated according to Brett (1964) as: $U_{crit} = U_F + (t_F U_i) t_i^{-1}$, where U_F is the highest completed current speed, t_F is the time spent swimming until fatigue was reached, U_i is the change in swimming speed between each increment and t_i is the time period of each current speed increment.

Since swim tunnels have fixed cross sectional areas, an object within the tunnel obstructs the flow, which causes the current velocity to increase around the object. This is termed solid-blocking effects and formulae have been developed to approximate the magnitude of this effect as well as to correct for it (Bell & Terhune, 1970). In the small swim-tunnel respirometer, the cross-sectional area of the fish was 4.8 ± 0.4% (mean ± SE) of the swim section, which theoretically only causes a minor effect (Bell & Terhune, 1970). In the large swim-tunnel set-up, fractional cross sectional areas of the fish were less than in the smaller tunnel, as fish rarely overlapped while swimming. Solid blocking effects were therefore not corrected for in either set-up.

The condition factor (K) of the fish was calculated as $100\ M(L_F^3)^{-1}$ (Fulton, 1904; Nash & Valencia, 2006).

2.5 | Statistical analyses

To test for differences between experimental groups in measured parameters (Size, SMR, MMR and U_{crit}), a one-way ANOVA was used, followed by Tukey's *post hoc* test, after equal variance and normal distribution of the data had been confirmed with Levene's test and Shapiro–Wilk test, respectively. A log transformation was necessary in a few cases to adhere to test assumptions. Statistics and figures were made in Sigmaplot (Systat Software; www.systatsoftware.com). P-values < 0.05 were considered significant and data are presented as mean ± SE, unless otherwise specified.

3 | RESULTS

Live mass (M), and K of *S. salar* were statistically similar between groups, but L_F was significantly lower in the chase group compared with the two groups tested in the larger swim tunnel (ANOVA, df = 68, $P < 0.01$; Table 1).

3.1 | Standard metabolic rate

In *S. salar* measured in the smaller swim-tunnel respirometer, the SMR was 123 ± 4 mg O_2 kg^{-1} h^{-1} when calculated as the average of the lowest 10% $\dot{M}O_2$ values, after removing outliers, over a *c.* 20 h period and 124 ± 7 mg O_2 kg^{-1} h^{-1} when back extrapolating to a swim speed of zero. When measured on groups in the larger swim-tunnel respirometer by back extrapolating to zero swim speed, the SMR was 137 ± 7 mg O_2 kg^{-1} h^{-1}. These three SMR estimates were all statistically similar (ANOVA, df = 23, $P > 0.05$; Figure 1(a)). The increase in $\dot{M}O_2$ as a function of swimming speeds with corresponding exponential regressions and derived SMR at 0 cm s^{-1} for both *S. salar* tested alone and in groups are shown in Figure 1b.

3.2 | Maximum metabolic rate

In the smaller swim-tunnel respirometer, the MMR of fish swimming at their highest speed prior to fatigue was 511 ± 15 mg O_2 kg^{-1} h^{-1}, while the initial confinement stress from being moved into the swim chamber resulted in a $\dot{M}O_2$ of 425 ± 21 mg O_2 kg^{-1} h^{-1} during the first 5 min. Furthermore, the $\dot{M}O_2$ measured on fish following the chase protocol was 337 ± 9 mg O_2 kg^{-1} h^{-1}. For fish tested in groups in the larger swim-tunnel respirometer, the MMR was 531 ± 6 mg O_2 kg^{-1} h^{-1}. These different approaches for measuring the MMR of *S. salar* gave significantly different results (ANOVA, df = 33, $P < 0.001$) (Figure 2). Specifically, the MMR obtained from the chase protocol was 174 mg O_2 kg^{-1} h^{-1} lower than in fish swimming to exhaustion in the same set-up ($P < 0.001$). This difference corresponds to a 52% higher estimate of the MMR when performing the U_{crit} test. Consequently, by using the SMR from fish at rest, the derived absolute AS will increase from 218 to 393 mg O_2 kg^{-1} h^{-1} and the factorial AS from 2.8 to 4.3 mg O_2 kg^{-1} h^{-1}, when performing the swim trial instead of relying on a chase protocol. Confinement stress also resulted in significantly lower MMR estimates compared with the U_{crit} protocol ($P < 0.001$), while MMR estimates were statistically similar between groups and individuals when swimming until exhaustion in the two different set-ups ($P > 0.05$).

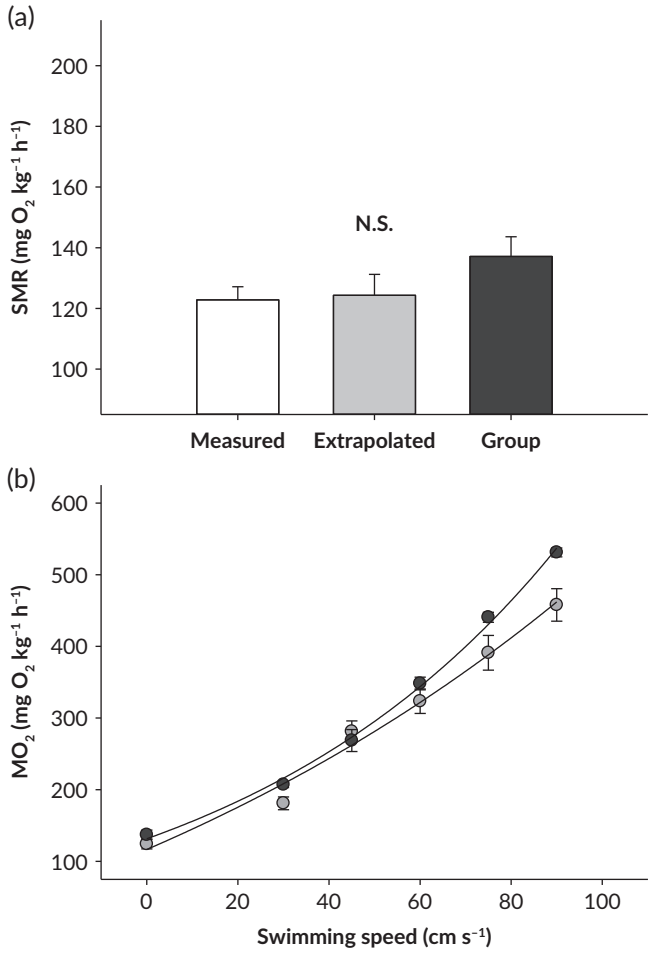

FIGURE 1 (a) The mean (+ SE) standard metabolic rate (SMR) of resting *Salmo salar* measured at 13°C (Measured) and from extrapolation to zero swim speed (Extrapolated), when tested individually in a small respirometer, and from extrapolation to zero swim speed using groups in a larger respirometer (Group). N.S., Non-significant differences. (b) The mean (±SE) oxygen uptake rate ($\dot{M}O_2$) *v.* swimming speed and the derived SMR at zero swim speed of fish tested individually (–○–) in a smaller respirometer or in groups (–●–) in a larger respirometer

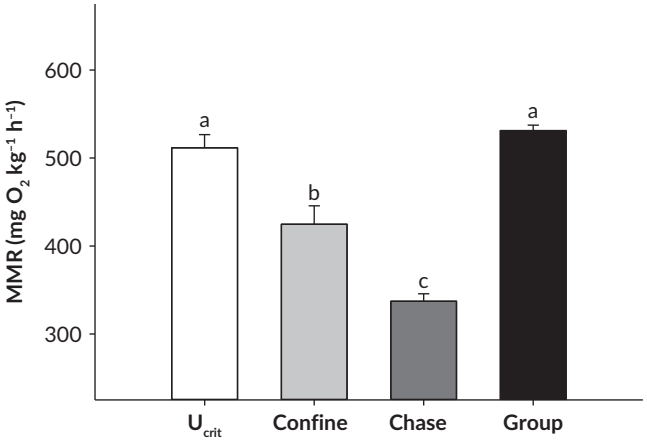

FIGURE 2 The mean (±SE) maximum metabolic rate (MMR) of *Salmo salar* measured at 13°C tested individually while swimming at their critical swimming speed (U_{crit}), after initial movement into the setup (Confinement), or after a 3 min exhaustive chase protocol (Chase) and for fish tested in groups while swimming at their U_{crit} (Group). Different letters above columns indicate significant differences ($P < 0.05$)

3.3 | The critical swimming speed

The U_{crit} was statistically similar between fish swimming alone in the smaller set-up, swimming alone in the larger set-up and swimming in groups in the larger set-up (ANOVA, df = 57, $P > 0.05$; Figure 3a). A scatterplot including individual fish from all groups is shown in Figure 3b to illustrate that U_{crit} expressed in L_B s^{-1} tended to decrease with fish size.

4 | DISCUSSION

4.1 | Standard metabolic rate

In the present study, the SMR of *S. salar* was first determined using intermittent-flow respirometry (Clark *et al.*, 2013). This method represents a direct $\dot{M}O_2$ measurement of an inactive and unfed fish subjected to a stable thermal history and may serve as a control from which indirect methods can be compared and evaluated such as back extrapolating to zero swim speed. Here, we found that back extrapolation provided a statistically similar estimate, confirming that it can serve as a valid method for the determination of SMR in *S. salar*. However, back extrapolation may become faulty in cases where fish show excessive spontaneous activity at low to moderate swimming speeds, as $\dot{M}O_2$ then reflects a higher energetic expenditure rather than what is required for swimming at those specific speeds (Chabot *et al.*, 2016). Measurements during continuous and steady swimming should ideally also be obtained at several speeds spanning the entire aerobic swimming capacity to provide a robust regression line. Furthermore, the final swimming speeds prior to fatigue represents a substantial anaerobic component in salmonids (Kiceniuk & Jones, 1977; Wilson & Egginton, 1994) and consequently $\dot{M}O_2$ may plateau over the last two swimming speeds coinciding with MMR (Hvas *et al.*, 2017a; Lee *et al.*, 2003). Hence, $\dot{M}O_2$ can underestimate the actual energetic requirement for sustained swimming at the final speed assessed and should therefore be omitted when drawing the regression line for extrapolating back to SMR. In other species of fish, obtaining reliable SMR estimates from extrapolation to zero activity will depend on their ability and willingness for continuous steady swimming over a range of speeds in a confined experimental set-up.

4.2 | Maximum metabolic rate

For the *S. salar* tested in the smaller swim tunnel, three methods were used to determine MMR. Of these, the U_{crit} protocol provided the highest estimate by a substantial margin (Figure 2), demonstrating that this is the best method for measuring MMR in *S. salar*. It could even

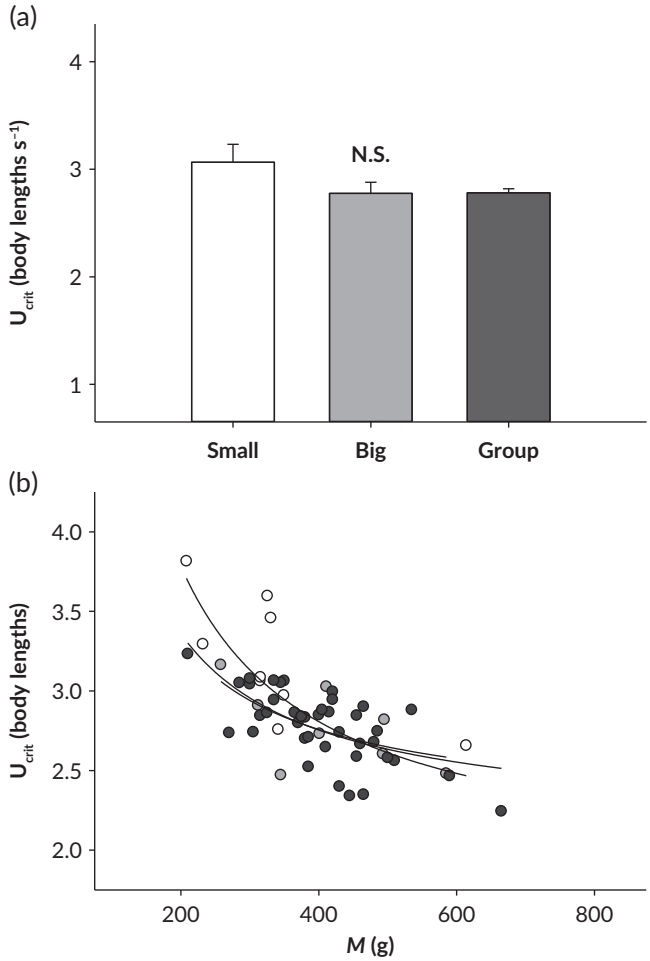

FIGURE 3 (a) The critical swimming speed (U_{crit}) of *Salmo salar* measured at 13°C in a smaller swim tunnel on individual fish (Small), in a larger swim tunnel on individual fish (Big), and in groups of fish in a larger swim tunnel (Group). N.S., Non-significant differences. (b) A scatterplot of U_{crit} values *versus* live mass (*M*) of each fish measured with an inverse first order regression lines for each group. (○) Small tunnel, (◍) big tunnel, alone, and (●) big tunnel, group

be argued that the U_{crit} protocol is the only valid method due to the large underestimates made when using the other methods.

A core assumption in aquatic respirometry is that rates of oxygen uptake is proportional to the rates of oxygen consumption and is therefore proportional to aerobic metabolic rates (Fry & Hart, 1948; Wood & Perry, 1985; Zhang & Gilbert, 2017). For this to be true steady-state conditions are required (Nelson, 2016). In the U_{crit} protocol, $\dot{M}O_2$ is measured on fish swimming continuously for several minutes and is therefore more likely to approximate steady-state oxygen consumption rates. In contrast, the chase protocol provides the highest measured $\dot{M}O_2$ in a shorter amount of time and represents a non-steady-state condition during initial recovery from exhaustion. Hence, imminent rates of oxygen uptake at the gills may not reflect rates of oxygen consumption in the mitochondria (Zhang & Gilbert, 2017) and meaningful physiological and ecological interpretation of what is actually measured is obscured. Nevertheless, chase protocols are currently widely used to estimate MMR, even on salmonids that are willing to swim in respirometers (Bowden *et al.*, 2018; Wood *et al.*, 2017; Zhang *et al.*, 2018). This could be because chase protocols in static set-ups are faster to complete and easier to perform compared with U_{crit} protocols in swim tunnels. However, if MMR is substantially underestimated this will also result in underestimation of AS, which often is the core parameter in respirometry studies. Whether these underestimations systematically become biased during certain experimental manipulations such as different water temperatures would be relevant to explore in future studies.

An additional possible estimate of MMR is the $\dot{M}O_2$ measured following initial movement into the respirometer on fish that have not been exhausted beforehand. This situation closely resembles the conditions of a typical confinement stress trial, which is a commonly used method when studying the acute stress response in salmonids (Pottinger & Carrick, 1999; Vindas *et al.*, 2016). Acute handling stress alone is expected to momentarily increase $\dot{M}O_2$. In addition, *S. salar* also showed brief hyperactivity after movement into the respirometer, which probably represented escape behaviour. The resulting $\dot{M}O_2$ in the first 5 min was higher compared with an exhaustive chase protocol, perhaps partly owing to avoidance of any lag-time before measurements could be started. Hence, a better estimate of MMR may be obtained by simply avoiding the chase protocol on species such as *S. salar* that have a nervous nature and are easily agitated when subjected to an unfamiliar situation. However, $\dot{M}O_2$ was still lower than swimming until fatigued.

4.3 | Group respirometry

A major objective of this study was to compare SMR and MMR estimates obtained from individual *S. salar* tested in a small swim-tunnel respirometer to those obtained in groups of fish in a larger swim-tunnel respirometer. Estimates between set-ups in both SMR and MMR were statistically similar, illustrating that group respirometry can serve as a viable method for *S.salar*.

A concern with group respirometry is the underestimation of the MMR as individual fish may be in different physiological states at the time of measurement (Hvas *et al.*, 2017a). However, MMR was similar to fish measured individually in the smaller set-up (Figure 2). Assuming an inherent underestimation with the group approach would therefore imply that MMR from the smaller set-up also was underestimated, perhaps as a consequence of limited space to express natural high-speed swimming behaviour (Tudorache *et al.*, 2007). Hence, a larger swim section may have alleviated some of the issues with individual variation within test groups. Furthermore, since the *S. salar* used in the present study were farmed fish of similar sizes from the same genetic origin, less individual variation was expected compared with if one had used groups of wild-caught fish. However, the true MMR could prove to remain elusive if the concerns of underestimation in both swim-tunnel set-ups are legitimate, especially as the alternative methods were found to provide substantially lower values.

Limitations with group respirometry can include measurements of excess post-exercise oxygen consumption and specific dynamic

action, as it is difficult to control for similar levels of experienced exercise and feed intake between individuals simultaneously. Additionally, group respirometry would be useless in experiments with a focus on individual variation in metabolic traits, or where more detailed measurements of individual fish are required. Furthermore, if only a limited number of fish are available for experimentation, devoting the time to study each individual thoroughly would be ideal. However, several notable advantages do exist with group respirometry. A larger swim section allows fish to express natural swimming behaviours and observations should therefore be more ecologically relevant (Remen *et al.*, 2016). Presumably, it is also less stressful to be tested in groups in a larger space, as smaller respirometers resemble confinement stress trials and farmed salmonids are conditioned to be in groups. Furthermore, group respirometry is more time efficient as less time is spent collecting data as $\dot{M}O_2$ measurements represent a treatment replicate average.

Because many fish can be tested at the same time while still obtaining the U_{crit} of individual fish, it is possible to do a large number of various non-repeated supplementary samplings in relation to exhaustive exercise stress and the subsequent recovery trajectories in substantial less time. For instance, previous studies performed histology and PCR analyses on gill samples from fish tested with amoebic gill disease, correlated ventricle mass with swimming capabilities, assessed exercise induced changes to gill Na^+-K^+-ATPase activity and sampled blood to measure plasma ions and various stress parameters (Hvas *et al.*, 2017b, 2018b; Hvas & Oppedal, 2017).

The suitability and relevance of group swim-tunnel respirometry in other species of fish will depend on their life styles and group dynamics. Schooling species with prolonged swimming capabilities should do well, while species with pronounced antagonistic behaviours in group settings may not provide representative $\dot{M}O_2$ measurements at moderate swimming speeds. Finally, it is imperative that the fish being tested belong to a similar size class and have been subjected to a similar acclimation history beforehand, as $\dot{M}O_2$ scales with size and is sensitive to environmental conditions (*e.g.* Hvas *et al.*, 2017a; Killen *et al.*, 2006; Oldham *et al.*, 2019).

4.4 | Critical swimming speed

Contrary to expectations, neither swimming in groups nor swimming in a larger tunnel was found to improve U_{crit} in *S. salar*. Earlier studies with other fish species have reported advantages when swimming in groups at moderate swimming speeds such as improved costs of transport, lower tail-beat frequencies and favourable hydrodynamics (Hartwell & Otto, 1978; Herskin & Steffensen, 1998; Marras *et al.*, 2015; Svendsen *et al.*, 2003). When measuring the U_{crit}, fish are eventually forced to swim at their maximum capacities where anaerobically powered burst-and-glide gaits becomes necessary. Maintaining an organised group structure can therefore become more difficult. Hence, unorganised and chaotic swimming at the final test speeds could explain why U_{crit} was not improved in the present study. However, it is still possible that *S. salar* are able to gain hydrodynamic advantages from swimming in schools at moderate speeds. This may be the case in marine sea cages, where *S. salar* can form circular schooling structures in slack waters or increase their schooling densities and swim tightly together in strong water currents (Hvas *et al.*, 2017c; Johansson *et al.*, 2014). In open waters, wild salmonids are mainly solitary and the extent of a group benefit is likely less than in naturally schooling species such as mackerels (Scombridae) and herrings (Clupeidae). Even so, tighter swimming and its benefits may be present when shoals of salmonids are approaching and entering their native rivers.

Longer and larger swim tunnels have previously been found to improve U_{crit} in species such as common carp *Cyprinus carpio* L. 1758, brook trout *Salvelinus fontinalis* (Mitchill 1814) and shortnose sturgeon (*Acipenser brevirostrum* LeSueur 1818 due to improved conditions for burst-and-glide swimming (Deslauriers & Kieffer, 2011; Tudorache *et al.*, 2007, 2010). For *S. salar*, more vigorous burst swimming was observed in the larger tunnel, while powerful bursts in the small tunnel sometimes caused the fish to collide with the upstream front end of the swimming section. This suggests that natural swimming behaviours were compromised in the smaller set-up.

Poorer laminar flow properties in the rectangular shaped smaller set-up compared with the circular cross section of the larger set-up could explain why U_{crit} was not lower. In the larger tunnel, *S. salar* were evenly spread out and did not appear to prefer specific sides or sections when swimming. However, in the smaller set-up, they consistently preferred the downstream left side when swimming at higher speeds, which is indicative of uneven current conditions. Since flow velocities in this study were only calibrated in the midsection, currents experienced by the fish were likely lower than reported, which may have resulted in U_{crit} being overestimated. Exploitation of flawed laminar flow properties is also suggested by a slightly lower $\dot{M}O_2$ compared with the groups in the larger set-up at supposedly similar swimming speeds (Figure 1b). Hence, while the obtained U_{crit} values were similar between the two swim tunnel designs, the actual swimming performance could have been compromised in the smaller set-up. To improve laminar flow properties in the smaller set-up in future studies it may be possible to adjust the collimators to slightly reduce current speeds in the middle of the swim section.

In conclusion, we compared the effects of experimental set-ups and protocols on the measurements of SMR, MMR and U_{crit} in *S. salar*. Group respirometry provided similar estimates as individually assessed fish, illustrating that it can be a valid method in some experiments. While SMR estimates were similar between measurements at rest and from back extrapolation to zero swim speed, MMR was found to be severely underestimated when using a chase protocol. A swim-tunnel respirometer that allows for $\dot{M}O_2$ measurements at high swimming intensities is therefore necessary for accurate MMR estimates and subsequent calculations of AS in *S. salar*. Contrary to expectation, the U_{crit} was neither improved by group swimming nor a larger swim section. However, the latter may be an artefact of flawed laminar flow properties in the smaller set-up.

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AUTHOR CONTRIBUTIONS

This work was conceived and designed by both authors. M.H. performed experiments, data analyses and wrote the first draft, while F.O. provided valuable and critical input before approving the final version.

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