REVIEW Field relevant metabolic measurements in fish: CBPA special issue contribution

Estimates of metabolic rate and major constituents of metabolic demand in fishes under field conditions: methods, proxies, and new perspectives

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Abstract

Metabolic costs are central to individual energy budgets, making estimates of metabolic rate vital to understanding how an organism interacts with its environment as well as the role of species in their ecosystem. Despite the ecological and commercial importance of fishes, there are currently no widely adopted means of measuring field metabolic rate in fishes. The lack of recognized methods is in part due to the logistical difficulties of measuring metabolic rates in free swimming fishes. However, further development and refinement of techniques applicable for field-based studies on free swimming animals would greatly enhance the capacity to study fish under environmentally relevant conditions. In an effort to foster discussion in this area, from field ecologists to biochemists alike, we review aspects of energy metabolism and give details on approaches that have been used to estimate energetic parameters in fishes. In some cases, the techniques have been applied to field conditions; while in others, the methods have been primarily used on laboratory held fishes but should be applicable, with validation, to fishes in their natural environment. Limitations, experimental considerations and caveats of these measurements and the study of metabolism in wild fishes in general are also discussed. Potential novel approaches to FMR estimates are also presented for consideration. The innovation of methods for measuring field metabolic rate in free-ranging wild fish would revolutionize the study of physiological ecology.

Keywords: energetics, respirometry, ecophysiology, environmental change, telemetry

1. Introduction

An organism's energy metabolism can be subdivided into supply ($Energy_{in}$), transformation or use ($Energy_{out}$) and accretion of tissue mass for growth or storage ($Energy_{retained}$) and reproductive effort which may be in the form of gonadal investment ($Energy_{retained}$) or may be $Energy_{out}$ with the release of gametes (Fig. 1). However, the interaction between the environment and an individual's energetic costs are complex and vary according to species, developmental stage, season and even subpopulation/geographic region. This complexity may confound direct extension of laboratory-derived estimates of energetic parameters to field-relevant questions. As such, robust means of estimating metabolic rate that can be extended for field use are critical to understanding the energy balance in individuals. Knowledge at the individual or population level can then be applied to study how variation in energetics may influence the species' role in the ecosystem. The interdisciplinary extension of laboratory-level techniques to field level questions represents an opportunity for significant advancement, as long as the assumptions and limitations of these approaches are recognized.

In many, if not most, aquatic ecosystems fish are critically important consumers. Fishes are often high level predators and, within the same ecosystem, smaller forage species may be key energy conduits between trophic levels. Moreover, fishes are well recognized for their susceptibility to environmental disturbances, including anthropogenic alterations, and are of worldwide economic and cultural importance. However, despite such ecological and sociological significance of fishes, there is a dearth of direct information for metabolic rate (MR) in free swimming fishes under field conditions. The limited information on MR for fish under truly natural conditions leaves an important information gap in the ability to relate fish energy demands with, for instance, environmental change or anthropogenic challenges. The aim of this article is to synthesize many of the strategies that can be applied to estimate MR (e.g. energy expenditure) or alternatively, that can provide proxy measures of major components of energy balance in fishes. Our goal is to cover several levels of investigation from the currently available approaches that predominate in this area of research, telemetry and respirometry, to longer term or integrative methods as well as more indirect proxies at the organ and tissue level. Each of these levels of investigation could warrant a review unto themselves but our task is to consolidate options in one place to encourage further discussion, development and inquiry.

It is also worth adding that while we refine our focus to specifically consider fishes, the majority of the following may be applicable to other organisms, including aquatic and non-aquatic species. We also emphasize that while it is simpler to complete metabolic studies under controlled laboratory conditions, and much excellent work has done so, it is difficult, if not impossible, to fully replicate truly environmental conditions and stochasticity in a controlled setting. As such, we focus here on approaches with potential for extension to field conditions or wild sampled fishes. We will first address some key definitions and broad scale aspects important to all metabolic work on fishes, including some specific areas of relevance. This is followed by brief review of several approaches to measuring MR, or major components that contribute to metabolic demands.

2. Definitions, relevance and caveats

There are several terms that must be defined and aspects that ubiquitously influence metabolism in fishes and therefore should be considered regardless of the experimental approach.

2.1 Definitions

2.1.1 What is metabolic rate?

Metabolic rate (MR), the energy expenditure by an organism under a given condition, is defined as a measurement of energy usage (in *J*, although often *kJ* or *kcal* are used) over time and can be quantified by direct or indirect calorimetry. Direct calorimetry measures MR by the heat released during metabolic energy transformation. Anything not using direct calorimetry to measure energy use is a proxy of MR and thus requires some form of conversion to be a measurement of MR. These proxies would include measurements of oxygen consumption or carbon dioxide production, termed $\dot{M}O_2$ or $\dot{M}CO_2$ by us below, even though gas exchange rates are frequently, and incorrectly, referred to as MR.

To convert a gas exchange rate to a MR is not trivial because it requires some knowledge of the metabolic fuel being oxidized, be it lipid, carbohydrate or protein as the carbon source. The fuels being oxidized can be determined empirically using a respiratory exchange ratio, which is the ratio of moles of CO_2 produced per mole of oxygen (O_2) consumed, or a respiratory quotient (RQ) if the animal is in a steady state; RQ values of 1.0, 0.7 and 0.8 are typically used for complete oxidation of carbohydrate, lipid and protein, respectively (Frayn 1983). However, unless an organism is effectively oxidizing either solely lipid or solely carbohydrate it becomes difficult to estimate MR with the RQ alone because the contribution of protein oxidation will be unclear.

Although the contribution from protein is sometimes ignored, since nitrogen is liberated in order to oxidize protein for ATP synthesis the RQ can be combined with a nitrogen quotient (NQ), moles of nitrogen produced per mole of O_2 consumed, to account for protein oxidation. Caution is required when calculating the NQ for fish because simply measuring the N-excretion products ammonia and urea to estimate total N-excretion, and thus net protein oxidation, may introduce errors, the degree to which may depend on the physiological state of the fish (Lauff and Wood, 1996; Kieffer et al., 1998; Kajimura et al., 2004). Alternatively, but far from ideal, assumptions on the fuels may be made.

With the proportional contribution of the major oxidative fuels the metabolic rate can be calculated with the energy contained per mole, or mass, of fuel used (typical values for glucose, palmitate and amino acid oxidation are 2818 kJ $mole^{-1}$, 10039 kJ $mole^{-1}$ and 1989 kJ $mole^{-1}$, respectively (Ferrannini, 1988)). Of note, these values of energy use are somewhat misleading because the efficiency of energy conversion in metabolic systems is not perfect, with substantial amounts of the energy 'available' being lost as heat rather than being coupled to metabolic or physical work.

2.1.2 Defining metabolic states

The main focus of this article is on field metabolic rate (FMR), which is considered to be the energy expenditure of free-ranging animals in their natural environment. In this regard it differs substantially from most other types of metabolic rate, which are generally measured on restrained animals or under a given set of conditions. Standard metabolic rate (SMR), for example, is the minimal metabolic costs of maintaining organismal homeostasis and integrity and corresponds with the term ***Basal costs*** *(Energy out)* in Fig. 1. SMR is measured in the post-absorptive state and at rest and is somewhat analogous to the basal metabolic rate (BMR) in endotherms, but since temperature influences MR, a SMR value also requires knowledge of the temperature at which it was measured, rather than simply being in the thermal neutral zone for BMR. Routine metabolic rate (RMR) is another estimate of metabolism commonly measured in fishes, referring to baseline costs plus the costs of voluntary, routine activity. Ideally, the amount of activity being performed by individuals should be quantified when performing measures of RMR. Maximum metabolic rate (MMR) is the upper limit of metabolic capacity. Generally the MMR is constrained to maximum aerobic MR even though organisms can have higher absolute metabolic energy use under short-term anaerobic burst locomotion. However, this high relative intensity anaerobic state in most animals, including fishes, is generally ephemeral with duration varying under the influence of

many factors including, but not limited to, species, life-stage and condition. An additional term, active metabolic rate (AMR), can be found in the literature; however, its intended meaning can vary. Sometimes AMR is used to replace MMR when MMR is measured during maximum sustained exercise (Jobling 1995) or after exercise-induced exhaustion (Norin and Malte 2011) as opposed to during feeding, for example). Other times it is used to mean any level of metabolism during activity (Ohlberger et al. 2005). Given this inconsistent definition of AMR we urge caution to the reader when this term is encountered in the literature.

2.2 The need and relevance of metabolic rate estimates applicable to field conditions

Fish have served as important models in our understanding of the proximate and ultimate drivers of variation in MR and its ecological importance (Conrad et al., 2011; Metcalfe et al., 2016a). This is despite the fact that almost all of this work has depended on MR data collected on animals in a laboratory setting or confined within an experimental apparatus such as a respirometer. As elaborated below, the innovation of methods for measuring FMR in free-ranging wild fish would revolutionize the study of physiological ecology as well as our understanding of the impacts of anthropogenic environmental disturbance.

2.2.1 Behavioural and ecological studies

Some of the greatest insights on the importance of intraspecific diversity have come from studies using fish and this area could be opened even further with the advent of methods for measuring FMR. During the last decade, there has been a tremendous increase in research examining intraspecific variation in MR and its links with the behavioural ecology of individual animals (Biro and Stamps, 2010; Burton et al., 2011; Killen et al., 2013). In general, animals with a higher BMR or SMR are more bold, active, aggressive, or exploratory. It has so far been extremely difficult to place such links into a true ecological context because we lack reliable means for measuring energy expenditure in free-ranging fish. Most studies compare behaviour measured during one time period, to estimates of MR measured during another time period, although occasionally behaviour can be quantified while the animal is in a respirometry chamber (Killen et al., 2007; Seebacher et al., 2013). Under these conditions, however, the animal is spatially constrained with unknown effects on behaviour. Some other researchers have used indirect proxies, such as opercular beat rate to estimate $\dot{M}O_2$ during the performance of behaviour (Millidine et al., 2009; Reid et al., 2012).

There are a number of specific behavioural contexts in which the ability to measure FMR would be extremely insightful. The energy spent during predator-prey and social interactions are difficult to estimate using traditional respirometry since these situations are notoriously difficult to replicate in the laboratory (e.g. Sloman and Armstrong, 2002). The ability to measure FMR alongside behaviour would increase our understanding of causal associations between MR and behaviour and provide insight into the potential for correlated selection on life-history traits (Hoffmann and Merilä, 1999; Sgro and Hoffmann, 2004; Killen et al., 2013). These methods would also facilitate tests of the allocation and production models of energy budgeting (Nilsson, 2002; Careau et al., 2008), which have so far been impossible to directly examine in fish because they depend on measures of daily energy expenditure.

2.2.2 Ecophysiology and toxicology

Extending detailed measurements of energetics or FMR to wild fish under natural conditions could be invaluable to assessing the adaptation of physiological phenomena to ecologically relevant variability or challenge under truly environmentally relevant conditions. For example, in a lab setting, SMR can vary according to food availability (O'Connor et al. 2000) and there is no reason to believe the situation is different in wild fish, but what consequence this has on overall energy budgets is largely speculative. A number of teleosts decrease or cease feeding activity over winter and SMR is depressed by a variety of mechanisms during that time. For example, Atlantic cod (*Gadus morhua*) and cunner (*Tautogolabrus adspersus*) exhibit seasonal changes in rates of protein synthesis (Treberg et al., 2005 and Lewis et al,. 2007, respectively) which are likely linked to substantial changes in MR. Changes in food availability may also influence contaminant uptake from prey items or even from water, as gill ventilation is adjusted to match energy demand. An increased reliance on lipid stores during periods of fasting could mobilize existing burdens of hydrophobic contaminants (Paterson et al., 2007) and modulate their toxicity. Reduced food intake is also associated with parental care, and in species such as the largemouth bass, activity levels can double during this time (Cooke et al. 2002). The capacity to measure FMR could test the actual metabolic consequences of these responses.

A variety of aquatic toxins alter MR in fish, including metals (Waiwood and Beamish, 1978), PAHs (Gerger and Weber, 2015) and pesticides (Lunn et al., 1976; Beyers et al., 1999) and responses can be bidirectional. For example, in largemouth bass (*Micropterus salmoides*), short term exposure to the pesticide dieldrin decreases $\dot{M}O_2$, while longer exposures increase $\dot{M}O_2$ in a

dose-dependent manner (Beyers et al., 1999). Environmentally relevant mixtures of toxins and physicochemical factors are difficult to reproduce in the lab so understanding how contaminants influence energetics or FMR under natural conditions will allow more accurate toxicokinetic modeling and estimations of ecological impacts.

2.2.3 Energetic consequences of environmental disturbance

Perhaps the biggest breakthroughs provided by measures of FMR in fish would be an enhancement of knowledge on how species are affected by environmental disturbance. Metabolic rate changes in response to a number of environmental factors including thermal fluctuations, oxygen availability, water pH, and contaminants, and all of these are expected to worsen in aquatic habitats over the next several decade in response to global climate change and anthropogenic activity. Although the effects of these factors on metabolism have been studied in the laboratory, we have no knowledge of how overall energy expenditure is impacted. Another major form of environmental alteration is the construction of dams, wave energy converters and other structures that alter flow regimes in freshwater and marine habitats. These are believed to have a major effects on activity specific metabolic demands in fish (Hanson et al., 2008), but the exact consequences are unknown because we have no direct measures of energy throughput in the field.

2.2.4 Stock management

Measures of species' energy demand at different trophic levels would also permit a more precise understanding of aquatic food webs and the prey requirements of economically and ecologically valuable fish stocks. Current fisheries models that utilise energy budget parameters rely on laboratory-derived estimates of MR or bioenergetics simulation (e.g. from the dynamic energy budget model), and would undoubtedly be refined by the use of actual field energy expenditure. Measures of FMR would also tell us how species (or individuals) alter their energy expenditure during key life-history periods such as migrations, spawning, or overwintering.

2.3 Considerations and caveats for metabolic rate determinations

Any approach to measuring MR, or the major constituents of MR, will have limitations and logistical constraints. Extended details are beyond the scope of this review, but these constraints range from the need for, and nature or degree of laboratory validation, to animal recapture and large scale data integration. Moreover, the nature of the scientific question may influence what approaches are appropriate. For instance, what could be valid for intraspecific comparisons may

be confounded by interspecific studies or introduce excess uncertainty and variation. Temporal variation in MR, or demand, may also occur in fishes and thus 'snapshot' techniques will only reflect the short-term leading up to the measurement, whereas measurements incorporating the long term integration of energetics, like growth as size at age, provide poor resolution over short time scales. As such, there is no clear 'one test fits all' approach to extending metabolic research to the field. Beyond just experimental conditions, the MR in fishes varies in response to a variety of environmental traits. For example, both SMR and MMR may be influenced by temperature and this presumably leads to the potential for seasonality in FMR. Likewise, food conversion efficiency, minimum and maximum ration and subsequently growth potential are all influenced by temperature in fishes (Brett et al., 1969; Jobling, 1988; Ojanguren et al., 2001; Handeland et al., 2008). Seasonal changes in locomotory activity, foraging effort and success, allocation to growth versus reproduction, along with potential seasonality in SMR may all need to be considered when trying to apply laboratory level strategies and data to the study of fishes in the field.

A major factor when discussing any estimate of MR is the effect of body size (Glazier, 2005; Killen et al., 2010). Absolute energy demand increases allometrically with biomass. Consequently, estimates of MR may need to be adjusted for differences in size, particularly if measures are made over long time periods during which the fish may either grow or lose mass. An indirect effect of changes in body size on field estimates of metabolism are potential changes in tissue concentrations of any injected reagents. This could limit the duration over which fish can remain at large and still provide useful measures of FMR. Further, smaller and younger fish tend to grow faster. Therefore, any confounding effects of growth or size on measures of FMR may be disproportionately problematic for particular life-stages.

Another limitation or constraint on many hypothetical methods for measuring FMR would be the ability to recapture individuals for re-assessment of tissue biochemistry, or retrieval of bio-loggers (e.g. accelerometers). In general, recaptures will be more feasible in stream-dwelling fishes (e.g. juvenile salmonids) or site-attached species (e.g. many coral reef fishes) but can be a barrier or challenge for the study of pelagic species or species with large home ranges. Recapture rates for particular species may also vary among environments (e.g. recapture may be affected by temperature effects on activity). Finally, the actual methods used for recapture could bias which phenotypes can be collected. For example, techniques such as trawling, trapping, or angling could

select for particular phenotypes (e.g. bolder individuals or those with a higher SMR (Philipp et al., 2009; Wilson et al., 2011; Killen et al., 2015)), potentially leading to recapture-associated bias in which phenotypes are ultimately included in estimates of FMR.

3. Approaches to metabolic rate estimates applicable to field conditions

3.1 Why doubly labelled water is a dead-end for FMR in fish

There is no consensus 'gold standard' technique for measuring FMR in terrestrial animals but the doubly labelled water (DLW) technique (Butler et al., 2004; Speakman et al., in the current issue) has been widely applied and may be as close as it comes for many animals less than ~ 100 kg in size. Briefly, the DLW method monitors the disappearance of labelled oxygen and hydrogen (enriched with stable isotopes) following injection of a known dosage of labelled water. Since oxygen can leave the body as CO_2 or H_2O while hydrogen is predominantly lost as H_2O the difference in the disappearance of the two tracers can be used to estimate CO_2 production. While attractive for estimating FMR for many animals, the DLW technique is not effective in aquatic species that have high whole body water turnover rates. For instance, teleost fishes have unidirectional water influx rates that indicate whole body water turnover rates from ~ 5-10% per hour to well over 100% of total exchangeable body water turnover per hour (Evans 1969). Osmoconforming animals appear to have equal or even higher rates of water turnover (Rudy, 1967; Haywood, 1974). Given such high water turnover rates, it would seem implausible to accurately monitor metabolic carbon dioxide CO_2 production with the DLW approach in these aquatic organisms. Since the majority of inhabitable space is aquatic, alternatives to the DLW approach are required to glean representative information on the FMR of a vast number of species.

3.2 Biotelemetry

There have been substantial advances in linking telemetry, accelerometry and other methods to estimate metabolic costs in fishes. We will only briefly touch upon some of the major concepts and components and direct the interested reader to Cooke et al. (this issue) for further details.

3.2.1 Heart rate

Tissue oxygen demand and metabolic waste removal are supported by blood flow, and heart rate (f_h) is an important determinant of total cardiac output in fish. To varying degrees, f_h is sensitive to feeding state, activity, physiological and social stress and water quality, all of which are closely tied to MR. Although there are limitations in the use of f_h as a proxy for MR in the field (see

below), it has shown promise as an indicator of energy expenditure in fish. Various logging and telemetry methods are now available to assess f_h in free swimming fish and eliminating the confinement and disturbances associated with lab-based measurements can greatly improve data quality. For instance, f_h is lower when measured in free swimming fish compared to confined animals (Gräns et al., 2010) and a similar pattern is evident for MR (Clark et al., 2010). Tag size and surgical constraints generally render this approach more appropriate for relatively large fish (>575 g), but lab trials have been successful for animals as small as 100 g (Snelderwaard et al., 2006).

There are a number of limitations to consider when using f_h as a proxy for MR in the field, many of which can be addressed by rigorous validations in the lab. The major concern is that the proportional influence of f_h on cardiac output can change according to the stimulus influencing MR and effects on the relationship between f_h and MR may be difficult to predict (Thorarensen et al., 1996). The use of f_h to estimate energy expenditure may be best applied over longer time scales and in combination with temperature logging as a more accurate predictor of MR than activity based methods (Clark et al., 2010).

3.2.2 Locomotory activity and accelerometry

Activity-specific energy expenditure represents a key part of the overall energy budget of a fish (Fig. 1) and techniques are available for quantifying activity in free swimming fish (reviewed by Metcalfe et al., 2016b). Locomotory activity can be assessed using electromyography (EMG) tags, which quantify contractile activity in specific muscles, or it can be estimated from accelerometry data. Accelerometry tags quantify acceleration of the animal in two or three dimensions and can provide very high resolution data on activity and behaviour patterns. Environmental variables can influence swimming kinematics, consequently, the relationship between EMG output and MR may vary between different environmental conditions. For example, fish may vary tail-beat frequency and amplitude independently as water temperature changes (Lea et al., 2016), so the characteristics of the EMG output may differ at similar swimming speeds.

As with f_h tags, these approaches are best deployed in relatively large fish where the additional volume and mass of the tag will be less burdensome. Tags can be either logging or transmitting, with or without the ability to simultaneously record environmental variables like temperature. Although activity-specific energy expenditure estimates do not account for the influence of

environmental variables on MR, the addition of temperature data could provide at least some capacity to estimate relative changes in metabolic demand over the recording period. Accurately assessing activity-specific energy expenditure from accelerometry data requires high sampling rates, which are more suitable to archival tags. Transmitting tags have a limited capacity to transmit high resolution data in real time but data integration techniques are becoming available to address this issue (Metcalfe et al. 2016b). As discussed above, the unnatural conditions imposed by lab-based studies can influence heart function in fish and the situation is no different with activity-related parameters. Confinement in a typical swim tunnel respirometer restricts movement and can prevent energy saving behaviours (e.g. schooling (Marras et al., 2015)) and the use of different swimming gaits (e.g. burst burst-and-coast swimming (Videler and Weih, 1982) or Kármán gaiting (Taguchi and Liao, 2011)). Relationships between EMG or accelerometry data and $\dot{M}O_2$ may therefore be somewhat different between lab and field studies, but this should not greatly diminish the power of these approaches for assessing activity-specific energy expenditure.

3.3 Respirometry

While direct calorimetry has been used for laboratory held fishes (for instance Smith et al., 1978; Van Waversveld, 1989; van Ginneken et al., 1996; Regan et al., 2013), this approach has not been commonly applied in field conditions or wild-captured fish. Instead, fish metabolism is usually indirectly estimated by measuring $\dot{M}O_2$ of fish in a respirometer (Brett, 1962; Beamish, 1978), although $\dot{M}CO_2$ has also been used for indirect calorimetry on fishes (Kutty et al., 1971; Kieffer et al., 1998). Respirometry encompasses introducing an organism into a sealed static chamber or swim tunnel and, in the case of $\dot{M}O_2$, measuring the decrease in oxygen concentration over time. Three different respirometry techniques are generally used: closed, flow-through and intermittent-flow systems (Steffensen, 1989; Clark et al., 2013; Svendsen et al., 2016)

The majority of respirometric experiments have been conducted in controlled laboratory settings following strict experimental procedures and with minimal environmental variation (e.g. constant water temperatures and velocities). A few studies have tried to incorporate environmental variations into the laboratory experiments by fluctuating temperature (Beauregard et al., 2013; Oligny-Hebert et al., 2015), flow (Enders et al., 2003; Taguchi and Liao, 2011), salinity and hypoxia. To fully incorporate natural environmental settings or to work with species at risk where regulations may prevent removal of fish from the river system, a few studies have attempted to

353 perform respirometric experiments in the field where native fish can be tested in their natal waters
354 under ambient light and temperature regimes (Farrell et al., 2003; Rodnick et al., 2004). Some of
355 these studies used very simple closed (Rasmussen et al., 2012; Warnock and Rasmussen, 2014) or
356 continuous flow-through respirometers (Hammer and Purps, 1994), while others employed state-
357 of-the-art intermittent-flow systems (Gamperl et al., 2002; Farrell et al., 2003). Some of the most
358 extreme examples of measuring $\dot{M}O_2$ in wild fishes come from the study of deep-sea fishes;
359 pressurized respirometers and baited-trap based *in situ* respirometers have demonstrated very low
360 MR in many deep-living species (Smith, 1978; Drazen et al. 2005; Drazen and Yeh, 2012).
361 Collectively, these studies on fishes recently collected or captured in the field have measured
362 variations of MR (i.e. SMR, routine (RMR), active (AMR) and MMR) as well as derivatives of
363 MR (e.g. aerobic scope), applying a wide range of different respirometric technologies. The size
364 of the employed equipment ranged over several scales from small 600 ml static chambers
365 (Warnock and Rasmussen, 2014) to a 26 000 l 'seagoing mega-flume swim tunnel' (Payne et al.,
366 2015).

367 When respecting habituation and fasting periods, field-based $\dot{M}O_2$ measurement generally
368 compare well to laboratory estimates. For example, field-based $\dot{M}O_2$ results for Sockeye salmon
369 (*Oncorhynchus nerka* Walbaum 1792) assessed with a mobile Brett-type respirometer swim tunnel
370 (Farrell et al., 2003) were comparable to laboratory-based $\dot{M}O_2$ results by Brett and Glass (1973),
371 strengthening the argument that reliable respirometry can be performed in field locations.

372 The available tools that allow for reliable field measurements of $\dot{M}O_2$ are of particular interest for
373 fish species that are too fragile for transportation and endangered species that cannot be removed
374 from their natural environment. While considerable effort has been spent to develop respirometric
375 methods to measure metabolic rates in the field, technical challenges remain for off-road, remote
376 locations without access to electrical power. It is also important to remember that any attempt to
377 use respirometry on animals in the field will not be estimating FMR because, by definition, FMR
378 can only be measured on unrestrained animals. However, using estimates of SMR or MMR derived
379 from respirometric experiments could be combined with some of the other methods, we describe,
380 to construct reasonable estimates of the fish's total energy expenditure in the natural environment.

381

3.4 Isotopic tracer turnover methods

Along with protein synthesis, see below, isotopic precursors have been used extensively for metabolic study of fishes and other animals. For instance, ^{14}C and ^{3}H labelled carbon substrates can be invaluable for measuring the rate of substrate oxidation/preferenda (van den Thillart, 1986) and blood-borne metabolic fuel turnover (Haman et al., 1997). However, these experimental approaches require extensive validation or the capacity for repeated sampling over time to establish either decay curves for turnover or stable-steady state conditions for calculating fluxes. These validation requirements seem to have thus far precluded the use of radioisotope, or parallel stable isotopic, tracer methods on fish under field conditions (the authors are unaware of any such studies). Interestingly, the Haman et al. (1997) study demonstrates an important caveat that is highly applicable for field sampling. By manipulating temperature and oxygen levels it was shown that plasma glucose and free fatty acid levels in rainbow trout (*Oncorhynchus mykiss*) were not necessarily reflective of metabolic flux or demand for a metabolic fuel (Haman et al., 1997). Therefore, differences or lack thereof in plasma metabolites from field sampled fishes should be interpreted with caution.

Recently rubidium turnover has become a possible alternative to the DLW technique for free-ranging small animals with whole body turnover paralleling the DLW estimate of MR and the $\dot{M}CO_2$ by respirometry (Tomlinson et al., 2013). It appears that rubidium turnover is likely due to rubidium acting as a potassium analogue, with whole body potassium losses being a function of MR (Tomlinson et al., 2014). Given the high environmental potassium exchange in fishes, which varies markedly with salinity (Eddy, 1985), it would seem that application of rubidium clearance approach may suffer from similar problems of isotopic turnover that preclude using the DLW technique in fishes.

3.5. Long term assimilation approaches

There are several means of evaluating energy use, or demand, over long time periods in fishes that are applicable to field sampling and use. These will have lower resolution compared to direct measurements on individuals, and may be better suited to the study of populations, but these long term estimates may have particular utility for some studies on metabolic costs in fish under field conditions. We will focus on two strategies, a bioenergetics balance model and isotopic enrichment and discuss them only briefly.

3.5.1. Energetic balance estimates

Taking a bioenergetics model approach has led to some important findings about environmental differences in fishes in the wild as well as the role fish have in the energy budgets of ecosystems. This generally takes the form of using estimates of the terms that make up typical bioenergetics models (see Fig. 1) or deriving these estimates based on field collected data. Often key terms must be assumed, such as losses as nitrogenous waste, digestion efficiency and the magnitude contribution of the costs of digestion, or are taken from laboratory studies on the same or closely related species. For the latter point, this is often done for estimates of SMR if a value is to be used. Estimates of food intake for wild fish are complicated but can be quantified from gut contents, although to assess $Energy_{in}$, this requires determining the rate of gut evacuation or assuming a value for this (Elliot and Persson, 1978; Hyslop, 1980).

A value for $Energy_{retained}$ can be determined using growth estimates based on the size at age combine with the energy content of somatic tissues, or their proximal composition (content of lipid, protein and carbohydrate) with reproductive investment determined based on the energy content of the gonad. The reproductive investment may also require correcting for past spawning activity if the species is iteroparous. If no estimates of metabolic energy expenditure are available, be it SMR or the energy used in activity, it is possible to estimate the combined total metabolic costs based on the difference between $Energy_{in}$ (as food consumption) and $Energy_{retained}$ (as tissue growth).

The need for robust comparison or ‘corroboration’ between laboratory and field-based bioenergetics models has been appreciated for over two decades (Hansen et al., 1993). Some datasets, however, failed to match laboratory and field results. This illustrates the need for cautious extension of the assumptions and simplifications that may come with a bioenergetics model approach. A more recent analysis found continued variable, and often poor, agreement between actual and modelled values (Chipps and Wahl, 2008). Moreover, physiological variation amongst distinct populations in response to local environmental conditions (local adaptation) is one of the recognized potential confounding factors along with uncertainty about feeding rates (Chipps and Wahl, 2008), the latter of which will be intimately linked to prey density and swimming activity. Moreover, conditions leading to compensatory growth (Whitledge et al., 1998) and the known

441 wide intraspecific differences in growth and SMR (Tyler and Bolduc, 2008) common in many
442 fishes may also lead to complications in fine scale resolution for individual fishes.

443 Despite the above considerations, using the concept of energetic balance, combined with data on
444 growth, estimates of energy intake and possible reproductive investment has led to some important
445 findings on the partitioning and use of energy in fishes. These include the remarkably high energy
446 investment in 'metabolism' in some deep-living, active swimming seamount fishes, who expend
447 large amounts of energy due to ocean currents. This corresponds to a much higher food
448 consumption but low food conversion efficiency compared to other deep-sea fishes with low
449 metabolic capacity (Koslow, 1996; 1997). Likewise, using energy budget estimates, it has been
450 shown that congeneric marcourids (rattails or grenadiers) with overlapping distributions may adopt
451 very different life-history strategies, or at least marked differences in energy allocation between
452 growth, activity (SMR and locomotion) and reproduction (Drazen, 2002). Thus, despite the
453 challenges of using an energetic balance approach to field studies of fish energy metabolism,
454 important clues to the adaptation to environmental factors can come from this approach.

455 ### 3.5.2. Otoliths

456 There have been attempts to link the rate of otolith growth to MR. For example, support for a
457 linkage in Atlantic salmon (*Salmo salar*) was found beyond simple somatic growth; the otolith
458 increment was linked to inter-individual differences in SMR but not growth (Wright, 1991).
459 Follow-up studies indicated that the metabolic response to changing temperature was more
460 pronounced than the observed otolith response (Wright et al., 2001) raising concerns about the
461 broad field applicability of this technique. Some more detailed approaches may support otolith
462 accretion as an indicator of growth, at least in Atlantic cod (*Gadus morhua*; Hüssy and Mosegaard,
463 2004). This is still an active area of study and the architecture of otoliths may ultimately prove as
464 a useful tool in estimating relative differences in MR across fishes.

465 An alternative use of otoliths comes from the partitioning of stable isotopes, namely ^{13}C and ^{12}C.
466 Metabolically derived CO_2/HCO_3^- in the blood is expected to be depleted in ^{13}C compared with
467 the environmental dissolved inorganic carbon and this decline in the $^{13}C/^{12}C$, or $\delta^{13}C$, should be
468 more pronounced as the rate of metabolic CO_2 production increases (Kalish, 1991; Gauldie, 1996).
469 The carbon being fixed within the otolith as calcium carbonate ($CaCO_3$) is thought to be a mix
470 between that in equilibrium with the environmental dissolved inorganic carbon pool and the

metabolically produced (^{13}C depleted) CO_2/HCO_3^- and, despite the large net efflux of CO_2, > 80% of the fixed carbon in otoliths may be from the dissolved inorganic carbon from the environmental pool (Solomon et al., 2006). Shifts in the $\delta^{13}C$ in otoliths have been shown to relate to estimated MR, even at the microscale where annual variation in MR may occur (Dufour et al., 2007). Adding to the potential utility of otolith isotope chemistry in field estimates of MR, the levels of ^{18}O may also provide an estimate of environmental temperature (Kalish, 1991) and determination of the $\delta^{18}O$ ($^{18}O/^{16}O$) and $\delta^{13}C$ in young-of-the-year Arctic charr (*Salvelinus alpinus*) shows support for a latitudinal gradient in growth and MR (Sinnatamby et al., 2015). The $\delta^{13}C$ and $\delta^{18}O$ have also been used to infer seasonal temperature cycles and MR in fossilized otoliths (Wurster and Patterson, 2003), suggesting this approach could be invaluable for archived samples. These isotopic approaches may be a valuable addition to the tools available for comparative biochemists and physiologists to study FMR in fishes, although many require further validation and may be limited in their capacity for fine temporal resolution (scale of less than months) or for precise comparisons between individuals.

3.6. Integrating methods

It is our position that there is currently no robust and widely applicable approach for assessing FMR in fishes; however, we feel that methods that could confidently estimate FMR in fishes would be highly beneficial. From the discussions above, it should be appreciated that while it may be possible to quantify FMR in free swimming fishes, estimates will be laden with assumptions and approximations. Validation and calibration is laborious and requires the assumption that laboratory results will recapitulate 'field relevant' conditions. Ideally, to assess FMR in fishes, a complete integrated value of all energy usages must be assembled. To do so would likely require combining indirect calorimetry for understanding basal costs, as well as some form of telemetry to integrate activity (locomotory) costs and possibly f_h measurements, which could be compared to lab-validated correlations to MR.

A general strategy would be to measure the MR of individuals, then release them into a natural or semi-natural environment for behavioural observation using video recordings. Mark-recapture studies are possible but they provide a relatively coarse quantification of space use and face the potential problem of low recapture rates. Currently, the most promising approach for aligning measurements of MR with behaviour in the natural environment for fish may be to measure MR

in respirometers and then release fish into an acoustic telemetry array for spatially tracking the movements of individuals (Baktoft et al. 2016). Modern telemetry technology can provide high resolution data for inference of activity level, habitat preference, territory size and even feeding frequency.

There are several potential issues common to all of these methods for attempting to correlate behavioural measures with measures of MR performed in the laboratory, even in cases where telemetry is used for measuring behaviour. First, these approaches only reflect how estimates of specific types of MR extracted from laboratory data (such as SMR) may be correlated with behaviour in free-ranging animals. They would provide no insight into the animal's moment-to-moment energy expenditure on physical activity or digestive costs. Further, and perhaps more importantly, all types of MR in fish will vary as a function of temperatures encountered in the wild (and perhaps oxygen availability in severe hypoxia (Claireaux and Lagardere, 1999)). If reaction norms for a measure such as SMR vary among individuals in response to changes in temperature (Brommer, 2013; Killen et al., 2016), relative rankings within a measured population in the laboratory at a single common temperature will not carry over to the wild in situations where there are spatial or temporal thermal fluctuations. This effect could greatly complicate attempts to relate estimates of SMR or other metabolic traits to free-ranging behaviour even in cases where the temperatures encountered by the fish are known from extrinsic or intrinsic temperature loggers.

In many cases it is likely that the suggested 'ideal' condition of respirometry and telemetry will not be possible (though see Bakstoft et al. 2016 and Cooke et al. this issue). Nevertheless, it may still be possible to glean insight into some of the major energetic costs in field conditions based on simple 'snap-shot' data, even if it is not possible to get integrated estimates of actual FMR. For instance, biochemical markers (discussed below) may give insight into intraspecific growth potential, or 'shore-based' respirometry may allow for comparisons of SMR and MMR if the hypothesis being tested can tolerate some degree of introduced error. Measures of SMR or MMR could also be combined with swim-flume calibrated accelerometry data to understand the costs of routine activity in the field (Murchie et al. 2011). Similarly, estimates of growth and tissue/energy accretion combined with gut contents and prey energy density could provide information on the metabolic responses and energy allocation of fishes in the field, albeit this would give only a partial picture.

3.7 Expanding the energetics toolbox with indirect proxies and indices of major energy requiring processes

Even if true FMR estimates are not currently possible for fish, there are several biochemical and physiological measurements that may provide a window into major energetic processes or overall energy balance in wild sampled fishes. In this section we examine several biochemical markers and techniques that may be useful as relative indices of metabolic capacity, especially under conditions where feeding success or growth rate may vary. Since basal metabolic costs (SMR) and growth are major components of the energy balance of an organism, we limit this discussion to correlates of these specific contributions to MR.

3.7.1 Organ and tissue energy metabolism enzymatic indices

For studies where many individuals must be sampled, for instance when comparing across populations over a wide geographical gradient, simple indicators of relative metabolic demand or capacity may be particularly useful due to high throughput and readily standardized methodologies across research groups. There has been some investigation into if the relative organ mass and tissue specific activities of energy metabolism enzymes or biochemistry could provide useful correlation to MR in fishes.

It is intuitively appealing to anticipate that individuals with higher MR may also have larger organs to support metabolically demanding processes. For instance, large livers for greater allocation to biosynthesis, increased renal mass for improved clearance capacity, elevated digestive organ size and complexity to process food either more quickly or in greater bolus quantities, or enhanced cardiovascular capacity to meet increased oxygen demand. Indeed, there is some evidence for organ or muscle size being linked to MR in endotherms and this may have some utility for intraspecific comparisons (Chappell et al., 2007), but taken as a whole the data do not support a generalized relationship. Recently, it has been shown that interspecifically relative liver size relates to SMR, with the latter estimated by respirometry (Killen et al., 2016). Many species accumulate hepatic lipid stores (Pelster, 1997; Phelger, 1998) so correlations between liver size and SMR must be made cautiously, since variations in the size of those stores may confound relationships with SMR. Moreover, the intraspecific data on fishes is equivocal with some support for a correlation between MR and the summed contribution of several organs to overall mass in eels (Boldsen et al., 2013), but with no such correlation in brown trout (Norin and Malte, 2012).

Similar to relative organ mass, it may seem intuitive that key enzymes of energy metabolism should correlate with tissue level adenosine triphosphate (ATP) demand. As noted above, interspecifically there are correlations between MR and depth of occurrence, at least across benthic and benthopelagic fishes, and several muscle enzyme activities parallel that MR trend (Drazen and Seibel, 2007; Drazen et al., 2015). Generally, lactate dehydrogenase and pyruvate kinase activity in white muscle correlate with depth-related declines in MR. The activity of the mitochondrial matrix marker enzyme citrate synthase also correlates with MR but is more variable and appears to be influenced by general locomotory capacity more so than these other enzymes (Drazen et al., 2015). Species lifestyle (benthic, benthopelagic or pelagic) is a potential confounding factor which should be considered in interspecific comparisons with all of these muscle metabolic enzymes. While mitochondrial enzyme activities like citrate synthase (a Krebs cycle marker enzyme) and cytochrome c oxidase (electron transport chain constituent) appear *a priori* as obvious choices for correlation with MR, empirical results for oxidative enzymes are mixed. Intraspecific investigations testing this hypothesis have shown results ranging from little (Norin and Malte 2012) or no correlation within a population with variable intraspecific MR (Boldsen et al. 2013) to some evidence of support across fishes where MR is manipulated at the whole animal level (Mathers et al., 1992; Pelletier et al. 1993). Importantly, although oxidative enzymes may correlate with growth (and thus presumably MR), fish size and seasonality may be more significant drivers of enzyme activity (Pelletier et al., 1993). In muscle, the activity of enzymes associated with glycolysis, including phosphofructokinase, pyruvate kinase and lactate dehydrogenase, often show good correlation when growth rate of fish is manipulated by ration size and thermal regime (Mathers et al., 1992; Pelletier et al., 1994; Pelletier et al., 1995). Overall, the activity of these enzymes may be more related to the capacity of a tissue to sustain high energy demand rather than energy needs *per se*.

The development of organ level indices that correlate with MR in fishes may be appealing due to their simplicity, but these will generally have low resolution and require species-specific laboratory validation. For developing enzymatic indices that may correlate with metabolic capacity or demand, it is important to consider what denominator to use, with per gram of tissue mass, per unit protein or per unit DNA, all being potential candidates. For further discussion see Pelletier et al. (1994; 1995). Caution is also warranted in attempts to develop relative organ mass or enzyme activities as proxies of MR because these traits may scale with body mass (Huang et al., 2013),

592 with relationships for muscle enzyme activities being at times complex and dependent on species
593 and developmental stage (Somero and Childress, 1980; 1990; Hinterleitner et al., 1987).

594 Along with the data on tissue enzyme activities, the RNA and DNA contents of tissue like white
595 muscle may also be a useful means of estimating the growth potential and status of a fish (Sutcliffe,
596 1965; Haines, 1973; Grant, 1996; Buckley et al., 1999, Chícharo and Chícharo, 2008), which may
597 be linked to their MR. Indeed, in some cases, it would appear that combined measurements of
598 these nucleic acids with enzyme activities may provide the best overall proxy of current growth
599 potential and/or feeding status in fishes (Mathers et al., 1992; Dahlhoff, 2004). Although these
600 patterns may not always reflect growth or feeding in all species, at least on the scale of less than
601 several weeks (Dutil et al., 1998). By combining multiple tissue biochemical and relative mass
602 indices, it is possible to construct models that may be sufficiently predictive of growth or condition
603 in wild fish or open water housed fish (Guderley et al., 1996; Couture et al., 1998) that they may
604 have utility in field-based studies.

605 ### 3.7.2 Whole animal and tissue rates of protein synthesis

606 Along with the ion-motive ATPases, protein synthesis represents the most prominent consumer of
607 cellular energy. The costs of protein synthesis have been estimated to account for 15-25% of basal
608 metabolic costs (Carter and Houlihan, 2001; Fraser and Rogers, 2007) and possibly as much as
609 42% in juvenile fish (Houlihan et al., 1988 but see Fuery et al., 1998). The whole-body rate of
610 protein synthesis is strongly correlated with SMR or BMR, in endothermic and ectothermic
611 animals respectively (Houlihan, 1991). Various biotic and abiotic factors, such as temperature,
612 pollution, seasonality and food consumption also have a similar effect on the rate of protein
613 synthesis and SMR (Fraser and Rogers, 2007). Finally, the rate of protein synthesis is one, if not
614 the most responsive biological process to limited energy supply, as elegantly demonstrated by
615 Buttgereit and Brand (1995). It is therefore appealing to consider the use whole-body protein
616 synthesis rate as a proxy to FMR.
617
618 Historically, measuring the rate of protein synthesis required the use of radioactive tracers, which
619 is not realistic in field situation. In the last two decades, however, alternative approaches to
620 measure the rate of protein synthesis were published and thus opened the possibility of transporting
621 this measurement to the field with minimal complexity. Notably, three of these approaches bear
622 great promises for use in field situation. The first approach consists in a modification of the

623 flooding dose technique for using stable isotope tracers. The flooding dose technique, as the name
624 implies, consists in injecting the fish with a bolus of a labelled amino acid. After the injection, the
625 fish is *released* and recaptured following a certain incorporation period. The subsequent
626 incorporation of the tracer in the animal's protein pool is measured. The technique originally
627 described by Garlick et al. (1980) involved the injection of a bolus dose of phenylalanine
628 containing tracer amounts of radioactive phenylalanine (^{3}H-phenylalanine). Modifications of this
629 technique to be used with stable isotopes were first published and validated in rats by Krawielitzki
630 and Schadereit (1992) and in fish by Owen et al. (1999). These two modified techniques are based
631 on the injection of a flooding dose of ^{15}N-labelled amino acid tracers and subsequent determination
632 of the incorporation rate of the tracer in the protein pool. These techniques were shown to produce
633 results that are undistinguishable from those obtained using the original radioactive approach.
634 However, the ^{15}N-amino acids are seldom used in fish physiology; probably because of their
635 inherent requirement of an isotope ratio mass spectrometer (IRMS) for the determination of the
636 tracer's enrichment in the protein pool. IRMS is not always readily available or accessible. More
637 recently, a variant of the flooding dose technique using ring-D_5-phenylalanine as a tracer was
638 described (Lamarre et al., 2015). The advantage of this tracer over the ^{15}N-tracers is that it only
639 requires the nearly ubiquitous gas chromatography-mass spectrometry (GC-MS) to perform the
640 measurements. Using the flooding dose technique, the rate of protein synthesis can be measured
641 over a relatively short period of time varying from less than one hour up to several hours.
642
643 The second approach that shows potential in the field is a non-isotopic technique that is based on
644 the use of the antibiotic puromycin; the SUnSET approach (Schmidt et al., 2009). Puromycin is a
645 structural analog of tyrosyl-tRNA that, when incorporated in the nascent protein, prevents
646 elongation. It was demonstrated that, when used at a very low dose, puromycin incorporation into
647 proteins is directly proportional to the rate of protein synthesis (Hansen et al., 1994; Nemoto et al.,
648 1999). Just like in the flooding dose technique, the animals must be captured, receive an injection
649 of puromycin and then be *returned* to the field for a predetermined incorporation period. The
650 animal is then recaptured for tissue sampling and the puromycin-labeled proteins detected by
651 western blotting using a puromycin-specific antibody (Goodman and Hornberger, 2013). The
652 SUnSET approach was shown, in rodents, to be as sensitive and accurate as the flooding dose
653 technique but this approach remains to be tested and validated in fish. The major advantage of

SUnSET is that it does not involve the use of isotopes and consequently, does not require mass spectrometry. The main limitation of this technique, however, is that it can only be used to measure relative rates or relative changes in protein synthesis (Goodman and Hornberger, 2013). A strategy to measure the absolute or fractional rate of protein synthesis has yet to be developed.

The third approach uses deuterated water (^2H_2O) as a tracer. This approach was first proposed by Ussing (1941). Briefly, when ^2H_2O is administered to an animal, the tracer quickly equilibrates with the body water. Extensive labelling of the free amino acids occurs rapidly mainly via transamination reactions. These labelled amino acids can then become incorporated into the protein pool. Alanine is generally the amino acid being followed since it has a very high turnover and can be labelled at four sites (Gasier et al., 2010). The use of ^2H_2O as a tracer to measure the rate of protein synthesis in fish was recently described (Gasier et al., 2009). The fish *simply* need to be maintained in water containing ~2-4% ^2H_2O for a period of at least 24 h. Following this period, the tissues are sampled and analyzed using a GC-MS or preferably IRMS for the incorporation of ^{15}N-alanine into the proteins. One advantage of this technique is that the rate of protein synthesis is measured over a long period of time (24 h or more) compared to the techniques described above. This longer incorporation period ensures that short-term changes and diurnal cycles of the rate of protein synthesis, and hence of the MR, are incorporated in the measurement. There is also minimal intervention on the animal since the label is added to the water surrounding the fish instead of being injected. On the other hand, the fish must be maintained in this labeled water for an extensive period of time, which is certainly challenging in the field but not impossible.

To our knowledge, the rate of protein synthesis has never been measured in fish in the field. The recent developments in non-radioactive techniques to measure the rate of protein synthesis should stimulate field biologists to consider applying it in their field studies. Of course, all of the techniques described here are only robust when they are properly validated in the species and the context of the questions being asked. It is beyond the aim of this paper to describe the proper validation of the techniques described but this information is readily available in the references provided above. Given the usefulness and biological value of the rate of protein synthesis as a proxy for MR, we speculate that it is only a matter of time before we start seeing the rate of protein synthesis of fish being measured in field studies.

685

686 ### 3.8 Tracer-based FMR estimate: perspective approaches

687 In the spirit of furthering discussion, we have derived a strategy that may be applicable to
688 addressing FMR in free-swimming fishes based on isotopic tracers. The concept revolves around
689 implanting osmotic pumps, which can deliver a volumetric payload at a constant rate of delivery
690 up to the scale of days-to-weeks. The osmotic pump could be filled with a solution of labelled
691 metabolic fuels, which may include glucose, palmitate, amino acids or a combination thereof.
692 Initially, in the laboratory this would likely use ^{14}C labelled fuels for simplicity and to avoid the
693 natural background of stable ^{13}C isotope that could obscure the physiological patterns we seek to
694 quantify (rate of metabolic CO_2 production or the steady-state enrichment of metabolite pools).
695 However, the use of ^{13}C labelled fuels could be rapidly envisioned provided the natural enrichment
696 of ^{13}C is measured on a blood sample taken at T_0 (just before the insertion of the osmotic pump).
697 The osmotic pump could be implanted into the peritoneal cavity (Fig. 2), which would facilitate
698 the larger pumps required for long-term delivery of the precursors. Once active, the pump would
699 infuse a constant supply of the labelled precursor, which would be absorbed by the fish as is seen
700 with other intraperitoneal applications of tracers (Cowey et al., 1975; Hemre and Kahrs, 1997;
701 Lewis et al., 2007; Lamarre et al 2015). During the initial validation, this constant tracer supply
702 combined with serial blood sampling for plasma could facilitate determining the rate of
703 disappearance and turnover of the tracer (Fig. 2B). This will also allow testing the impact of
704 feeding and other biotic and abiotic influences on metabolite flux, which could be combined in
705 some cases with indirect calorimetry.

706 Once the temporal pattern of roughly stable systemic metabolic enrichment is established, this
707 provides the potential window for the next phase of development; long-term collection methods
708 that may be transferrable to the field. We propose two possible solutions, one based on plasma
709 collection, the other the long-term integrated capture of metabolic CO_2 (Fig. 2A), that in concert
710 could lead to FMR estimates in free swimming fishes. It should be appreciated that both are
711 completely theoretical but should be experimentally plausible. In both cases, the recapture of the
712 fish would be essential.

3.8.1. Plasma collection

The positive pressure generated by osmotic influx of water is how osmotic pumps work to deliver solutions. Therefore, it should be possible to create negative pressure within the inner impermeable chamber by inverting the osmotic gradient established within the pump. By filling the pump's 'osmotic layer' with a solution that is hypoosmotic to the organism's body fluids it could be possible to establish a fluid collection vessel, rather than a delivery mechanism. By addition of a layer of dialysis membrane or similar selectively permeable material over what is usually the delivery opening, the system would prevent collection of blood cells and proteins thereby minimizing metabolic activity within the internal chamber. By implanting several pumps, with differing collection volumes and manipulation of capacity for osmotic exchange and regulation of the opening size of the inner chamber it should be possible to have differentially timed collections of body fluid (on the scale of days or possibly weeks). If these 'reverse' osmotic pumps can be implanted with their opening in the systemic blood supply, then serial, long-term, sampling could be achieved to assess if the integrated specific enrichment of tracers change over time, which should reflect the metabolic turnover of the compounds of interest (Fig. 2B).

3.8.2. In situ collection of CO_2

The second approach would capitalize on enclosing a solution of strong base (e.g. 9M NaOH) within a thick membrane that is partially permeable to gaseous CO_2 and implanting this either with a small region exposed to the blood (ideally in the ventral aorta) or within the peritoneal cavity. The membrane material should be relatively inert, for example silicone, and be designed to become a kinetic limitation to CO_2 diffusion to the internal reservoir by being thick enough and possibly partially enclosed by gas impermeant material. The rationale of this device and its design constraints would be to slowly subsample the metabolic CO_2 in circulation as the gas diffuses into the alkaline 'trap' within the internal reservoir on the scale of days-to-weeks. The osmotic pump would provide a constant infusion of labelled tracer, oxidation of which will lead to $^{14}CO_2$ or $^{13}CO_2$ in equilibrium with the rest of the body fluid CO_2 pools. Thus, the accumulation of labelled-CO_2 in the reservoir would be a function of the metabolic oxidation of the tracer precursors. By appropriate tracer selection, it should be plausible for this collection of the labelled-CO_2 to reflect actual whole body metabolic labelled-CO_2 production, which could be confirmed in lab via indirect calorimetry. The enrichment of labelled-C in the CO_2 pool could then be measured by a scintillation counter in the case of ^{14}C in the lab or with an IRMS when the tracer is ^{13}C (of course

744 correcting for the natural abundance of organic ^{13}C measured at T_0). Altered enrichment of ^{13}C in
745 the otolith (3.5.2) may provide a biological alternative or validation of this alkaline trap approach.

746 ### 3.8.3. Challenges

747 As noted in section 3.3 the validation of tracer turnover and kinetics studies are laborious and the
748 above field strategies would be limited to a small number of sampling time points per individual
749 fish once released. This low sampling could limit resolving power but given the complexities of
750 other options to assess FMR in fishes, our speculations on following tracer carbon kinetics could
751 be a viable alternative worth exploring. Nevertheless, even if the technological challenges of the
752 sampling devices described in 3.8.1 and 3.8.2 were solved, there would be additional cautions and
753 assumptions with these techniques, only a few of which we will address. Some are logistical, such
754 as regulatory agency approval for the release of animals laden with tracers, but many are
755 methodological. For example, can the collection devices be reasonably implanted with access to
756 appropriate blood pools (e.g. ideally the ventral aorta prior to the gas exchange at the gills for
757 labelled-CO_2) and if not, are other body pools of fluid comparable? For the capture of labelled-
758 CO_2, the peritoneal cavity may be useful since several devices might be implanted. However, this
759 body cavity would not necessarily be acceptable for the steady-state labelled-C-tracer enrichment
760 approach, since this will also be the point source for the tracers prior to distribution and dilution.
761 Will the collection devices be prone to differential collection rates? This could be a significant
762 concern and will depend on materials selection and quality control in the manufacturing process.
763 For instance, the amount of CO_2 diffusion into the alkaline trap will be a function of the pCO_2
764 gradient across the membrane as well as the membrane thickness and total surface area exposed
765 for gaseous capture. Likewise, can the collection devices accumulate sufficient tracer or product
766 to be quantifiable? This could only be assessed empirically.

767 ## 5. Future directions: a call to action

768 In summary, we feel that there is currently a lack of widely accepted and straightforward means
769 of measuring FMR in fishes. Comparative biochemists and physiologists are well suited to build
770 upon the existing framework of approaches, which we have briefly reviewed, to develop robust
771 strategies to address this important methodological gap. We anticipate that to do so will require
772 novel technologies and the integration of multiple metabolic and physiological proxies. This will
773 certainly increase the complexity of experimental validation and execution, but these new

techniques have the potential to greatly enhance research capacity across multiple disciplines, from metabolic biochemistry to behavioural physiology. Accurate estimates of FMR will promote a better understanding of the intricate relationships between energy and intra- and interspecific variation in fishes, and how the environment influences metabolic demands, energy allocation and life-history strategies.

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Legends

Figure 1. ***Illustration of the energy budget in a fish***. Energy intake as ***Food*** requires energetic costs as specific dynamic action (***SDA***) and some energy will be lost from the animal as ***Egestion*** (indigestible material and carbon not assimilated) or as nitrogenous ***Excretion***. The remaining energy will be used to meet the costs of life (***Basal costs*** such as maintenance of ion gradients, protein and DNA repair etc.) with the energy in ***Excess*** of basal requirements being allocated to ***Growth/storage***, ***Locomotion and physical work*** or ***Reproduction*** which can be either output as gametes or retained as gondal investment (which can also be viewed as ***Growth/storage***). The $Energy_{in}$, $Energy_{out}$ and $Energy_{retained}$ nomenclature are described in the text.

Figure 2. ***Graphical illustration of proposed FMR strategy for fishes***. ***A.*** linkage between labelled substrates in surgically implanted osmotic pump and putative collection strategies including ***1.*** 'reverse' osmotic pumps for sampling steady-state tracer enrichment (specific activity) and ***2.*** An alkaline 'trap' based measurement of integrated substrate oxidation, measured as labelled CO_2 in the reservoir trap. It would be expected that the rate of labelled CO_2 appearance in the trap, following an initial 'loading phase' of the whole body metabolite pool, should reflect the integrated rate of metabolic substrate oxidation. ***B.*** Illustration of laboratory validation strategy for plasma enrichment measurements. Following a time-lag for tracer distribution to all tissue pools there should be a linear rate of appearance due to influx of the labelled substrate. As the labelled

substrate(s) are oxidized the enrichment (specific activity or *SA*) in the plasma will reach a plateau over time. At this plateau the rate of appearance = the rate of disappearance by oxidation. The exponential curve that describes this time-dependent progression towards a plateau in plasma enrichment will determine the rate constant (***k***) for tracer clearance. Following along the Time axis it is illustrated how the plateau level (e.g. ~ steady state enrichment) will respond to changes in ***k*** where clearance rate i) increases, ii) decreases or iii) is unchanged.

* The body total CO_2 pool (tCO_2) will be an equilibrium between ionized (e.g. HCO_3^-) and non-ionized (predominantly gaseous CO_2) forms of carbon dioxide. For simplicity we do not discuss this in detail; however, since the alkaline trap strategy will only collect a fraction of the gaseous CO_2 it is assumed that the metabolically derived carbon dioxide pools have been fully equilibrated due to spontaneous and enzymatic reactions.

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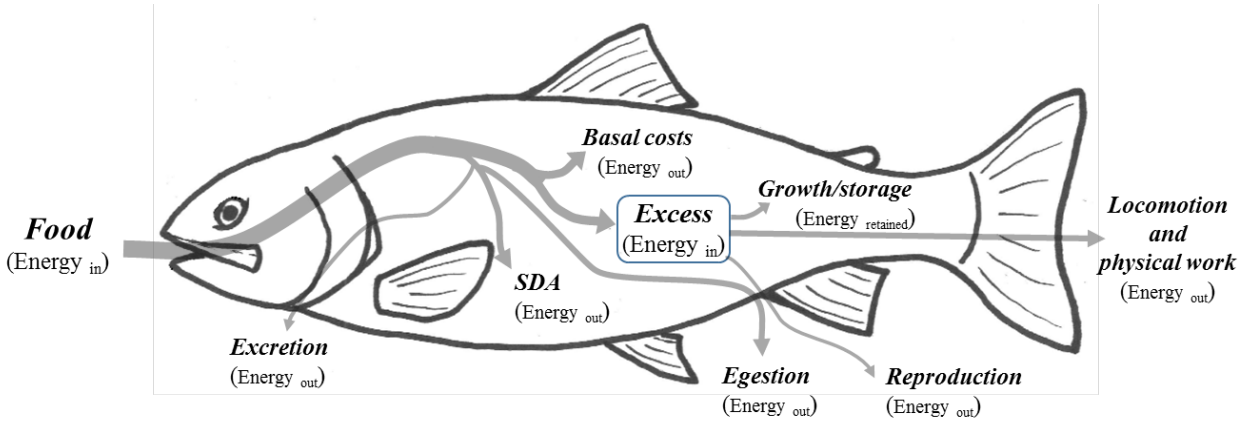

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1364 **Figure 1.** ***Illustration of the energy budget in a fish***.

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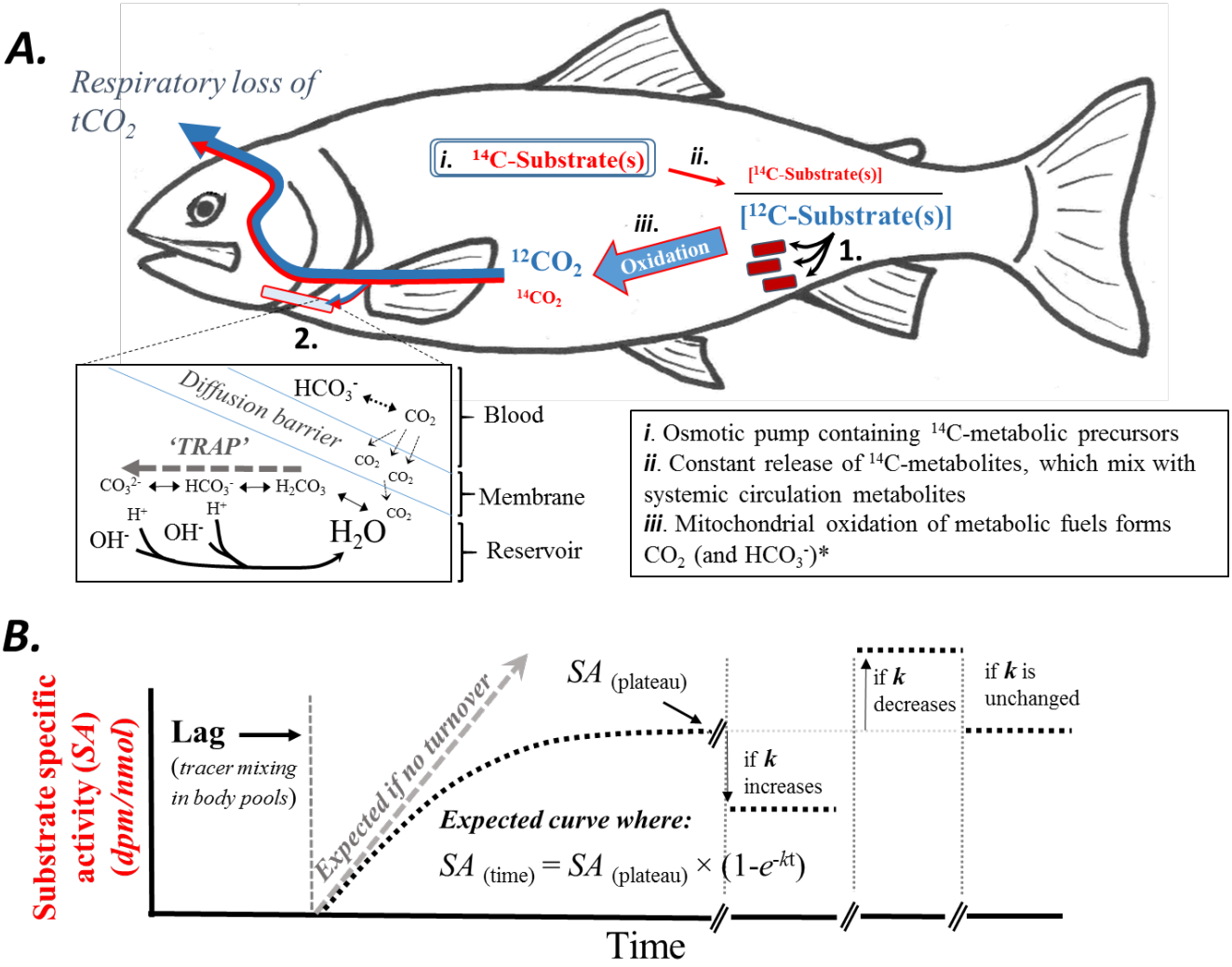

Figure 2. ***Graphical illustration of proposed laboratory validation of FMR strategy for fishes***.