The determination of standard metabolic rate in fishes

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This review and data analysis outline how fish biologists should most reliably estimate the minimal amount of oxygen needed by a fish to support its aerobic metabolic rate (termed standard metabolic rate; SMR). By reviewing key literature, it explains the theory, terminology and challenges underlying SMR measurements in fishes, which are almost always made using respirometry (which measures oxygen uptake, \( \dot{M}O_2 \)). Then, the practical difficulties of measuring SMR when activity of the fish is not quantitatively evaluated are comprehensively explored using 85 examples of \( \dot{M}O_2 \) data from different fishes and one crustacean, an analysis that goes well beyond any previous attempt. The main objective was to compare eight methods to estimate SMR. The methods were: average of the lowest 10 values (low10) and average of the 10% lowest \( \dot{M}O_2 \) values, after removing the five lowest ones as outliers (low10%), mean of the lowest normal distribution (MLND) and quantiles that assign from 10 to 30% of the data below SMR (\( q_{0.1}, q_{0.15}, q_{0.2}, q_{0.25} \) and \( q_{0.3} \)). The eight methods yielded significantly different SMR estimates, as expected. While the differences were small when the variability was low amongst the \( \dot{M}O_2 \) values, they were important (>20%) for several cases. The degree of agreement between the methods was related to the c.v. of the observations that were classified into the lowest normal distribution, the c.v. MLND (C,\(_{\text{MLND}}\)). When this indicator was low (\( \leq 5\% \)), it was advantageous to use the MLND, otherwise, one of the \( q_{0.2} \) or \( q_{0.25} \) should be used. The second objective was to assess if the data recorded during the initial recovery period in the respirometer should be included or excluded, and the recommendation is to exclude them. The final objective was to determine the minimal duration of experiments aiming to estimate SMR. The results show that 12 h is insufficient but 24 h is adequate.

A list of basic recommendations for practitioners who use respirometry to measure SMR in fishes is provided.

Key words: minimum metabolic rate; oxygen consumption; quantile; SMR.

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INTRODUCTION

Whole-animal metabolic rate (MR) is influenced by a variety of factors such as activity level, physiological state, body size, temperature, food intake and anabolism. Indeed, MR can vary upwards of 10 fold in ectothermic animals. Yet, there is a minimum MR (MR\(_{\text{min}}\)) for the subsistence of an organism (Hulbert & Else, 2004), which is called basal (BMR) or standard MR (SMR). This terminology is discussed below.

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Subsistence living allows for no activity, digestion, growth or production of sexual products, but merely supports essential homeostatic activities for cells and whole organisms (Brett, 1962; Frappell & Butler, 2004). In mammals, it is estimated that 90% of cellular oxygen consumption takes place in the mitochondria (Rolfe & Brown, 1997), and this can be further separated into the cost of counteracting mitochondrial proton leak (c. 20%) and ATP production (c. 80%) (Rolfe & Brown, 1997). These authors further estimated that at a whole-animal level, ATP production is used about equally for Na\(^+\), K\(^+\)-ATPase activity and protein synthesis (20–25% each), with significant contributions for Ca\(^{2+}\)-ATPase activity, gluconeogenesis, ureagenesis and actinomyosin-ATPase activity, and c. 6% for all other ATP-consuming processes. This is similar in other higher vertebrates (Hulbert & Else, 2004).

If MR is below MR\(_{\text{min}}\), physiological function is in some way impaired and for most species (including fishes), life cannot be sustained for long (Job, 1957; Smit, 1965; Priede, 1985; Claireaux & Chabot, 2016), unless the animal can suppress some of these metabolic energy requirements, such as during aestivation or exposure to anoxia or severe hypoxia (Delaney et al., 1974; Stecyk et al., 2008; Richards, 2010; Seibel, 2011). Replacing aerobic metabolism with anaerobic glycolysis is only a temporary solution because of a less efficient use of fuel and an accumulation of toxic wastes.

The objectives of this study are to review the terminology and methods associated with the concept of MR\(_{\text{min}}\). As there is no accepted method in the specific case when activity level is unknown during the measurement of MR, the review part of the paper is followed by an experimental part that aims at comparing the most often used methods and make recommendations.

### TERMINOLOGY

Because MR has almost always been measured as O\(_2\) removal from the water by a fish, MR is actually oxygen uptake (\(\dot{M}O_2\)) rather than oxygen consumption by tissues. Oxygen uptake is also the most widely used method for mammals, birds and reptiles, and both terrestrial and aquatic invertebrates. The advantages and disadvantages of using oxygen uptake instead of direct calorimetry are discussed in Brett & Groves (1979), Cech (1990) and Nelson (2016). Also, \(\dot{M}O_2\) expressed in oxygen units rather than energy units is best called respiration rate instead of MR. This review of the terminology in the field must, however, by necessity, use the original terms of the literature cited.

As early as the end of the 18th Century, Séguin & Lavoisier (1789) established that physical activity and digestion increased \(\dot{M}O_2\). They also determined that their subjects had to be exposed to a comfortable temperature, otherwise they would increase their rate of energy expenditure to cool or warm themselves. They defined the basic conditions to measure MR in humans: the subjects must be at rest, fasted and at an ambient temperature of 26° C. MR measured in such conditions became known as BMR.

Krogh (1914, 1916) subsequently reserved the term BMR for the lowest rate of oxygen consumption of an organ that is not performing any work. In his view, BMR was impossible to measure for a whole organism, because the circulatory and respiratory functions required to maintain an animal alive involved some expenditure of energy above BMR. Instead, he preferred the term SMR for whole organisms exhibiting minimal functional activity, i.e. in total absence of voluntary muscular movements and when no food was being digested or absorbed (Krogh, 1914). SMR was considered
to be the ‘nearest attainable approximation’ of basal metabolism, which he argued would be attained if all organs were absolutely at rest (Krogh, 1914). Krogh (1914) even attempted to estimate the cost of processes such as circulation and breathing, to evaluate the difference between BMR and SMR. His stringent condition of absolutely no voluntary activity, however, required immobilizing non-human subjects with curare or, for fishes, sedation, although in some cases there was no difference in \( \dot{M}O_2 \) between sedated and quiescent fishes (Ege & Krogh, 1914). This demonstrates that when conditions are right, some species of fish can become essentially motionless in a respirometer, a fact that still influences the design of respirometers and protocols used to estimate SMR today. It bears repeating that for Krogh (1914), it was theoretically impossible to measure BMR of a live fish. Only SMR measurements were possible, in part because BMR was more a property of cells rather than animals that rely on organs to perform external work, and SMR required a completely motionless fish.

The possibility of obtaining absolutely quiescent or resting states with undomesticated animals, or animals susceptible to excitement from handling or experimental confinement, has been questioned frequently. Instead of immobilizing their subjects pharmacologically, other authors adopted the term SMR to mean the lowest \( \dot{M}O_2 \) commensurate with appropriate experimental techniques but could not ensure that there was strictly no contribution from activity (Fry & Hart, 1948; Brett, 1962). In this context, SMR became a practical approximation of BMR, with the possible inclusion of some minimal level of activity, so being different from Krogh’s (1914) SMR that excluded all external work.

Whenever subjects show some minor activity in a respirometer (swimming or maintaining station in the case of fishes) many authors prefer the term routine MR (RMR), which includes a minor cost of activity (Winberg, 1960; Brett, 1962; Dall, 1986). This term is necessarily vague because activity is undefined and not quantified, and RMR can be at any level between SMR and the maximum MR (MMR), although good respirometer design and protocol should insure that it is closer to SMR than to MMR. When activity is known to be very limited, such as the small fin movements to maintain position in a respirometer, authors have used terms such as ‘low routine’, ‘resting’ or ‘fasting’ MRs as practical approximations of BMR and SMR (MacKinnon, 1973; Howell & Canario, 1987; Blaxter, 1989; Plaut, 2000). Schmidt-Nielsen (1984) also objected to the term BMR but for semantic reasons, because the term implies that MR cannot fall below BMR. In humans, \( MO_2 \) is lowest during sleep, but BMR is measured when subjects are resting but not asleep. He proposed the term maintenance metabolism instead of BMR. This is not, however, an improvement as Krogh (1916) had dismissed this term for semantic reasons of his own: an animal cannot maintain itself on BMR or SMR for any length of time, it will start losing mass to sustain its MR if fasted continuously. Jobling (1994) promoted the pragmatic use of ‘minimal’ or ‘fasting’ metabolism because of the many different interpretations of SMR.

Tradition is, however, hard to break and BMR and SMR are the recommended terms to represent MR\(_{\text{min}}\), measured in specific conditions, in homeotherms and ectotherms, respectively (McNab, 1997; Frappell & Butler, 2004). A few mammals do not regulate their body temperature and their BMR cannot be measured, so their SMR at a given temperature is measured, just as in ectotherms (McNab, 1997). Both BMR and SMR measurements include the cost of the external work of organs such as those involved in circulation and respiration, but not activity. To reiterate, SMR is the preferred terminology for fishes.
The ecological significance of SMR might be thought of as limited, because fishes in the wild are rarely in such states. Exceptions might include periods of food shortage or in environmental extremes such as hypoxia and supra-optimal temperatures, when the oxygen delivery system is taxed to the extent that routine activities beyond SMR become almost impossible. SMR does, however, have pervasive ecological relevance, with important potential implications for maximum performance, growth rate, lifestyle and social interactions (Clarke, 1991; Metcalfe et al., 1995, 2016; Clarke & Johnston, 1999; McCarthy, 2000; Cutts et al., 2002; Millidine et al., 2009; Killen et al., 2010). A valid SMR measurement is needed to determine aerobic scope properly, defined as MMR minus SMR (Fry, 1947, 1971). Aerobic scope and the Fry paradigm, with its derivative the oxygen and capacity-limited thermal tolerance hypothesis, are useful frameworks to define limits and optima for temperature tolerance of fishes (Fry, 1971; Claireaux & Lefrançois, 2007; Pörtner & Farrell, 2008; Farrell, 2009; Cucco et al., 2012). Also, an accurate SMR is a prerequisite to measure hypoxia tolerance as $O_{2\text{crit}}$ (Claireaux & Chabot, 2016). Furthermore, SMR is an essential input variable for energetic models that predict energy portioning and resource allocation (Armstrong et al., 1992; Hansson et al., 1996), even if reliable quantitative information on the energy cost of specific activities is sadly lacking, beyond swimming and digestion.

Before addressing the issue of how to measure SMR in fishes, it is useful to consider how BMR is measured in homeotherms. The conditions for a BMR measurement in human subjects are particularly precise: (1) the individual must be awake and supine, (2) in a state of complete muscular repose, (3) after a 20–30 min period of rest, (4) fasting for 12–14 h following a last meal at about the maintenance level and that excluded caffeine and any subsequent nicotine, (5) isolated from external stimuli in a thermoneutral and dark environment, and acclimated to such an environment, (6) free from emotional stress and (7) in women, the measurement should not be made immediately before or during the menses, or during pregnancy (Harris & Benedict, 1918; Blaxter, 1989; McNab, 1997; Hulbert & Else, 2004; Johnstone et al., 2005).

For other homeotherms, the main conditions are acclimation to the experimental setup, fasting, rest (no activity), a thermoneutral environment and regulation of body heat (to exclude animals in torpor) (Kleiber, 1975; McNab, 1997). Of course, states of hibernation, aestivation or torpor add a further but rather specialized layer of complexity to defining $M_{\text{Rmin}}$, but these states are not considered BMR or SMR. Some authors restrict the measurement of BMR to adult subjects in a non-reproductive state, to exclude the cost of growth and reproduction (McNab, 1997). The requirement of using only non-reproducing adult subjects is mandatory when dealing with interspecific comparisons of BMR, in particular the effect of body size on BMR.

The conditions to measure SMR in ectotherms are similar to those for BMR in mammals and birds, and are designed to produce low values of $MO_2$. In the case of fishes, SMR at a given temperature is typically measured in post-absorptive (but not starving), inactive individuals, except for minimal fin movements to remain stationary in the water column for fishes that do not rest on the bottom, after acclimation to the experimental temperature and setup, and during the inactive part of the fish’s circadian cycle (Fry & Hart, 1948; Brett, 1962). Nonetheless, there are many possible variations in procedures and analytical techniques.
In this regard, it is important to recognize the advances that have been provided by developments in respirometer design and in technologies to measure dissolved oxygen (DO). Prior to around 1970, DO was almost always measured by Winkler titration (Winkler, 1888; American Public Health Association American Water Works Association & Water Environment Federation, 1999), an extremely accurate but time-consuming method that does not yield instantaneous results. Practically, and at best, only one \( \dot{M}O_2 \) measurement was possible every 30–60 min (Keys, 1930; Wells, 1932; Fry & Hart, 1948). Consequently, these measurements integrated \( \dot{M}O_2 \) over a long time period, increasing the risk of activity but decreasing the risk of obtaining a value of \( \dot{M}O_2 \) below SMR. The lowest value of \( \dot{M}O_2 \), obtained during the time of day when the species was known to be less active, was as close to SMR as could be hoped for (Fry & Hart, 1948).

Since the 1970s, DO has been measured mostly with electrodes (Clark, 1956; Gatti et al., 2002), and more recently with optodes (Holst & Grunwald, 2001; Glazer et al., 2004; Tengberg et al., 2006). Both offer short time lags in detecting changes in DO and immediate results. Along with the advent of intermittent-flow and open-flow respirometers (Muir & Niimi, 1972; Duthie, 1982; Bushnell et al., 1984; Steffensen, 1989; Svendsen et al., 2016) and data storage on computers, more frequent \( \dot{M}O_2 \) measurements became the norm, along with real-time analysis and display with commercial software, such as AutoResp (Loligo Systems; www.loligosystems.com) or open-source software such as AquaResp (www.aquaresp.com/oxygen). Moreover, a shorter measurement period (5–20 min) might allow comparison with prevailing fish behaviour, potentially monitored with video (Crocker & Cech, 1997). The effect of activity and daily cycles on \( \dot{M}O_2 \) are not precluded, therefore, just better managed and accounted for. The shorter time frame unfortunately introduces a new concern. An individual’s oxygen uptake is the sum of many demands made at tissue and organ level (Darveau et al., 2002). The instantaneous oxygen consumption of organs such as the heart and gills would reflect each contraction (Priede, 1985). Brief periods of anaerobic activity can temporarily suspend oxygen consumption by some tissues or organ, followed by greater oxygen consumption during recovery. The lag between oxygen pickup at the gills and its utilization in tissues buffers some of these short variations of oxygen consumption at the individual level, but an integration time of c. 30 min better reflects steady-state in whole-animal oxygen uptake (Priede, 1985). Therefore, with the short measurement periods that are now common, consecutive \( \dot{M}O_2 \) measurements are unlikely to be strictly constant even when a fish is in a state corresponding to SMR. Instead, some variation about an average value corresponding to SMR is expected. It cannot be assumed, therefore, that the single (or several) lowest \( \dot{M}O_2 \) values are estimates of SMR. Instead, the values associated with SMR should be observed repeatably and represent a reasonable proportion of the \( \dot{M}O_2 \) values, unless the experiment is very unsuccessful at getting fishes to become inactive in the respirometer. The key question then becomes, what is a reasonable proportion? This question is addressed below, by analysing data from fish respirometry experiments, much of which is new.

**Adequate respirometer design**

Any determination of \( \dot{M}O_2 \) requires an appropriate respirometer design, an issue thoroughly considered elsewhere (Steffensen, 1989; Svendsen et al., 2016). A few key points are offered here. Estimating SMR requires numerous determinations of \( \dot{M}O_2 \) to accommodate acclimation, daily \( \dot{M}O_2 \) cycles and variable activity patterns. Thus,
intermittent-flow respirometry or continuous-flow (but suitably corrected for wash-out) respirometry is required. Respirometer volume should be sufficient to avoid stress, but small enough to minimize unwanted activity and ensure that variations in oxygen levels in the system occur and can be accurately quantified within a reasonable time period. It is essential to take precautions to ensure that fishes calm down inside the respirometer (Clausen, 1936; Cech, 1990), such as controlling water quality (e.g. DO, pH and ammonia), temperature, light, noise and vibration levels.

A species’ natural habitat and behaviours should be considered too because unfamiliarity can increase $\dot{M}O_2$. The common sole *Solea solea* (L. 1758) has a lower $\dot{M}O_2$ when there is substrate in the respirometer; sand was needed to properly estimate SMR (Howell & Canario, 1987). This may be true of other burrowing species such as sand lances (*Ammodothyidae*) (J. F. Steffensen, unpubl. data). Some species require access to a shelter within the respirometer to exhibit their lowest $\dot{M}O_2$, such as juvenile salmon *Salmo salar* L. 1758 (Millidine *et al.*, 2006). Similarly, some gregarious species such as Atlantic menhaden *Brevoortia tyrannus* (Latrobe 1802) may become hyperactive, stressed and even die when tested alone (Hettler, 1976) such that testing in groups is necessary. Some researchers recommend the use of ‘conditioned water’, or water that previously contained fishes (Winberg, 1960) or water from the tank where the fish was previously held (Fry, 1971), but it has never been shown that these measures lower $\dot{M}O_2$ when respirometer design is appropriate and fishes are properly acclimated to experimental conditions.

**Duration of experiments**

Perhaps, the greatest initial challenge of measuring SMR is that handling stress and excitement may elevate $\dot{M}O_2$ considerably (Smit, 1965; Fry, 1971). Minimally, several hours are needed to recover from the stress of being moved to the respirometer, and ideally several days are needed to then identify any diurnal patterns. The repayment of an oxygen debt, such as incurred by chasing the fishes or when fishes struggle as they are placed into a respirometer, elevates $MO_2$ (Keys, 1930). Therefore, $\dot{M}O_2$ usually remains elevated for several hours once fishes are in a respirometer (Keys, 1930; Steffensen *et al.*, 1994; Herrmann & Enders, 2000; Cheng & Farrell, 2007) and SMR cannot be determined during this period. This period can be shortened by reducing initial stress, such as by coaxing the fish into a plastic bag with a minimum of chasing to transfer it into the respirometer (McKenzie *et al.*, 2007; Dupont-Prinet *et al.*, 2013b), because chasing and air exposure are stressful to fishes (Zahl *et al.*, 2010). Without such precautions, the duration of this period can be 24 h or more (Wells, 1932; Sundnes, 1957a, b). There is no accepted minimum duration for the acclimation period, however, because of species differences. The best solution probably lies in measuring $MO_2$ over a few days and inspecting data to demonstrate that each fish (or minimally a sub-sample of fishes, or a pilot experiment; Jobling & Spencer Davies, 1980) regularly achieve a low and relatively constant $\dot{M}O_2$ compared with the elevated initial $\dot{M}O_2$. Minimally, fishes are typically placed in respirometers for 10–48 h before starting to measure $\dot{M}O_2$ to minimize handling stress (Clausen, 1936; Fry & Hart, 1948; Clark *et al.*, 2013). This is often the best solution when experiments are conducted in the field and there are other constraints on holding the fishes.

Similarly, there is no accepted minimum duration of experiments aiming to determine SMR in fishes. Some species have a circadian cycle of $MO_2$ (Clausen, 1936; Fry, 1947; Fry & Hart, 1948; Mehner & Wieser, 1994; Svendsen *et al.*, 2014). While many
species are day-active, some are active during the night, including the Pacific hagfish *Eptatretus stoutii* (Lockington 1878) (Cox *et al.*, 2011) and juvenile Greenland halibut *Reinhardtius hippoglossoides* (Walbaum 1792). Unless a diurnal cycle can be ruled out by prior knowledge or pilot experiments, MO₂ should be measured regularly over at least 24 h (Cech, 1990), preferably 48 h, after the fish is acclimated to the respirometer. If shorter experiments are desired, it must be demonstrated that they are timed so that the quiescent part of the cycle occurs after fishes are acclimated (MacKinnon, 1973).

The duration of the fasting period, which usually precedes placement in the respirometer, has to be sufficient to ensure that fishes are in a post-absorptive state. Strictly speaking, this requires knowledge of gut evacuation time. This can be determined experimentally or from the literature before the start of respirometry experiments. Ammonia excretion can also be used to verify that a fish is in a post-absorptive state (Brett & Zala, 1975). In general, digestion time decreases with increasing temperature (Andersen, 1999) and increases with ration and prey quality (energy density) (Andersen, 1998; Andersen & Beyer, 2005; Fu *et al.*, 2005a, b). Digestion proceeds more slowly in hypoxia (Jordan & Steffensen, 2007; Zhang *et al.*, 2010). A very large single meal (>10% body mass), especially at a cold temperature or low DO level, can increase MO₂ for >5 days (Jordan & Steffensen, 2007; Dupont-Prinet *et al.*, 2013b; Eliason & Farrell, 2014), and even 16 days at −0.5 °C (Secor, 2009). Fishes fed on a regular basis may require even a longer fasting period because repeated meals increase MO₂ more than a single meal (Soofiani & Hawkins, 1982; Fu *et al.*, 2005c), although gut evacuation time after the last meal does not always increase (Fu *et al.*, 2005c). For some species, prolonged fasting progressively lowers SMR (Mehner & Wieser, 1994; Fu *et al.*, 2005d; Van Leeuwen *et al.*, 2012; McKenzie *et al.*, 2014). Thus, a progressive decrease in MO₂ over days could be related to factors ranging from a postprandial decline, through to habituation and recovery from handling stress, and a transition to a starvation-related suppression of MR if fasting is too long. The duration of the fasting period should be explained and justified when reporting SMR of fishes. When it is impossible to fast the fishes (e.g. fishes captured from the wild for limited observations), RMR should be reported.

Test temperature must be controlled and reported because of the strong influence of temperature on SMR of ectotherms (Fry, 1971; Hulbert & Else, 2004). The necessity to work with thermally acclimated fishes has been emphasized (Fry, 1947; Winberg, 1960; Brett, 1962; Beamish, 1964a). While SMR will vary exponentially with an acute temperature change, often with a quotient of around 2 for every 10 °C difference, thermal acclimation tends to shift MR back towards that of the previous temperature. Thus, in a thermally acclimated fish, the quotient for a 10 °C difference will be lower, between 1 and 2, than the quotient measured following an acute temperature change. Thermal acclimation can require days to weeks, depending on the extent and direction of the temperature change (Wells, 1935; Barrionuevo, 1998). This consideration is particularly important in aerobic scope. For example, the shape of the relationship between aerobic scope and temperature differed between killifish *Fundulus heteroclitus* (L. 1766) acclimated to each temperature or acutely exposed to that temperature (Healy & Schulte, 2012). Similarly, the shape of the aerobic scope curve with an acute temperature change varied when goldfish *Carassius auratus* (L. 1758) were tested at different acclimation temperatures (Ferreira *et al.*, 2014). When considering adaptability or susceptibility to a slow temperature change, such as global warming, aerobic scope needs
to be measured in thermally acclimated fishes (Clark et al., 2013). This is not to say that measurements of $\dot{M}O_2$ in fishes subjected to acute temperature changes (Lee et al., 2003a; Sokolova & Pörtner, 2003; Zakhartsev et al., 2003; Clark et al., 2008, 2011; Steinhausen et al., 2008; Eliason et al., 2011) are not of interest. For example, they help understand how the circulatory and respiratory functions of fishes cope when moving through thermally stratified water. A prerequisite to a proper SMR measurement, however, is thermal acclimation and the duration of thermal acclimation should be reported with SMR.

**Body mass and life stage**

SMR is influenced by body mass, with large fishes using more oxygen per unit time, but less oxygen per unit mass per unit time. Within a species (an ontogenic effect), SMR is linearly related to body mass after logarithmic transformation of both variables (Sundnes, 1957a; Winberg, 1960; Beamish, 1964a; Beamish & Mookherjii, 1964; MacKinnon, 1973; Duthie, 1982; Ishibashi et al., 2005), but the relationship can differ for larval and post-larval states (Oikawa et al., 1991; Yagi et al., 2010). Therefore, comparisons of SMR should acknowledge the need to treat mass as a covariate or scale SMR for a standardized mass (Newell et al., 1977; Schurmann & Steffensen, 1997). This scaling is only possible for individuals of the same life stage but with different masses, as the scaling exponent may change for different life stages (Post & Lee, 1996; Killen et al., 2007a, b).

Even though SMR does not include growth-related expenditures, it can be argued that it can be measured in fast-growing juvenile fishes, as long as the usual conditions to measure SMR are met, including an appropriate fasting period. Most fishes have indeterminate growth and, strictly speaking, it would be difficult to meet a no growth requirement even in adult fishes. The required post-absorptive state should ensure that little growth occurs during the determination of SMR. A recent study, however, has shown that the nutritional plane or prevailing nutritional context (long-term food availability) can influence SMR of juvenile fishes, with better-fed fish having a greater SMR than poorly fed fish (Van Leeuwen et al., 2012), even though SMR was determined after an appropriate fasting period. Similarly, fishes that were food deprived and then given ad libitum access to food often go through a phase of growth compensation. This has been shown to alter SMR in the common minnow *Phoxinus phoxinus* (L. 1758) (Killen, 2014). Ideally, juvenile fishes should be on a maintenance ration for a few weeks prior to SMR determination (Rosenfeld et al., 2015), but at minimum, feeding conditions during the last few weeks leading to the SMR test should be reported. Larval fishes of many species are always active and fast growing and cannot be fasted for long periods. It is preferable to report RMR (Bochdansky & Leggett, 2001; Moran & Wells, 2007; Peck & Moyano, 2016).

Adult fishes are characterized by reproductive phases and invest energy into gonad maturation and gamete production, which by necessity increases $\dot{M}O_2$ (Wells, 1935; Brett, 1962; Beamish, 1964b). These energy investments can continue during fasting because migratory Pacific salmon (*Oncorhynchus* spp.) cease eating for at least a month before spawning and yet their gonads continue to grow (Farrell, 2009). Similarly, female Atlantic cod *Gadus morhua* L. 1758 cease feeding c. 1 month prior to the start of spawning (Fordham & Trippel, 1999). Thus, measuring SMR is impossible in a reproductively developing or spawning fish. Instead, RMR must be reported and used
to estimate aerobic scope and, for example, the net cost of transport (Lee et al., 2003a, b; Steinhausen et al., 2008; Eliason et al., 2011, 2013).

Thus, body mass, ontogenic state (if not obvious from body mass), feeding regime and, for adults, reproductive status of the fishes and season or date should be provided when reporting SMR or RMR.

Locomotor activity

Dealing with spontaneous activity is almost an intractable challenge when measuring SMR. The problem is two-fold. First, the potential error due to spontaneous activity is very large because locomotion can increase $\dot{M}O_2$ up to three to five-fold in most fish species, and up to nine-fold in tunas (Thunnini) (Brill & Bushnell, 1991). Second, inactivity is not a natural state except for lethargic species and ambush predators, although possibly the latter are spending more energy for muscle tone to be ready to pounce. Some researchers have used sedation (Ege & Krogh, 1914; McFarland, 1960; Piiper & Schuman, 1967; Edwards et al., 1972; Brill, 1987; Houlihan et al., 1995; Hove & Moss, 1997; Leonard et al., 1999; Dowd et al., 2006). Sedation, however, can interfere with functions that are now considered part of SMR (e.g. ventilation) and fishes can accumulate an oxygen debt while under sedation (Keys & Wells, 1930; Spoor, 1946). Another approach is spinal blockade and artificial ventilation to immobilize a fish (Bushnell et al., 1990; Bushnell & Brill, 1992). Sedation and immobilization should be considered a last recourse because of the potential to underestimate SMR.

DETERMINING SMR WHEN ACTIVITY LEVEL IS MEASURED

It is possible to statistically remove the effect of controlled and spontaneous locomotion on $\dot{M}O_2$. In the first case, different steady-state speeds in a swimming respirometer (an aquatic treadmill) create a relationship between $\dot{M}O_2$ and swimming speed that can be extrapolated to zero speed, which is assumed to be SMR (Brett, 1964; Brett & Groves, 1979). Indeed, when SMR has been measured in a conventional static respirometer and by extrapolation to zero speed, the two measurements can agree (Schurmann & Steffensen, 1997; Roche et al., 2013), although not always (Duthie, 1982; Roche et al., 2013). Concerns include the need to prevent spontaneous locomotor activity at slow, sustained speeds when it is easily accommodated within the fish’s aerobic scope (Brett, 1964; Smit, 1965; Fry, 1971; Bushnell et al., 1994; Reidy et al., 2000). This problem can be reduced by training (acclimation to the experimental setup and to different swimming speeds). Further, anaerobic metabolism increasingly fuels locomotion at intermediate and high speeds (Puckett & Dill, 1984; Lee et al., 2003b; Svendsen et al., 2010), and this cost should be added before calculating the relationship between $\dot{M}O_2$ and swimming speed. Also, this extrapolation method implicitly assumes that sustained swimming only translates to an increased $\dot{M}O_2$, but routine gut blood flow progressively decreases below the normal allocation of 30–40% of the cardiac output with increased swimming speed in some fasted fishes (Thorarensen et al., 1993; Farrell et al., 2001). Thus, $\dot{M}O_2$ need not necessarily increase appreciably in the early stages of swimming (Thorarensen & Farrell, 2006) and may underestimate the true cost at the highest swimming speeds (Lee et al., 2003b; Farrell, 2007). Other considerations include fishes that normally orient into water currents (e.g. salmonids), that continuously swim to assist gill ventilation (e.g. ram ventilation in sharks), or transition to ram ventilation. A gentle water current that does not induce swimming has been
used with great success to minimize spontaneous activity in salmonids using swimming respirometers (Farrell et al., 2003). All of these considerations influence the slope and intercept of the relationship between $\dot{MO}_2$ and swimming velocity, as does the choice of the correct mathematical function relating $\dot{MO}_2$ to swimming speed (Korsmeyer et al., 2002; Roche et al., 2013). Lastly, swimming respirometry systems are more complex and costly than static respirometers, and some species refuse to swim in swimming respirometers (Kolok & Farrell, 1994; Roche et al., 2013).

With a static respirometer (no strong, directed water flow) that is large enough to allow spontaneous movement, it is possible to improve SMR measurement by visually selecting periods when the fish is inactive (Armstrong et al., 1992), perhaps aided by video observation (Crocker & Cech, 1997). Others have regressed $\dot{MO}_2$ against an index of activity level, with SMR corresponding to the extrapolation to zero activity (Spoor, 1946; Beamish, 1964a; Torres & Childress, 1983; Lucas & Priede, 1992; Carlson et al., 1999; Zimmermann & Kunzmann, 2001; Tudorache et al., 2009). Such a system is usually less costly than a swimming respirometer. The challenge is the large range of $\dot{MO}_2$ values observed with zero spontaneous activity (Zimmermann & Kunzmann, 2001) as well as high variability around the regression line (Tudorache et al., 2009), probably because of measurement error resulting from the greater water volume relative to fish mass in respirometers where fishes can move spontaneously (Svendsen et al., 2016), the imprecision of quantifying activity and the cost of excitement in absence of activity (Winberg, 1960; Brett, 1962; Smitt, 1965; Puckett & Dill, 1984; Millidine et al., 2006). This variability adds to the inherent variability of SMR.

DETERMINING SMR WHEN THERE IS NO INFORMATION ON ACTIVITY

Most measurements of $\dot{MO}_2$ in fishes have not concurrently measured activity. In this case, species knowledge may help identify when it is normally quiescent (Fry & Hart, 1948; Dall, 1986; Crear & Forteath, 2000; Ferry-Graham & Gibb, 2001; Fu et al., 2005d). Alternatively and perhaps with less subjectivity, the frequency distribution of the $\dot{MO}_2$ values and their distribution in time are very valuable sources of information to identify SMR. There is no universally accepted method, however, among these three previously used approaches (Nelson & Chabot, 2011), i.e. taking the average of a variable number of the lowest $\dot{MO}_2$ values, taking a variable quantile from the values of $\dot{MO}_2$, and assigning $\dot{MO}_2$ values to a mixture of normal distributions.

By using the lowest values of $\dot{MO}_2$ the first approach assumes that they correspond to periods of inactivity or a minimal level of spontaneous activity. The concern here is underestimating SMR with a small number of the lowest $\dot{MO}_2$ values measured over a short interval because of the temporal variability in SMR and measurement error associated with short-term measurements of $\dot{MO}_2$, and the expectation that $\dot{MO}_2$ values observed when fishes are at SMR should be approximately normally distributed. This is one, but not the only, explanation for SMR estimated by extrapolation to zero spontaneous activity being larger than the lowest $\dot{MO}_2$ values (Zimmermann & Kunzmann, 2001). Another explanation is that the fishes in these examples may have been nervous or stressed during some of the $\dot{MO}_2$ measurements made at zero activity, and the two explanations are not mutually exclusive. The risk of underestimating SMR decreases, of course, as the number of $\dot{MO}_2$ values used to estimate SMR increases. This number is highly variable among studies, e.g. one (Wieser & Medgyesy, 1991), three
MEASURING SMR IN FISHES

(Crear & Forteath, 2000; McKenzie, 2001; Roche et al., 2013), five (Lefevre et al., 2012), six (Cruz-Neto & Steffensen, 1997; Schurmann & Steffensen, 1997) or 10 (Norin & Malte, 2011; Boldsen et al., 2013; Svendsen et al., 2014). Recognizing the potential for aberrant low $\dot{M}O_2$ values, others have used 10% of the values (Herrmann & Enders, 2000; Rosewarne et al., 2014), or all $\dot{M}O_2$ values obtained during the quiet part of the daily cycle (Castanheira et al., 2011), or after a specific time since the beginning of the experiment (Cutts et al., 2002). Other variants integrate more data by averaging two quiescent periods (Cheng & Farrell, 2007), or averaging continuous readings into block means and taking the mean value of a given number of the lowest block means (Eliason et al., 2007, 2008). In some cases, outliers have been subjectively (Herrmann & Enders, 2000; Boldsen et al., 2013) or statistically ($>2$ s.d. from the average of the low values, Boldsen et al., 2013) removed. The main problems with selecting the lowest $\dot{M}O_2$ values are determining the number of values to be used and the fact that the lowest values of $\dot{M}O_2$ are assumed to represent SMR, whereas an assumption of this paper is that values of $\dot{M}O_2$ should be approximately normally distributed and the lowest values may be far from the mean.

The second approach to estimate SMR when activity is not measured assumes that when the animal is in the state corresponding to SMR, $\dot{M}O_2$ values vary around the real SMR, as explained above, with half of them below and half above SMR. These $\dot{M}O_2$ values are interspersed with other $\dot{M}O_2$ values that lie variably above SMR due to spontaneous activity. This assumption fits the concept of a quantile, which splits a data set into the $p$ smallest and the $1-p$ largest values, where $p$ is a proportion chosen by the experimenter. Van den Thillart et al. (1994) used $p$ values of 0·05 and 0·1 to estimate SMR in S. solea. There was very little difference between these two values and they adopted 0·05. Dorcas et al. (2004) used a $p$ of 0·25 to estimate SMR of the eastern diamondback rattlesnake Crotalus adamanteus. Others have used intermediate values ($p = 0·1$: Daoud et al., 2007; $p = 0·15$: Dupont-Prinet et al., 2013a, b). This then leads to the quandary of choosing the correct $p$. Van den Thillart et al. (1994) suggested a graphical method, i.e. finding a steep gradient of the cumulative frequency distribution, but such a steep gradient is not always present. Instead, the analysis of data that follows compares different $p$ values for the same fish as well as across fish species and temperatures.

The third approach acknowledges that the frequency distribution of a large set of $\dot{M}O_2$ values measured over a long period is often bimodal (or multimodal). Steffensen et al. (1994) fitted two normal distributions to $\dot{M}O_2$ data for Greenland cod Gadus ogac Richardson 1836. One distribution accounted for the elevated $\dot{M}O_2$ values associated with the acclimation period and spontaneous activity, while the other distribution accounted for the low values of $\dot{M}O_2$ obtained after the fish settled down. The mean of this lowest normal distribution (MLND) was proposed as a robust estimate of SMR. This method has a strong theoretical appeal because of the assumption that SMR measured for short time periods shows temporal variability around an average whenever the fish is calm, immobile and post-absorptive. Variability around SMR is then the sum of measurement error and biological variability, which are unknown but should be small with a well-designed respirometry system. This statistical approach has been adopted in many recent studies of fish SMR (Behrens & Steffensen, 2007; Jordan & Steffensen, 2007; Dupont-Prinet et al., 2010; Skov et al., 2011; Svendsen et al., 2011; Frisk et al., 2012; Roche et al., 2013; Rosewarne et al., 2014).
A possible drawback of the MLND method, especially for excitable species, is that bouts of low levels of excitement or activity could inflate many of the $\dot{M}O_2$ values. There may be too much overlap between the distribution of $M\dot{O}_2$ values corresponding to SMR conditions and those corresponding to low levels of activity or excitement, resulting in a combined distribution that will overestimate SMR. This problem may not be noticeable by observing fish behaviour, because excitement can increase $M\dot{O}_2$ without observable movements (Winberg, 1960; Puckett & Dill, 1984; Millidine et al., 2006). Examples of this problem are shown below.

**OBJECTIVES**

Without clear support for any single method to estimate SMR when animal activity is not measured, these three approaches are compared for the first time using examples from many species of fishes and one crustacean, with the aim of identifying the more robust method or methods. Because animals are stressed and often agitated immediately after being introduced into the respirometer, these observations are removed before calculating SMR (Herrmann & Enders, 2000). A second objective is to assess if this step is necessary or if at least some of the methods yield the same SMR estimates when data from the acclimation period are retained in the calculation of SMR, as in Steffensen et al. (1994). SMR measurements should minimally last 24 h beyond the acclimation to account for possible circadian activity cycles. To verify this, the data were truncated after 12 and 24 h post-acclimation and SMR was estimated for each of these two scenarios. The effect of experimental duration on SMR estimation was then assessed.

**MATERIALS AND METHODS**

**DATABASE AND SMR METHODS**

The database contained $M\dot{O}_2$ values from c. 300 individual animals belonging to four species of fish [R. hippoglossoides, G. morhua, spotted wolffish Anarhichas minor Olafsen 1772 and brook trout Salvelinus fontinalis (Mitchill 1814)] and one crustacean (northern shrimp Pandalus borealis). All data were obtained using the same technique, intermittent-flow respirometry in a static respirometer (Steffensen, 1989; Svendsen et al., 2016) without measuring animal activity. All animals had been without food for usually 4 days but from 2 to 7 days, before being transferred into the respirometer. Raw $M\dot{O}_2$ data were also obtained from the published literature for a G. ogac (Steffensen et al., 1994), a horse mackerel Trachurus trachurus (L. 1758) (Herrmann & Enders, 2000), a few E. stoutii (Cox et al., 2011) and for three perch Perca fluviatilis L. 1758 (E. A. F. Christensen, pers. comm.) to provide greater fish diversity.

$M\dot{O}_2$ was plotted as a function of time for each animal and a sub-set of 85 cases were selected for analysis. The collection of data formed a gradient from a clearly defined, narrow horizontal band of low $M\dot{O}_2$ values to a very diffused horizontal band on the $M\dot{O}_2$ plots. Because SMR is not expected to result in strictly constant $M\dot{O}_2$ values for measurements lasting 5–30 min, these bands of low $M\dot{O}_2$ values were assumed to represent SMR, at least when they were well defined. The sample size for each species is shown in Table I. The average duration of data set for an individual fish was 53.5 h (range: 13.7–116.4 h). For each animal, SMR was estimated with the following eight methods using a single R script: MLND, quantiles with a range of $p$ values (0·1, 0·15, 0·2, 0·25 and 0·3, also referred to as $q_{0.1}$, $q_{0.15}$, etc.), the average of the 10 lowest values of $M\dot{O}_2$ (low10) and the average of the lowest 10% of the $M\dot{O}_2$ values, after removal of the five lowest, considered probable outliers (low10%). All these methods, except $q_{0.3}$, have been used to estimate the SMR of fishes in published studies. The R script is shown in Appendix SI.
Table I. Sample size (n), test temperature (T), experiment duration, acclimation duration and mean fasting period for each species represented in the collection of 85 examples used to compare eight methods of standard metabolic rate (SMR) estimation

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>T (°C)</th>
<th>Experiment duration (h)</th>
<th>Acclimation duration (h)</th>
<th>Mean fasting period (h)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anarhichas minor</td>
<td>17</td>
<td>8</td>
<td>60.7</td>
<td>12</td>
<td>96</td>
<td>a</td>
</tr>
<tr>
<td>Eptatretus stoutii</td>
<td>1</td>
<td>10</td>
<td>45.9</td>
<td>5</td>
<td>n/a</td>
<td>b</td>
</tr>
<tr>
<td>Gadus morhua</td>
<td>26</td>
<td>12</td>
<td>45.6</td>
<td>12</td>
<td>92</td>
<td>a</td>
</tr>
<tr>
<td>Gadus ogac</td>
<td>1</td>
<td>4.5</td>
<td>13.7</td>
<td>5</td>
<td>124</td>
<td>c</td>
</tr>
<tr>
<td>Pandalus borealis</td>
<td>12</td>
<td>5</td>
<td>46.5</td>
<td>12</td>
<td>98</td>
<td>d</td>
</tr>
<tr>
<td>Perca fluviatilis</td>
<td>3</td>
<td>10</td>
<td>44.8</td>
<td>10</td>
<td>504</td>
<td>e</td>
</tr>
<tr>
<td>Reinhardtus hippoglossoides</td>
<td>17</td>
<td>5</td>
<td>72.2</td>
<td>10</td>
<td>142</td>
<td>f</td>
</tr>
<tr>
<td>Salvelinus fontinalis</td>
<td>7</td>
<td>12</td>
<td>44.6</td>
<td>10</td>
<td>56</td>
<td>a</td>
</tr>
<tr>
<td>Trachurus trachurus</td>
<td>1</td>
<td>13</td>
<td>37.5</td>
<td>5</td>
<td>24</td>
<td>g</td>
</tr>
</tbody>
</table>

a. D. Chabot (unpubl. data).

c. Steffensen et al. (1994).
d. Dupont-Prinet et al. (2013a).
e. F. E. A. Christensen (pers. comm.).
f. Dupont-Prinet et al. (2013b).
g. Herrmann & Enders (2000).

(Supporting Information). Because chasing and handling and of the stress caused by the new surroundings, \( \dot{M}O_2 \) values are elevated after fishes are introduced into a respirometer, the data sets were shortened by removing \( \dot{M}O_2 \) values measured during an acclimation period before calculating SMR. These are the main data sets. As a compromise between a single duration of acclimation period for all animals and a different duration for each animal, one duration was chosen for each species so as to remove the descending part of the \( \dot{M}O_2 \) values for most individuals in the sample (Table I).

The MLND was calculated using the function Mclust of the R package mclust (Fraley & Raftery, 2002; Fraley et al., 2012). Mclust fits a mixture of normal distributions to the data. The optimal number of distributions is automatically chosen between one and nine, but the user can choose a narrower range of distributions. For each animal, the number, mean (MLND) and c.v. MLND (C.V. MLND) of the \( \dot{M}O_2 \) values assigned to the lowest normal distribution were calculated. To facilitate the comparison of SMR methods across animals with very different absolute values of SMR, an overall average of all eight estimates was calculated (\( \text{SMR}_{\text{MEAN}} \)) for each animal and used to obtain scaled deviations of each method from this mean value \[ e.g. \text{deviation}_{\text{method}} = \left( \text{SMR}_{\text{method}} - \text{SMR}_{\text{MEAN}} \right) \div \text{SMR}_{\text{MEAN}} \].

The MLND method is of particular interest because it is the only one of the eight methods that does not assume that a fixed number (low10) or proportion (low10% and all quantile-based methods) of the observations are at or below the true SMR. Unlike the other methods, it has the potential to adjust to the data without bias. The appropriateness of the MLND method was assessed semi-qualitatively for each of the 85 animals. Up to a C.V. MLND = 7, the criterion was in fact quantitative. The MLND was scored as unacceptable if 75% or more of the observations assigned to the lowest normal distribution by the Mclust procedure were below the MLND for a period of at least 6 h \[ e.g. \text{case 6; Appendix SII (Supporting information)} \]. Variability in short-term \( \dot{M}O_2 \) when fishes are calm and resting is assumed to be the sum of changes in the functions of different organs and to experimental error. Although different fish species are likely to differ in the proportion of time spent calm and resting in a respirometer, variability about the SMR should be relatively low. This amount of variability is unknown and must be estimated from the data. Some animals had a C.V. MLND as low as about 2% in this study. The
qualitative part of the criterion is that MLND was considered unsuccessful when $C.V_{\text{MLND}}$ exceeded 7%.

To assess if the acclimation period could be left in when calculating SMR and what duration of $MO_2$ measurements should be sufficient to measure SMR, all eight SMR methods were calculated three more times for each animal: when using the complete data sets (including the observations obtained during the acclimation period) and with data sets truncated to just 12 and 24 h post-acclimation (truncated 12 h and truncated 24 h). For each method, SMR for the complete and the two truncated data sets were compared with SMR calculated for the main data sets. Observations during the acclimation period were always excluded from the analysis except for the analysis of the complete data sets.

**Statistical Analyses**

Using the main data sets (acclimation period excluded), the eight estimates of SMR were compared with a univariate ANOVA for repeated measures. Each animal served as its own control, thereby alleviating the problem of pooling different species with different SMR values. SMR estimates were $\log_{10}$ transformed to obtain homoscedasticity amongst the different methods and animals. This pooling of species was done deliberately because any estimation method should be independent of the species being considered. The assumption of sphericity (homogeneity of variances of all differences between methods) was tested (Mauchly’s test), and appropriate correction to the d.f. effected, with the ez R package (Lawrence, 2013). Lack of sphericity invalidates post hoc comparisons based on a common error term (pooled variance) (Quinn & Keough, 2002). To assess which methods were different, $t$-tests for paired samples were performed for each pair of methods, correcting the $p$ level required for significance with the Šidák method to maintain the family-wise error rate to 0.05 (Abdi, 2007). Only significant ($P < 0.05$) differences are reported. To assess the effect of experiment duration, the same procedure was used to compare four different data sets (main, which excluded observations from the acclimation period, complete, which included them, and truncated to 12 and 24 h post-acclimation). In the case of experiment duration, not all possible pair-wise comparisons were of interest, but only the three comparisons involving the main data sets. When presenting results of repeated-measures ANOVA, mean values and their s.d. are shown for each treatment (method or duration). Significant differences were often detected despite large s.d. There are two reasons for this. Firstly, the s.d. values were smaller after logarithmic transformation, and secondly, what matters in this type of analysis are the s.d. of the differences between treatments, and these could not be shown on the tables.

A logistic regression (logit transformation) was fitted to the semi-quantitative assessment of the appropriateness of the MLND method and $C.V_{\text{MLND}}$. The $C.V_{\text{MLND}}$ yielding 50% success was estimated with the dose.p function from R package MASS (Venables & Ripley, 2002). Regression analysis was used to assess the relationship between the degree of similarity amongst methods and the variability in the data. All statistical analyses were performed with R (R Core Team; www.r-project.org).

**Results**

**Usefulness of the New MLND Algorithm**

Unlike the original algorithm for the MLND method, which fitted two normal distribution to the $\dot{MO}_2$ data (Steffensen et al., 1994), the Mclust function of the mclust R package (Fraley et al., 2012) automatically chooses the optimal number of distributions that best fit the data, from a minimum of one to a maximum of nine. With this new method, the optimal number of distributions was greater than two for almost half of the animals (39 of 85; Table II). When more than four distributions were fitted to the data, however, the lowest distribution often included only a few data points and failed...
Table II. Number of distributions found for three different settings of the Mclust function to calculate the mean of the lowest normal distribution (MLND) method: the maximum number of distributions was set to 2, 4 and 9. The total number of animals with a given number of distributions is reported.

<table>
<thead>
<tr>
<th>Number of distributions found</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
</tr>
</tbody>
</table>

Table III. Decrease in the standard metabolic rate (SMR) estimated by the mean of the lowest normal distribution MLND method when a maximum of four normal distributions is allowed, instead of two distributions only.

<table>
<thead>
<tr>
<th>Decrease in MLND (%)</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>0–2</td>
<td>9</td>
</tr>
<tr>
<td>2–5</td>
<td>12</td>
</tr>
<tr>
<td>5–10</td>
<td>11</td>
</tr>
<tr>
<td>&gt;10</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
</tr>
</tbody>
</table>

to represent the SMR. The best results were obtained by limiting the maximum allowable number of distributions to four. When four distributions were allowed, the SMR estimated by the MLND method was lower, compared with the original method, e.g. two distributions, for the 39 animals with more than two distributions (Table III). These lower SMR estimates were due to better discrimination of $\dot{M}O_2$ values corresponding to SMR from higher $\dot{M}O_2$ values that were likely caused by small amounts of activity or stress (Fig. 1).

Calculating SMR When the Acclimation Period is Excluded

Two measures of variability in the data are used: c.v. of the observations, called C.V. $\dot{M}O_2$ (C.V.$\dot{M}O_2$), and the c.v. of the observations assigned to the lowest normal distribution, called C.V.$\dot{M}O_2$MLND. Examples with low, intermediate and high C.V.$\dot{M}O_2$MLND are shown in Fig. 2. Another c.v. was calculated for the mean of the eight SMR estimates (C.V.$\dot{M}O_2$) and served as a measure of agreement between methods. It varied more than 10 fold (Fig. 3). As expected, agreement was better (lower C.V.$\dot{M}O_2$SMR) when there was less variability in the observations (C.V.$\dot{M}O_2$, range was 2–70%) [Fig. 3(a)]; the different SMR methods used different sub-sets of $\dot{M}O_2$ when estimating SMR, and these sub-sets were more similar when the observations were more similar overall (C.V.$\dot{M}O_2$SMR = 2.37 + 0.06 C.V.$\dot{M}O_2$; $F_{1,80} = 28$, $P < 0.001$, $r^2 = 0.26$). All methods but the MLND target the lowest observations by design when estimating SMR. Therefore, the methods should be more similar whenever the lowest observations are more
Fig. 1. (a, c) Oxygen uptake ($\dot{M}O_2$) plot and (b, d) frequency distribution of $\dot{M}O_2$ of a juvenile Salvelinus fontinalis [animal 46, Appendix SII (Supporting Information)], including the normal distributions fitted to the data ( ). The standard metabolic rate (SMR) is estimated by the mean of the lowest normal distribution (MLND). In (a) and (b), two normal distributions were fitted, as in Steffensen et al. (1994). In (c) and (d), up to four distributions were allowed and the algorithm chose three. This fish had many values of $\dot{M}O_2$ that were intermediate between the high values observed soon after it was placed into the respirometer and the many low values when the fish was assumed to be calm and inactive. In (a) and (c), the is the MLND and the symbols represent which normal distribution each $\dot{M}O_2$ was assigned to. Observations from the 10 h habituation period were included for comparison with Appendix SII (Supporting Information), where they were excluded.

similar. The $C.V_{\text{MLND}}$ is a measure of the variability amongst the low values of $\dot{M}O_2$. $C.V_{\text{MLND}}$ varied more than five-fold [Fig. 3(b)] and, as expected, the agreement between SMR estimates was strongly related to $C.V_{\text{MLND}}$ ($C.V_{\text{SMR}} = 1.07 + 0.47 \, C.V_{\text{MLND}}$; $F_{1,80} = 268$, $P < 0.001$, $r^2 = 0.77$). In contrast, the relationship between $C.V_{\text{MLND}}$ and $C.V_{\text{MO2}}$ was significant but weak ($C.V_{\text{MLND}} = 4.06 + 0.09 \, C.V_{\text{MO2}}$; $F_{1,80} = 14$, $P < 0.001$, $r^2 = 0.14$) [Fig. 3(c)]. The ratio of the largest to the smallest SMR estimate, another measure of agreement amongst estimates, was also strongly related to $C.V_{\text{MLND}}$ ($C.V_{\text{SMR}} = 1.021 + 0.018 \, C.V_{\text{MLND}}$; $F_{1,80} = 290$, $P < 0.001$, $r^2 = 0.77$).
Fig. 2. (a, c, e) Oxygen uptake ($\dot{M}_O_2$) plot and (b, d, f) frequency distribution of $M_O_2$ of (a–d) Gadus morhua and (e–f) Anarhichas minor. Horizontal lines on the $M_O_2$ plots and vertical lines on the frequency distributions represent different estimates of standard metabolic rate (SMR): —, the mean of the lowest normal distribution (MLND); ——, quantile with $p = 0.2$ ($q_{0.2}$); ---, the average of the lowest 10% of $M_O_2$ values after the removal of the 5 lowest ones; ,, the average of the 10 lowest $M_O_2$ values (low10). The normal distributions that best fit the frequency distributions of $M_O_2$ values are also shown (|). Variability amongst the low values of $M_O_2$, as measured by the c.v. of the $\dot{M}_O_2$ values assigned to the lowest normal distribution, was low (2.1%) in (b), intermediate (5.2%) in (d) and high (8.7%) in (f). Not shown to prevent the SMR indices from becoming undistinguishable on the figures, but available in Appendix SIII (Supporting Information) (IDs 4, 35 and 65), are $q_{0.1}$, $q_{0.15}$, $q_{0.25}$ and $q_{0.3}$. •, excluded from the calculation of SMR because they occurred too early after each animal was placed into the respirometer to represent SMR. —— indicate night-time.
Fig. 3. Effect of variability in the data on the agreement amongst the eight methods of standard metabolic rate (SMR) estimation. (a) c.v. for the eight SMR estimates of each example (C.V. SMR) as a function of the c.v. of all the oxygen uptake (MO$_2$) values ($y = 2.37 + 0.06x$; $r^2 = 0.26$, $F_{1,80} = 28$, $P < 0.001$), (b) C.V. SMR as a function of the c.v. of the MO$_2$ values assigned to the lowest normal distribution (C.V. MLND) ($y = 1.07 + 0.46x$; $r^2 = 0.77$, $F_{1,80} = 268$, $P < 0.001$), (c) C.V. MLND as a function of the c.v. of all the MO$_2$ values ($y = 4.06 + 0.09x$; $r^2 = 0.15$, $F_{1,80} = 14$, $P < 0.001$) and (d) ratio of largest to smallest SMR estimate for each example as a function of C.V. MLND ($y = 1.02 + 0.02x$; $r^2 = 0.78$, $F_{1,80} = 290$, $P < 0.001$). Three outliers for (b) are identified by their identification number and excluded from all regressions. Data are the 85 examples from Appendices SII and SIII (Supporting Information).

$r^2 = 0.78$) [Fig. 3(d)]. Therefore, the various methods of estimating SMR differed the most when the lowest normal distribution was dispersed. Conversely, SMR estimates were similar when the horizontal band of low MO$_2$ values was not dispersed. Consequently, if spontaneous activity is minimal (which is the primary source of MO$_2$ variability), SMR can be estimated using a variety of methods, such as those used here. In this study, the largest difference between estimates was <10% in 26 animals, presumably the most quiescent ones. The difference in SMR estimates, however, reached 50% for more active individuals and exceeded 20% in 14 animals in this study. The analysis that follows examines which of the methods provided a good SMR estimate even when the MO$_2$ values are variable due to activity.
Fig. 4. Scaled deviations of five of the standard metabolic rate (SMR) estimates calculated for each of 85 fish and shrimp in relation to the variability amongst measurements of oxygen uptake ($MO_2$) assigned to the lowest distribution of $MO_2$ ($C.V_{MLND}$, displayed on a log$_{10}$ axis). The methods are the mean of the lowest normal distribution, MLND (--), quantiles with $p$ set to 0.3 (---), 0.2 (--) and 0.1 (--), the average of the lowest 10% of $MO_2$ values (---) and the 10 lowest $MO_2$ values (--). Vertical lines indicate $C.V_{MLND}$ of 4.7 (●) and 5.7% (●), respectively. The deviations of two indices ($q_{0.15}$ and $q_{0.25}$) are omitted for clarity.

All 85 animals are plotted in increasing order of $C.V_{MLND}$ in Appendix SII (Supporting Information), and the eight SMR estimates for each animal are given in Appendix SIII (Supporting information). The scaled deviations as a function of $C.V_{MLND}$ for the different methods are presented in Fig. 4 and clearly illustrate the widening differences among SMR methods with increasing $C.V_{MLND}$. This figure also reveals relationships amongst different methods. As expected, low10 was the lowest estimate in all 85 animals. Low10% was similar to $q_{0.1}$ and both were below all other methods except low10. The MLND and $q_{0.2}$ estimates were similar when $C.V_{MLND}$ was low (Fig. 4). Starting at a $C.V_{MLND}$ of c. 4.7, the MLND estimates was equal to or greater than the $q_{0.2}$ most of the time, and became greater than $q_{0.3}$ most of the time starting at a $C.V_{MLND}$ of c. 5.7 (Fig. 4). The MLND was almost always judged appropriate when the $C.V_{MLND}$ was low [Fig. 5; see Appendix SIII (Supporting information) for the semi-quantitative assessment of the appropriateness of the MLND for each animal], and a 50% success rate for the MLND occurred when the $C.V_{MLND}$ was 5.4 (95% c.i.: 4.7–6.2). Therefore, the extensive data sets used to generate Figs 4 and 5 suggest that the MLND method becomes unreliable once the $C.V_{MLND}$ exceeds c. 5.4.

The eight methods were first compared on a sub-set of 34 animals with a low $C.V_{MLND}$ (≤5.4). The MLND was judged successful for 28 of them and for the six others, the difference between the MLND method and the centre of the horizontal band of low values never exceeded 2.7% [see IDs 5, 9, 20, 28, 31 and 33; Appendix SII (Supporting Information)]. Therefore, the MLND was generally a good estimate of SMR when $C.V_{MLND}$ was ≤5.4. Only the low10 method had an average difference >5% when compared with the MLND estimate. All the same, all but the $q_{0.2}$, $q_{0.25}$ and $q_{0.3}$ estimates differed significantly from the MLND estimate, and from each other, even if the differences in the SMR estimates were not large (Table IV).
When the MLND method was judged successful and C.V._MLND was between 5.4 and 7.0, three quantiles (q_{0.25}, q_{0.25} and q_{0.3}) were similar to the MLND method, but the other methods differed from each other, except that q_{0.1} was similar to low10% and low10, and q_{0.15} was similar to q_{0.2} and to low10%, despite the small sample sizes (Table V; n = 7). Within the same range of C.V._MLND, but when the MLND was rejected (n = 13), the MLND method was similar only to the two highest of the quantile methods (q_{0.25} and q_{0.3}). Except for the pair q_{0.1} and low10%, all methods differed from each other (Table V).

The MLND was never considered a valid estimate of SMR when C.V._MLND exceeded 7%. In other words, the variability in the lowest normal distribution appeared greater than the variability in the low horizontal band of MO_2 values on MO_2 plots. Further, in some cases (e.g. animals 63, 64, 67, 71 and others), there appeared to be another normal distribution to the left of the lowest distribution identified by Mclust, but the algorithm was unable to discriminate it. For animals with such high variability (n = 31), the MLND yielded such a high estimate of SMR that it was significantly above all other methods (Table VI). This high variability also caused all other methods to be significantly different from each other, except for q_{0.1} and low10% (Table VI).

**ACCLIMATION PERIOD AND EXPERIMENT DURATION**

For each method, the four possible durations (main, complete, truncated 12 h and truncated 24 h) were compared using only the 67 animals with at least 32 h of data post-acclimation. This was to ensure that the main data sets were longer than the truncated 24 h data sets. For all eight methods, duration was a significant effect (Table VII). The MLND method was expected to be impervious to the inclusion or exclusion of the acclimation period. For these 67 animals, the average MLND was indeed similar for the two durations (Table VII). For some individuals, however, the
Table IV. Comparison of eight different methods of standard metabolic rate (SMR) estimation: mean of the lowest normal distribution (MLND), quantiles with \( p \) values ranging from 0.1 to 0.3 \((q_{0.1} \text{ to } q_{0.3})\), mean of 10% of the lowest observations, after removing the lowest 5 (low 10%) and mean of 10 lowest observations (low 10). Only the 34 cases with low variability were included (coefficient of variation of values in the lowest normal distribution, \( CV_{\text{MLND}} \leq 5.4 \)). In addition to mean ± s.d. for each method, the deviations were calculated between methods 2–8 and method 1 (MLND) and the results were expressed as % of MLND. The s.d. as well as minimum and maximum values of these deviations are shown. SMR estimations were \( \log_{10} \) transformed before statistical analysis. Method is a significant effect \((F_{7,231} = 69.1, \ P < 0.001 \text{ with Greenhouse–Geisser } \varepsilon = 0.22)\). Mean values of SMR with different superscript lower-case letters are different \((t\text{-tests for paired samples with Šidák correction, } P < 0.05)\)

<table>
<thead>
<tr>
<th>Method</th>
<th>MLND</th>
<th>( q_{0.1} )</th>
<th>( q_{0.15} )</th>
<th>( q_{0.2} )</th>
<th>( q_{0.25} )</th>
<th>( q_{0.3} )</th>
<th>Low10%</th>
<th>Low10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± s.d. SMR</td>
<td>34.6(\text{abc} ) ± 19.1</td>
<td>33.5(c ) ± 18.5</td>
<td>33.9(d ) ± 18.7</td>
<td>34.2(c ) ± 18.8</td>
<td>34.6(b ) ± 18.9</td>
<td>35.1(a ) ± 19.1</td>
<td>33.2(f ) ± 18.4</td>
<td>32.4(e ) ± 18.0</td>
</tr>
<tr>
<td>Deviation from MLND (%)</td>
<td>−3.3 ± 1.4</td>
<td>−2.0 ± 1.6</td>
<td>−0.8 ± 2.3</td>
<td>0.4 ± 3.3</td>
<td>2.0 ± 5.4</td>
<td>−4.1 ± 1.6</td>
<td>−6.7 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>−6.8</td>
<td>−5.4</td>
<td>−4.3</td>
<td>−3.5</td>
<td>−2.8</td>
<td>−9.7</td>
<td>−17.1</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>−0.4</td>
<td>3.1</td>
<td>8.6</td>
<td>15.5</td>
<td>28.4</td>
<td>−1.9</td>
<td>−2.4</td>
<td></td>
</tr>
</tbody>
</table>
Table V. Comparison of eight different methods of standard metabolic rate (SMR) estimation: mean of the lowest normal distribution (MLND), quantiles with \( p \) values ranging from 0.1 to 0.3 (\( q_{0.1} \) to \( q_{0.3} \)), mean of 10% of the lowest observations, after removing the lowest 5 (low 10%) and mean of 10 lowest observations (low 10). Only cases with intermediate variability were included (coefficient of variation of values in the lowest normal distribution, \( C.V_{MLND} \leq 7.0 \)). (a) Animals with a successful MLND and (b) animals with unsuccessful MLND. In addition to mean \( \pm \) s.d. for each method, the deviations were calculated between methods 2–8 and method 1 (MLND) and the results were expressed as \% of MLND. The s.d. as well as minimum and maximum values of these deviations are shown. SMR estimations were log\(_{10}\) transformed before statistical comparison. Method is a significant effect when the MLND method is successful (\( F_{7,42} = 37.6, P < 0.001, \) with Greenhouse–Geisser \( \epsilon = 0.17 \)) as well as when it is not (\( F_{7,84} = 113, P < 0.001, \) with Greenhouse–Geisser \( \epsilon = 0.28 \)). Mean values of SMR with different superscript lower-case letters are different (\( t\)-tests for paired samples with Šidák correction, \( P < 0.05 \)).

<table>
<thead>
<tr>
<th>Method</th>
<th>MLND</th>
<th>( q_{0.1} )</th>
<th>( q_{0.15} )</th>
<th>( q_{0.2} )</th>
<th>( q_{0.25} )</th>
<th>( q_{0.3} )</th>
<th>Low10%</th>
<th>Low10</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Animals with successful MLND (( n = 7 ))</td>
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<tr>
<td>Mean ± s.d. SMR</td>
<td>( \mu \text{mol min}^{-1} \text{kg}^{-1} )</td>
<td>29.5( ^{\text{abc}} ) ± 17.1</td>
<td>27.9( ^{\text{d}} ) ± 16.4</td>
<td>28.5( ^{\text{cd}} ) ± 16.7</td>
<td>28.9( ^{\text{c}} ) ± 16.8</td>
<td>29.4( ^{\text{b}} ) ± 17.1</td>
<td>30.0( ^{\text{a}} ) ± 17.5</td>
<td>27.7( ^{\text{k}} ) ± 16.5</td>
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<tr>
<td>Deviation from MLND (% (mean ± s.d.)</td>
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<td>Minimum</td>
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<td>Maximum</td>
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<tr>
<td>(b) Animals with unsuccessful MLND (( n = 13 ))</td>
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<td></td>
</tr>
<tr>
<td>Mean ± s.d. SMR</td>
<td>( \mu \text{mol min}^{-1} \text{kg}^{-1} )</td>
<td>32.8( ^{\text{d}} ) ± 19.9</td>
<td>30.6( ^{\text{e}} ) ± 18.6</td>
<td>31.1( ^{\text{d}} ) ± 18.7</td>
<td>31.5( ^{\text{c}} ) ± 19.0</td>
<td>31.9( ^{\text{b}} ) ± 19.1</td>
<td>32.3( ^{\text{a}} ) ± 19.3</td>
<td>30.4( ^{\text{c}} ) ± 18.5</td>
</tr>
<tr>
<td>Deviation from MLND (% (mean ± s.d.)</td>
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<td>Minimum</td>
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<tr>
<td>Maximum</td>
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</tbody>
</table>
Table VI. Comparison of eight different methods of standard metabolic rate (SMR): mean of the lowest normal distribution (MLND), quantiles with p values ranging from 0.1 to 0.3 ($q_{0.1}$ to $q_{0.3}$), mean of 10% of the lowest observations, after removing the lowest 5 (low 10%) and mean of 10 lowest observations (low 10). Only cases with high variability were included (coefficient of variation of values in the lowest normal distribution, C.V.$_{MLND}$, C.V.$_{MLND} > 7$, $N = 31$). In addition to mean ($\mu$mol min$^{-1}$ kg$^{-1}$) and s.d. for each method, the deviations were calculated between methods 2–8 and method 1 (MLND) and the results were expressed as % of MLND. The s.d. as well as minimum and maximum values of these deviations are shown. SMR estimations were log transformed before statistical comparison. Method is a significant effect ($F_{7,210} = 142$, $P < 0.001$, with Greenhouse–Geisser $\varepsilon = 0.24$). Mean values of SMR with different lower-case letters are different ($t$-tests for paired samples with Šidák correction, $p < 0.05$).

<table>
<thead>
<tr>
<th>Method</th>
<th>MLND</th>
<th>$q_{0.1}$</th>
<th>$q_{0.15}$</th>
<th>$q_{0.2}$</th>
<th>$q_{0.25}$</th>
<th>$q_{0.3}$</th>
<th>Low10%</th>
<th>Low10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SMR</td>
<td>31.5$^a$</td>
<td>28.1$^f$</td>
<td>28.7$^e$</td>
<td>29.3$^d$</td>
<td>29.8$^c$</td>
<td>30.3$^b$</td>
<td>27.7$^l$</td>
<td>26.1$^g$</td>
</tr>
<tr>
<td>s.d.</td>
<td>26.2</td>
<td>23.2</td>
<td>23.6</td>
<td>24.2</td>
<td>24.5</td>
<td>24.9</td>
<td>22.4</td>
<td>20.1</td>
</tr>
<tr>
<td>Mean deviation from MLND (%)</td>
<td>-10.9</td>
<td>-8.9</td>
<td>-6.8</td>
<td>-5.0</td>
<td>-3.1</td>
<td>-11.6</td>
<td>-16.2</td>
<td></td>
</tr>
<tr>
<td>s.d.</td>
<td>3.4</td>
<td>3.1</td>
<td>2.7</td>
<td>3.1</td>
<td>3.6</td>
<td>3.5</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>-20.9</td>
<td>-16.4</td>
<td>-14.9</td>
<td>-13.4</td>
<td>-10.3</td>
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<td>-34.5</td>
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</tr>
<tr>
<td>Maximum</td>
<td>-4.9</td>
<td>-3.5</td>
<td>0.1</td>
<td>4.9</td>
<td>10.1</td>
<td>-3.9</td>
<td>-7.3</td>
<td></td>
</tr>
</tbody>
</table>
addition of the data in the acclimation period allowed the algorithm to distinguish two normal distributions instead of a single one amongst the low $\dot{M}O_2$ values. The new MLND and $C.V_{\text{MLND}}$ were lower, and seven animals with a $C.V_{\text{MLND}} > 5.4$ when the acclimation period was excluded had an acceptable $C.V_{\text{MLND}} \leq 5.4$ when these data were included (in particular, animals 40, 69 and 75; Fig. 6). Conversely, the addition of observations obtained during the acclimation period sometimes caused the algorithm to merge two different distributions into a single one, making the new lowest normal distribution more variable and MLND estimates higher (animals 3, 25 and 31; Fig. 6).

There was no clear advantage or disadvantage in excluding data for the acclimation period for the MLND method.

Using observations from the acclimation period entails adding a large number of observations, most of them with high values of $\dot{M}O_2$. The consequence on quantiles is that a greater number of observations had to be below the chosen quantile and therefore the SMR estimates were always significantly greater (Table VII). For instance, $q_{0.15}$ when the acclimation period was included resembled $q_{0.2}$ when the acclimation period was excluded. The low10\% method also uses a proportion of the data to estimate SMR, and like quantiles, the addition of a large number of high $\dot{M}O_2$ values resulted in a significant increase of this SMR estimate (Table VII). Low10 was expected to remain the same when data from the acclimation period were included because no very low $\dot{M}O_2$ value was expected to occur during the acclimation period. This was confirmed (Table VII).
Table VII. Standard metabolic rate (SMR) estimated with eight methods and with four sub-sets of the data sets representing different durations: main (observations from the acclimation period excluded), complete (observations from that period included), truncated after 12 and 24 h since the end of the acclimation period. Only the 67 animals with at least 32 h of recording post-acclimation were used. Means ± s.d. are reported. Duration was a significant effect for all methods ($F_{3,198} \geq 12.1$, Greenhouse–Geisser adjusted $P < 0.001$).

<table>
<thead>
<tr>
<th>Method</th>
<th>Main</th>
<th>Complete</th>
<th>Truncated 12 h</th>
<th>Truncated 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLND</td>
<td>33.1 ± 23.8</td>
<td>33.3 ± 23.9</td>
<td>35.6* ± 26.0</td>
<td>33.5* ± 24.0</td>
</tr>
<tr>
<td>$q_{0.1}$</td>
<td>31.0 ± 21.9</td>
<td>31.2* ± 22.1</td>
<td>32.4* ± 23.3</td>
<td>31.2 ± 22.1</td>
</tr>
<tr>
<td>$q_{0.15}$</td>
<td>31.5 ± 22.2</td>
<td>31.8* ± 22.4</td>
<td>33.2* ± 23.8</td>
<td>31.7 ± 22.4</td>
</tr>
<tr>
<td>$q_{0.2}$</td>
<td>32.0 ± 22.6</td>
<td>32.4* ± 22.8</td>
<td>33.8* ± 24.1</td>
<td>32.2 ± 22.7</td>
</tr>
<tr>
<td>$q_{0.25}$</td>
<td>32.4 ± 22.8</td>
<td>33.0* ± 23.2</td>
<td>34.4* ± 24.3</td>
<td>32.7 ± 23.0</td>
</tr>
<tr>
<td>$q_{0.3}$</td>
<td>32.9 ± 23.1</td>
<td>33.6* ± 23.5</td>
<td>35.1* ± 24.7</td>
<td>33.2 ± 23.5</td>
</tr>
<tr>
<td>Low10%</td>
<td>30.6 ± 21.5</td>
<td>30.7* ± 21.6</td>
<td>33.0* ± 23.3</td>
<td>31.1* ± 21.8</td>
</tr>
<tr>
<td>Low10</td>
<td>29.3 ± 20.2</td>
<td>29.3* ± 20.2</td>
<td>32.2* ± 22.6</td>
<td>30.1* ± 20.7</td>
</tr>
</tbody>
</table>

MLND, mean of the lowest normal distribution.

*Different from the estimate obtained with the main data sets ($t$-tests for paired samples with Šidák correction, $P < 0.05$).

For all methods, estimates of SMR obtained with the shortest data sets, truncated 12 h, were significantly higher than those obtained with the main data sets (Table VII). With the truncated 24 h data sets, however, SMR estimates for the quantile methods were indistinguishable from those obtained with the main data sets (Table VII). The MLND, low10% and low10 were still higher than when they were calculated with the main data sets.

**DISCUSSION**

**CHOICE OF METHOD TO ESTIMATE SMR**

The main objective of the experimental part of this paper was to compare methods to estimate SMR of fishes when $MO_2$ is measured frequently over short intervals in a static respirometer, and fish activity is not monitored, as there is no prescribed method to do so. The selection of $MO_2$ values associated with a calm, motionless animal and required to assess SMR must use characteristics of the data. Here, the frequency distribution of the $MO_2$ values, their distribution in time and an estimate of variance were used. The time dimension allows for a rough assessment of important considerations such as recovery from stress and diurnal rhythms, whereas the frequency dimension allows the application of statistical and cut-off techniques. The estimate of variance provided an additional statistical measure to compare the eight methods. Three broad methods have been used in the literature to calculate SMR, but the relative performance of these methods has not been thoroughly assessed until now. These methods are: (1) calculating the mean value of the lowest values of $MO_2$ observed for a fish, with many variations in the number of values that should be used, (2) taking a quantile from the values of $MO_2$, with variants as to which $p$ value to use and (3) assigning $MO_2$ values to a mixture of normal distributions and using the MLND or mean of the lowest normal distribution.
This study compared eight variants of these three methods on the same diverse data sets (85 fishes and crustaceans) that probably encompassed most situations encountered when attempting to measure SMR of fishes with intermittent-flow respirometry. The methods tested were two variants of the first approach (low10 and low10%), five of the second approach (quantiles with p values ranging from 0.1 to 0.3) and an improved version of the MLND approach, which fits optimally from one to four normal distributions, depending on the data, instead of always two normal distributions (Steffensen et al., 1994). The true SMR was unknown for any of these fishes or crustaceans. A key assumption of this study was that \( \dot{M}O_2 \) measured over short intervals (min) is not expected to be constant even in calm, inactive fish because whole-fish \( MO_2 \) is the sum of the oxygen usage of different organs, which can vary very quickly (Priede, 1985; Darveau et al., 2002). Also, the possibility of short bouts of anaerobic metabolism during measurement could underestimate SMR. Steffensen et al. (1994) have shown that the \( MO_2 \) measurements of a calm, inactive fish form an approximately normal distribution and the histograms generated in this study (Appendix SII, Supporting Information) confirm this. On a plot of \( MO_2 \) v. time, the observations corresponding to this normal distribution form a horizontal band, more or less pronounced depending on the proportion of the time that the fish was at SMR and how variable the measurements were when the fish was at SMR. Thus, an estimate of SMR was deemed acceptable when it was close to the centre of this horizontal band.

The MLND method is an objective statistical approach that works regardless of the proportion of the time the animal was at SMR. Thus, the MLND can reliably calculate SMR when the fully acclimated, quiescent animal is at SMR practically all the time, as in the G. ogac used in Steffensen et al. (1994) and also in this study (animal 1), but it should also work when the animal is at SMR much less often, and many examples of such situations were evident in the 85 data sets shown in Appendix SII (Supporting Information) (e.g. animals 7, 8, 15–18 and more). The change to the MLND method introduced in this study (e.g. allowing up to four normal distributions instead of two) represented an improvement because the lowest normal distribution was narrower, with a lower mean value, for nearly half of the 85 data sets. Even so, SMR estimated by the MLND method was sometimes amongst the highest or even the highest of all methods tested for an animal. When this happened, the normal distribution assigned to SMR covered a wide range of \( MO_2 \) values and had high variance, which does not agree with the low variation that is expected from temporal variability in organ and tissue oxygen demand when a fish is calm and inactive. As sophisticated as the algorithm used by the Mclust function is, it is not always successful at isolating \( MO_2 \) values associated with SMR when slightly higher \( MO_2 \) values are also abundant, i.e., those most likely associated with mild activity or perhaps stress. The fact that the MLND method does not always reflect SMR is a problem. The results presented in this study suggest that the MLND does represent SMR well when the variability of the data in the lowest normal distribution (C.V.\(_{MLND}\)) is ≤5-4. Therefore, C.V.\(_{MLND}\) should be used to screen a data set to determine if the MLND can be reliably used.

The seven other methods use a cut-off approach, which requires the experimenter to decide on a number or proportion of low \( MO_2 \) values that are acceptable. These values are then either averaged (low10% and low10) or considered to be below SMR (quantiles). The low10, however, cannot be reconciled with the assumption that \( MO_2 \) values in calm and inactive animal are normally distributed with a mean value that corresponds to SMR. On the contrary, it is based on the concept that the lowest values
observed during a trial represent SMR. This method was expected to find the lowest estimates of SMR, and it did. The results shown here do not support the use of the low10 to estimate SMR for controlled laboratory studies with fishes, even though this method or similar variants have been used in a considerable number of previous studies. Earlier, Roche et al. (2013) compared the mean of the lowest three $\dot{M}O_2$ values and the MLND for 10 individuals of the coral-reef fish Scolopsis bilineata (Bloch 1793) and found no difference between these two methods. The small sample size in Roche et al. (2013) resulted in lower statistical power than this study. Also, it is possible that $\dot{M}O_2$ variability was very low in their study because, as shown in this study, all methods provide similar SMR estimates when the variability is low (Fig. 4).

The number of $\dot{M}O_2$ values included in the low10% method depends on sample size. When $n > 105$, the low10% uses more data than the low10 method and produces larger estimates. Even with smaller samples, the low10% yields larger estimates than the low10 because the five lowest $\dot{M}O_2$ values are always rejected as outliers. The removal of 5 outliers is also why the low10% is often very similar to the $q_{0.1}$, even though one is taking the average of the lowest 10% of the data, and the other puts 10% of the data below SMR. Both methods were almost always below the horizontal band of low $\dot{M}O_2$ values that was assigned to SMR. This agrees with a limited comparison of low10% with MLND in a few (six) individuals of two freshwater crustaceans: low10% always produced lower estimates than the MLND (Rosewarne et al., 2014). The other quantiles put progressively more data below SMR, from 15 ($q_{0.15}$) to 30% ($q_{0.3}$). One advantage of the quantiles over both the low10% and low10 is robustness against variable numbers of outliers and no requirement for normality. Low10% and low10, on the other hand, calculate the arithmetic mean of what is often the left tail of a normal distribution, even though the mean is a measure of central tendency for normal distributions.

The MLND method provided a good estimate of SMR when the C.V. ($\dot{M}O_2$) was low (<5-4). For the 34 data sets with the lowest C.V. ($\dot{M}O_2$), the lowest normal distribution included on average 53-4% of the $\dot{M}O_2$ values obtained after the acclimation period (s.d. = 21-2; range = 17–100%). On average, 26% of the observations were below SMR, but the range was large (8.5–50%). It would appear that finding a single number to use with the cut-off methods is illusory. The quantile approach, however, is robust and the estimates provided by the $q_{0.2}$ and $q_{0.25}$ methods did not differ significantly from the MLND for the 34 data sets with a low C.V. ($\dot{M}O_2$). Estimates from these methods were again similar to those from the MLND method at intermediate variability, except when the MLND was judged to be a poor estimate of SMR. In this situation, as well as in the vast majority of cases when the C.V. ($\dot{M}O_2$) was very high (>7), the $q_{0.2}$ and $q_{0.25}$ continued to be close to the centre of the horizontal band of low values, when such a band could be detected.

It is recommended that in cases when the MLND cannot be considered reliable (C.V. ($\dot{M}O_2$) > 5-4), either the $q_{0.2}$ or $q_{0.25}$ be used instead. There is no statistical reason to strongly recommend one over the other. Instead, $\dot{M}O_2$ plots should be examined and these quantiles should be compared to the position of the horizontal band of low values before deciding which quantile should be considered the best substitute for the MLND method. In practice, D. Chabot (unpubl. data) has found the $q_{0.2}$ to work well with the vast majority of fishes and crustaceans. When $\dot{M}O_2$ variability is very large, and the animal may not have been at SMR often enough to assess SMR reliably, it may be safest to reject such cases. Some species will remain difficult to study in a
respirometer (e.g. *P. borealis* and *E. stoutii* in this study) and the $q_{0.2}$ or $q_{0.25}$ are likely the best estimates of SMR possible.

With this recommendation, the SMR is well estimated for the vast majority of the 85 animals included in this study. In a few cases, this solution is suboptimal. Thus, the MLND was recommended for animals 9, 20 and 28 because of low C.V$_{MLND}$ (Appendix SII, Supporting Information), but the MLND was ruled unsuccessful with the semi-quantitative assessment and the $q_{0.2}$ was closer to the centre of the horizontal band of low $MO_2$ values. On the contrary, the $q_{0.2}$ was less well positioned than the MLND for animals 39 and 57, but the latter was rejected because of the relatively high C.V$_{MLND}$ (5.7 and 7, respectively). The difference between the two methods was small and following the recommendation is unlikely to entail problems when studying a sufficiently large number of fishes. If a single strategy is desired instead of alternating between the MLND and a quantile method within the same study, one of the $q_{0.2}$ or $q_{0.25}$ methods, chosen using the same criterion as above, could be used for all animals, even those with a low C.V$_{MLND}$, because of the similarity between these quantiles and the MLND in conditions of low $MO_2$ variability.

A large number of variable data sets (85 animals) were used here, but the recommendations to estimate SMR may not apply to every fish study. If the pivotal C.V$_{MLND}$ of 5.4 calculated in this study does not appear to be valid for a given species and experimental setup, a quantitative or semi-quantitative method could be used to assess the usefulness of the MLND for each fish, or to calculate a new cut-off value of C.V$_{MLND}$ that is more appropriate. Examination of $MO_2$ plots may suggest that adjustments are required for the recommended quantiles as well. Most of the 85 animals used in this study were demersal species that were expected to become inactive in the respirometer often enough for a band of low values to be apparent. Also, the pelagic *S. fontinalis* were virtually motionless in the respirometers most of the time. But, other pelagic species may be active more often in a static respirometer (Cech, 1990; Herrmann & Enders, 2000), thus altering the ratio of measurements made when the fish is inactive or active. Animal 79, a *T. trachurus* from Herrmann & Enders (2000; Fig. 3) was considered by these authors to be active often. The MLND method placed most observations into a single normal distribution with a mean that likely represents RMR instead of SMR and this method is unlikely to estimate SMR well with active pelagic species. Even the $q_{0.2}$ was greater than the SMR calculated by Herrmann & Enders (2000) (114 v. 104 μmol min$^{-1}$ kg$^{-1}$), suggesting that quantiles with lower $p$ values may be more appropriate with pelagic species. More work is required to recommend $p$ values for the use of quantiles to estimate SMR with active pelagic species.

An important final consideration is to avoid *ad hoc* decisions, such as choosing which method to use (MLND or quantile or different quantiles) case by case, without a rigorous criterion. As long as a method works well for the majority of animals in a study, cases when the estimated SMR appears to be unacceptable should be rejected instead of bending the rules for them. Comparisons with other studies will only be possible if a small number of methods are used, and those recommended here should be valid for most studies of benthic and demersal fishes.

**ACCLIMATION PERIOD**

Although fishes are clearly not at SMR soon after being placed into a respirometer, it would be simpler if SMR could be calculated using the entire data sets, without the
need to determine the duration of an acclimation period and the removal of these data. All methods except MLND and low10, however, produced significantly different estimates of SMR when observations from the acclimation period were included. Further, even though the average SMR estimated by the MLND method was not influenced by the acclimation period, the results differed strongly for some animals. Therefore, only the low10 was truly unaffected by the inclusion of the acclimation period, which was expected because it is unlikely that one or more of the lowest \( \dot{M}O_2 \) values should occur during the acclimation period, unless the duration of this period is made too long. This being said, all methods used in this study can be adapted to work with complete data sets. The C.V.\(_{MLND} \) can still be used to decide if the MLND is reliable. The quantiles are influenced in a predictable manner by the inclusion of the elevated \( \dot{M}O_2 \) values observed during the acclimation period. Therefore, the \( q_{0.2} \) and \( q_{0.25} \), which performed best when the acclimation period was excluded, could be replaced by the \( q_{0.15} \) and \( q_{0.2} \), respectively, when the acclimation period is retained. Similarly, a method equivalent to low10\% \( \dot{M}O_2 \) values, could be used with complete data sets and the results would be comparable to those provided by the low10\% after removal of the data recorded during the acclimation period. Nevertheless, there is an advantage to the removal of data from a period when it is known that the fish cannot be at SMR: it sets a precedent for the exclusion of other periods, if any, when the animal is unlikely to be at SMR, without needing any adjustments to the methods. For example, periods when the experimental protocol provokes disturbances or lasting changes in \( \dot{M}O_2 \) (e.g. feeding the fishes to measure specific dynamic action, SDA), or periods when environmental variables (temperature, DO and pH) are modified, can be excluded for the calculation of SMR using the principle that the animal is unlikely to be at SMR, just as during the acclimation period.

**MINIMUM EXPERIMENT DURATION**

For 67 animals used to compare three durations (main, truncated 12 h and truncated 24 h data sets), the truncated 12 h data sets yielded significantly higher estimates of SMR with all eight methods compared in this study. This suggests that experiments lasting only 12 h after fishes have acclimated to the respirometer are too short to evaluate SMR reliably, and the error (overestimation) is likely to be particularly severe for species with a strong circadian activity pattern. Truncation after 12 h may have eliminated the period of the day when some of these 67 animals were most quiescent, and in some cases, quiescent \( \dot{M}O_2 \) values were still declining after 12 h, perhaps due to on-going SDA in cold water (e.g. animals 2, 8, 9, 13). This may have been avoided by setting the duration of the acclimation period for each animal, instead of for each species. The high SMR estimates obtained with the truncated 12 h data sets constitute a clear warning that 12 h is usually too short to assess SMR, with the onus on the experimenter to demonstrate the feasibility of such short experiments to measure SMR or report that as RMR.

A period of 24 h ensures that the quiet part of the day is included in the experiment, as long as the acclimation period was well set and the fish was acclimated for this entire 24 h period. This duration was expected to be sufficient for accurate determination of SMR. It was the case for all quantile methods. The MLND, however, was on average greater when the records were truncated after 24 h than when the entire record was used. It was shown above that the inclusion or exclusion of the acclimation period sometimes
changed the MLND considerably. The presence of a few additional $\dot{M}O_2$ values when the animal was at SMR can help this method to identify a distribution that corresponds to SMR, and conversely, the addition of a few $\dot{M}O_2$ values associated with low levels of stress or agitation can result in two distributions being merged into a single one that overestimates SMR. The addition or removal of data measured after 24 h can have the same effect. Because the MLND estimate of SMR is used only when the C.V.$_{MLND}$ is low, and when it is not, a quantile is used instead, and because quantile methods performed similarly with the main and truncated 24 h data sets, it is safe to recommend experiments lasting 24 h to estimate SMR. When possible, longer experiments increase robustness of the SMR determination and confirmation that $\dot{M}O_2$ values are similar on multiple days at the time of low activity. It should be noted that the commonly used low10 and low10% are particularly sensitive to experiment duration; both methods produced significantly greater estimates of SMR when data sets were truncated at 12 and 24 h.

Given the effect of experimental period on the SMR estimates, variants of the MLND and quantile ($q_{0.25}$) methods that made SMR calculation for every possible 24 h window of the experiment in 1 h increments, starting at the beginning, were tested but are not reported on. The lowest value for each method was retained. These sliding MLND and $q_{0.25}$ SMR estimates made it unnecessary to remove the data from the acclimation period and would, in theory, automatically remove periods when the animal is not at SMR, such as after a fish has been fed. The added complexity of these methods, however, did not result in a noticeable improvement, except for rare cases when the behaviour of an animal changed and the lowest $\dot{M}O_2$ values observed one day were consistently different from those observed the next or previous day [e.g. animals 27 and 33; Appendix SII (Supporting Information)] or when an animal was at SMR reliably at the same time each day, but for a very short period each time [animal 64; Appendix SII (Supporting Information)]. Given the improvements were marginal, it is simpler to reject extremely variable data sets, or to accept the error that comes with the recommended methods.

In summary, different variants of three different methods of estimating SMR were evaluated and compared statistically for the first time using 85 data sets that included a wide variety of fishes and one crustacean with very different metabolic states during intermittent respirometry experiments. While the best approach should be as objective as possible, the analysis revealed the importance of initial visual inspection of raw $\dot{M}O_2$ data for both trends and variance. Indeed, some data might be rejected on this somewhat subjective analysis alone. Beyond the initial screening of $\dot{M}O_2$ data, it was clear that, if the fish was largely quiescent after the initial acclimation period (typically up to 10 h), the differences between the various SMR estimations were quantitatively very small, which made the selection of the estimation method less critical, although methods that offer no advantage or are biased, such as low10, which always underestimates SMR, should be avoided. When fishes were more active in the respirometer, however, the differences among the methods became appreciable and some SMR estimation methods became unreliable. In these cases, a choice must be made among the SMR estimation methods. To make the decision about fish activity less subjective, a novel recommendation is to fit up to four normal distributions to the $\dot{M}O_2$ values and estimate the variance of the lowest distribution as a guide to the method. Lastly, a series of general recommendations are provided below as a guide to estimate SMR in fishes. In addition, by providing an R script that automatically calculates SMR with all the methods tested.
here, as well as the variability in the lowest normal distribution, individual researchers can make their own judgments and justifications for the SMR estimation method.

CONCLUSIONS

SMR is defined here as the rate of energy expenditure of a fish that is in post-absorptive, calm, inactive state after proper thermal acclimation. These conditions can be difficult to satisfy. The following recommendations that stem from the present review and analyses are offered to increase the reliability of SMR estimates in fishes. (1) Use an appropriate respirometry system (intermittent-flow, proper respirometer size, sensitive and reliable oxygen sensors). Swimming respirometers and static respirometers can both be used. In both cases, precautions need to be taken to avoid disturbing the fishes. (2) Fishes need to be fasted long enough to ensure that digestion and absorption of nutrients does not increase \( \dot{M}O_2 \). Postprandial metabolism in fishes can be elevated for close to a week under some conditions, especially at cold temperature and after a large meal or multiple meals. Justification for the duration of the fasting period should be provided. Feeding conditions for the weeks leading to experiments should be reported, at least in juvenile fishes, because they can influence SMR. (3) Fishes should be acclimated to the experimental temperature for a proper measure of SMR. It is necessary to report the duration of the thermal acclimation, and how quickly temperature was changed to reach the acclimation temperature. (4) SMR is influenced by body mass and ontogenic state, both should be reported. (5) The duration of the measurement period that follows acclimation to a static respirometer should be justified. Ideally, experiments should last a minimum of 24 h after the initial acclimation period to ensure that any period of low activity is incorporated. Longer experiments add robustness to all SMR estimation with the proviso that fasting for too long can lower \( \dot{M}O_2 \) below SMR. (6) Original \( M_O_2 \) data should be plotted as a function of time and by inspection the duration of the initial decline in \( M_O_2 \), the duration of the acclimation or recovery period can be determined. Report and justify the duration of the acclimation period, and then remove these data before SMR is determined. SMR can be compared with this plot to ensure that the calculated SMR makes sense and periods of activity are identified. (7) Adult fishes can exhibit elevated MR during gonad maturation or spawning, making it impossible to measure SMR. In this situation, RMR should be reported, along with season and reproductive status. (8) Locomotor activity is energetically costly. The cost of locomotion can be estimated by forcing fishes to swim at different speeds in a swimming respirometer. Then, SMR can be estimated by extrapolating the relationship between \( M_O_2 \) and speed back to zero speed. Possible sources of error with this approach are explained in this review. (9) Locomotor activity can be considered in a large static respirometer by measuring \( M_O_2 \) and fish activity simultaneously and statistically removing the effect of activity on \( M_O_2 \). (10) In the absence of information on fish activity, a mixture of up to four normal distributions should be statistically fitted to the \( M_O_2 \) values, and the mean (MLND) and c.v. (C.V. MLND) of the lowest normal distribution should be calculated. When the C.V. MLND is low (\( \leq 5.4\% \)) the MLND method is a reliable estimator of SMR but when this is not the case, see next recommendation. (11) In the absence of information on locomotor activity and when the MLND is unreliable, calculate the quantile of the \( M_O_2 \) values, with \( p = 0.2 \) or 0.25, to estimate SMR. Use \( M_O_2 \) plots to assess which
one is best for a given species and experimental setup. (12) If using the same method for all fishes of a study is preferable and the data for some fishes have high variability, use a quantile ($q_{0.2}$ or $q_{0.25}$, selected as in the previous recommendation) for all fishes.

Some of these recommendations, especially those dealing with the duration of the fasting period and of experiments, do not apply to fish larvae due to their high metabolic requirements (Peck & Moyano, 2016). New technologies are making it possible to measure $\dot{M}O_2$ in the field. The stringent requirements for SMR recommended here are likely to be impractical in the field, and results should be reported as RMR.

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Supporting Information

Supporting Information may be found in the online version of this paper: APPENDIX SI. R script to estimate the standard metabolic rate with multiple methods. APPENDIX SII. Oxygen uptake plots and frequency distribution of oxygen uptake values for 85 fishes and crustaceans. APPENDIX SIII. Estimates of the standard metabolic rate with multiple methods for 85 fishes and crustaceans.

References


Healy, T. M. & Schulte, P. M. (2012). Thermal acclimation is not necessary to maintain a wide thermal breadth of aerobic scope in the common killifish (Fundulus heteroclitus). Physiological and Biochemical Zoology 85, 107–119. doi: 10.1086/664584


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