Solving the conundrum of intra-specific variation in metabolic rate: A multidisciplinary conceptual and methodological toolkit

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PROBLEMS & PARADIGMS

Solving the conundrum of intra-specific variation in metabolic rate: A multidisciplinary conceptual and methodological toolkit

New technical developments are opening the door to an understanding of why metabolic rate varies among individual animals of a species

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Abstract

Researchers from diverse disciplines, including organismal and cellular physiology, sports science, human nutrition, evolution and ecology, have sought to understand the causes and consequences of the surprising variation in metabolic rate found among and within individual animals of the same species. Research in this area has been hampered by differences in approach, terminology and methodology, and the context in which measurements are made. Recent advances provide important opportunities to identify and address the key questions in the field. By bringing together researchers from different areas of biology and biomedicine, we describe and evaluate these developments and the insights they could yield, highlighting the need for more standardisation across disciplines. We conclude with a list of important questions that can now be addressed by developing a common conceptual and methodological toolkit for studies on metabolic variation in animals.

KEYWORDS
ATP, energetics, energy, mitochondria, nutrition, physiology, respirometry

INTRODUCTION

Metabolic rate provides a quantification of the energetic cost of living by telling us the rate at which an organism converts fuel into energy or heat. Its measurement is of great interest to biologists working at all levels, from molecules to communities. Metabolic rate is central to the energy flux within cells. Limitations on the rate at which individuals can generate adenosine triphosphate (ATP) will determine key life history traits, such as growth, self-maintenance, and reproduction and the trade-offs amongst them. The development of metabolic theories within ecology have advanced our understanding of broad scale variation in life histories across taxa.[1–3] There has also been some success in linking metabolic rate in animals to whole-organism performance[4–6] and even to colony size in colonial species.[7] Nonetheless, our understanding of how and why metabolic rate varies is incomplete, particularly within species (reviewed in refs.[8, 9]) where substantial (often ~2-fold) spatial and temporal variation can be found among individuals, sexes and life history stages.

While some of this variation is probably due to measurement artefacts or lack of standardisation (e.g., for the effect of body size on metabolic rate), it is still unclear how much of the remaining variation is adaptive or non-adaptive, or whether it is genetically or environmentally determined. Such information is essential if we are to predict how animals will cope with rapid environmental change and to understand the causes of metabolic disease. Very large differences in heritability estimates among studies have been reported[10] and we lack information on the role of developmental processes and short-term reversible changes in physiology. The proximate mechanisms determining variation in whole-body metabolic rate in animals remain disputed among researchers in the field. Much of this debate focusses on metabolic scaling,[11] but there are also issues surrounding mass-independent variation driven by changes in, for example, mitochondrial function,[12] size of cells, organs or tissues with different energy requirements.[13,14]

The evolutionary mechanisms driving this persistent variation in metabolic rate also remain largely unexplored. It might reflect fluctuating and/or context-dependent selection, with fitness peaks changing in space and time.[15,16] However, constraints on plasticity and indirect selection through genetically-correlated traits[10] are also probably at play. In particular, metabolic activity or efficiency can carry costs, such as oxidative damage or protein glycation.[12] Understanding how energetic efficiency is traded off against those costs is necessary to better describe evolutionary constraints on metabolic rate. Since whole-body metabolic rate is necessarily the sum of the respiration of separate tissues, we also need to identify the targets of selection that have the biggest impact on energy management.

The aim of this paper is to examine these gaps in our knowledge in animal metabolic rates and how they can be tackled through an interdisciplinary approach to methods and concepts. We highlight how recent technological and conceptual advances have opened new approaches, and list key questions that can now be addressed. We think these insights will be useful to a broad range of researchers from

diverse backgrounds and study systems, including human health and disease.

ALTERNATIVE MEASURES OF METABOLIC RATE

Throughout the history of scientific study, researchers have faced technological limitations that constrain the questions they can address. One of the oldest questions relating to energy expenditure and metabolism relates to the identification of the causes underlying variation in metabolic rates, both among and within species.[17] It has long been known that metabolic rate varies at the individual level with development, age, season, time of day, activity level, environment and sex, and can also differ within individuals at the tissue or cellular level.[9] Recent technological advances have expanded the scope of this research: for example, we can now measure mitochondrial function in wild animals sampled at high elevation field sites,[18] or using frozen tissue,[19] and even via miniscule tissue-punches from specific regions of the brain.[20] Given the rate at which technology is opening new avenues in this area, we should now identify the key questions we need to answer, rather than only focus on those we were able to address in the past.

However, despite the array of emerging technologies for estimating whole-animal metabolic rates, there is confusion surrounding the terminology for the various types of metabolic rate that are commonly studied (Table 1). Whole-animal metabolic rate, usually quantified indirectly through measurement of oxygen uptake rates during aerobic respiration, is not a single, fixed trait.[10,21] For example, while some estimates of whole-animal metabolic rate reflect solely maintenance costs, others include costs associated with physical activity, thermoregulation and other physiological functions including growth or digestion (Table 1). Furthermore, some types of metabolic rate are measured during relatively short time periods when the animal is in a constant state, while others represent longer timeframes that include spontaneous costs associated with changes in activity, internal processes or external conditions (Table 1). The most appropriate measurement depends on the question of interest. For example, routine metabolic rate – which typically includes maintenance costs, spontaneous activity and the short-term costs of an autonomic stress response – is generally calculated as an average metabolic rate throughout the measurement period.[22,23] While routine metabolic rate can be useful when measuring acute metabolic responses to a stressor (e.g., a temperature change or response to a predator), it is often rather loosely defined and can sometimes be used as a substitute for field metabolic rate (FMR).[22] This conflation of terminology is problematic, since routine metabolic rate is usually measured in food-deprived animals while FMR also includes additional energy expenditure such as food digestion. Further confusing the issue is that researchers studying different disciplines or taxonomic groups often use different terminology to refer to similar estimates of metabolic rate (Table 1), or use inconsistent abbreviation conventions when referring to specific estimates of metabolic rate or capacity (e.g., aerobic scope – the difference between maximum and minimum aerobic metabolic rates – being variously referred to as AS, absolute aerobic scope [AAS], or factorial aerobic scope [FAS]; Table 1).

Which is the most appropriate measure of whole-animal metabolism?

Due to the relative ease of standardising measurement conditions, animal physiologists have tended to focus on measuring the minimal (floor) and maximal (ceiling) metabolic rates, whether at the whole animal or cellular (mitochondrial) level. These measurements can reveal the physiological capacity and constraints acting on the animal. Analyses of minimal metabolism have led to insights into, for instance, the spatial distribution of ectotherms in relation to their thermal and hypoxia tolerance[24] and relationships between body mass and metabolic rate in mammals and birds.[25] However, the terminology needs to be clear: 'minimal metabolic rate' is perhaps a misnomer, since it usually refers to the animal in a resting but not torpid or hibernating state (when metabolism can drop even lower). Furthermore, the assumptions underlying the estimations of minimal metabolism may not always be met: the subjects may not be in a post-absorptive state, species with indeterminate growth are typically always growing, and, in endotherms, individuals are frequently found in conditions outside of the thermoneutral zone in the wild (and they may come in/out of heterothermy). Moreover, whether minimum metabolic rate should be measured during rest or the active phase of the day make the comparisons difficult because of circadian variations in energy expenditure.[26] These challenges make it hard to standardise and justify the conditions under which the measurements are made. Measures and derivatives of maximum metabolic rate pose similar problems: the peak rate of oxygen consumption can depend on the context in which metabolic rate is maximised (Table 1). It can be expressed as a multiple of resting or basal metabolic rate (BMR) to facilitate comparisons among groups of animals differing in body size; the resulting index can indicate the relative contribution of activity energy expenditure to total metabolic rate. However, there are a number of ways of calculating this index, depending on the period over which the energy expenditure is measured (e.g., FAS is usually measured as instantaneous rates whereas physical activity level (PAL) measures daily energy expenditure [DEE] – see Table 1 for definitions). Moreover, as with minimal metabolism, animals are rarely operating at their maximal rate of metabolism. Maximum FAS or PAL can reach values as high as 10 (e.g., in migrating birds) but only for brief periods since this rate is dependent on stored fuel and can carry other long-term costs.[27] The limit for maximal sustained FAS or PAL is set by an alimentary energy supply limit.[28–30] A typical value, that can be sustained for months while maintaining energy balance, is around 2.5 for birds and mammals, including humans.[31] A heightened energy intake allows a greater FAS or PAL over shorter time intervals of several weeks, as observed in nestling feeding birds and in professional endurance athletes during the 3-week Tour de France cycle race,[32,33] but the maximum declines curvilinearly with event duration.[29]

TABLE 1 There are many types of metabolic rates measured by scientists, with the list below representing those frequently appearing in the literature

Abbreviation	Type of metabolic rate	Definition
(A)AS	Absolute aerobic scope	Absolute difference between basal (or standard) and maximum metabolic rate; often referred to as just aerobic scope (AS)
BMR	Basal metabolic rate	Minimum resting metabolic rate required by an endotherm to survive at thermoneutrality, measured in an animal that is postabsorptive, non-growing, non-reproductive and resting (but not sleeping or in hibernation or torpor)
DEE	Daily energy expenditure	The total energy used by an individual during a full circadian cycle
DIT	Diet-induced thermogenesis	The energy dissipated as heat after food intake (often referred to as SDA – see below – in ectotherms)
FAS	Factorial aerobic scope	Maximum metabolic rate divided by basal (or standard) metabolic rate
FMR	Fasting metabolic rate	Metabolic rate measured in a food-deprived and post-absorptive individual
FMR	Field metabolic rate	Metabolic rate measured in a free-ranging individual
MMR	Maximum metabolic rate	Maximum aerobic metabolic rate, usually induced by sustained physical activity but sometimes also measured post-feeding in ectotherms. In some taxa and disciplines this is referred to as VO_2max (when measured as the maximum rate of oxygen uptake during physical activity)
M_{sum}	Summit metabolism	Maximum resting metabolic rate during acute cold exposure in endotherms
PAL	Physical activity level	DEE or field metabolic rate divided by BMR
PMR	Peak metabolic rate	Maximum metabolic rate that is induced by either exercise (MMR) or acute cold (M_{sum})
REE	Resting energy expenditure	Metabolic rate measured in a resting animal (synonymous with resting metabolic rate)
RMR	Resting metabolic rate	Metabolic rate measured in a resting animal (synonymous with REE). Is sometimes used to imprecisely refer to any of routine metabolic rate, basal metabolic rate, or standard metabolic rate
RMR	Routine metabolic rate	Average metabolic rate during spontaneous behaviour or a particular activity, under controlled conditions
SDA	Specific dynamic action	The energy dissipated as heat after food intake (see diet-induced thermogenesis)
SMR	Sleeping metabolic rate	The lowest stable metabolic rate over ~3 h measured in a sleeping individual
SMR	Standard metabolic rate	Minimum metabolic rate required to survive at a particular temperature, for an animal that is post-absorptive, non-growing, non-reproductive and resting (but not in torpor or diapause). Applied to ectotherms, and to endotherms outside of thermoneutrality
SusMR	Sustained metabolic rate	Metabolic rate over an extended period, with energy balance maintained via food intake

As definitions of these metabolic rates can vary among fields of study, we provide brief, simple definitions along with common abbreviations (note that the same abbreviation is sometimes used to mean different measures).

For some research questions, especially those with an ecological setting, a more relevant measurement is the average metabolic rate (e.g., FMR or DEE) since this represents overall energy (and hence food) requirements for a specified period. However, this can be more challenging to measure in a standardised manner,[34] although a historical record of FMR can now be estimated retrospectively even in deep sea fishes.[35] This approach has revealed differences in thermal performance curves between two ecotypes of cod *Gadus morhua*, consistent with temperature differences in the habitat in which they live.[36] Meanwhile some of the most detailed measures of DEE come from humans. The DEE of modern humans is similar to that of other mammals when accounting for body size differences.[37] Humans usually maintain a neutral energy balance during daily life, possibly controlled via homeostatic regulation of body mass.[38] However, environmental perturbations can change DEE and/or energy intake, potentially altering this balance. For example, at low environmental temperatures, cold-induced brown adipose tissue activation may contribute to a small, yet highly variable thermogenesis,[39] but humans have been shown to increase their energy intake to a greater extent than needed to offset the increase in DEE.[40] Circadian misalignment (i.e., chronically eating and sleeping at unusual times in the 24 h cycle) causes a higher sleeping metabolic rate and lower DEE, while energy intake is increased, potentially leading to weight gain.[41] These examples demonstrate the value of using FMR and/or DEE over simply floor/ceiling rates of metabolism in energy budget models.

Correlations between measures of metabolic rates

The ambiguity and multiplicity of metabolic rate measures is not the only issue we have to take into account when considering variation in metabolic rate. Another interesting question is to what extent

these different measures of metabolic rate are under independent control. According to the aerobic capacity model, there is a mechanistic link between minimum (BMR or standard metabolic rate [SMR]; see Table 1 for definitions) and aerobic maximum metabolic rates (MMR or M_{sum}; Table 1).[42] This relationship has been demonstrated for over 40 years using both artificial selection experiments in controlled conditions[43,44] and measurements on wild-caught animals taken into captivity.[45,46] A recent meta-analysis of phenotypic correlations between minimum and maximum metabolic rates across a large number of species from all five classes of vertebrates has found an overall significant positive relationship.[47] However, the correlation between BMR and M_{sum} was found to be non-significant in mammals, possibly due to the role of uncoupling mechanisms in thermogenesis.[47] This suggests an effect of phylogeny and evolution through differential selective pressures on the strength of relationships between metabolic traits. We should also take into account the level at which the correlation is measured,[45,47,48] its repeatability[49] and the circumstances of the measurements (e.g., whether made in the laboratory or in the wild[48,50]). For example, intraspecific variation in BMR and M_{sum} in birds suggests that these traits are under independent physiological control and may lack a functional link.[51] Since metabolism is a labile trait, which may change over time and with environmental conditions, our analyses should in the first instance standardise the measurement conditions before trying to assess the level and magnitude of correlation between different metabolic measures.

WHOLE-ORGANISM MEASUREMENTS OF METABOLIC RATE TELL US LITTLE ABOUT THE UNDERLYING PROCESSES

When seeking to understand the underlying processes linking metabolic rate to health and performance, important questions remain regarding how organism-level measures relate to the mitochondrial or tissue-organ measures, and about measures of O_2 consumption versus energy flux. There are at least four important caveats to carefully consider here.

First, measures of whole-body O_2 consumption are not the mere sum of O_2 consumed by each mitochondrion within the body. Non-mitochondrial O_2 consumption can be close to 10% of the total in many cells, due to the activity of various oxidases, desaturase and detoxification enzymes.[52]

Second, the O_2 used at the mitochondrial level by the electron transport chain is not perfectly coupled to oxidative phosphorylation (i.e., the production of ATP), since energy is partially dissipated as heat by proton leakage.[53] The efficiency with which mitochondria produce ATP (vs. heat) for a given amount of O_2 can vary among tissues and individuals and with the environmental context (e.g., changing in response to food intake), making it difficult to infer functional consequences from whole-body measurements of metabolic rate.[12,54] Moreover, whole-body O_2 consumption does not include the contribution of anaerobic metabolism to ATP production.[55]

Third, tissues and organs show considerable variation in mass-specific metabolic rates. For example, in humans, 70%–80% of resting energy expenditure is due to highly active organs that comprise only around 5% of body weight (e.g., liver, kidneys, heart and brain[56]). Differences in aerobic metabolic rate between tissues or organs can be due to differences in mitochondrial content[57] or reliance on anaerobic metabolism, and are dynamic, depending on the biological context (e.g., whether the body is at rest or engaged in aerobic or resistance exercise).

Fourth, although some tissues/organs make only a minor contribution to whole-body metabolic rate, the functioning of their mitochondria may have significant consequences for health, performance and fitness. For example, the mitochondria in innate immunity cells, which on the whole contribute little to metabolic rate, are nevertheless important in immune responses due to their production of reactive oxygen species (ROS) that both act as signalling molecules and attack pathogens.[58,59] Similarly, variation in mitochondrial metabolism within spermatozoa has major consequences for male fertility.[60]

Overall, making extrapolations from mitochondria to tissues/organs, whole-body metabolic rate and fitness is complex. Ultimately, an individual's survival and reproduction is likely to be determined by its total energy requirements in relation to fuel availability, which will vary across biological and ecological contexts. Hence, important new insights are likely to come from integrative research on whole-body metabolic rates and energy flow through different tissues/organs, the latter being assessed directly or indirectly via O_2 consumption at the mitochondrial, cellular or tissue-organ levels.

WE NEED TO KNOW HOW TO LINK WHOLE-ANIMAL METABOLIC RATE TO BIOLOGICAL FUNCTIONS

There is strong evidence that measures of metabolism (e.g., BMR, RMR, MMR, DEE; see Table 1) are responsive to selection, and can correlate with differences in survival and reproductive performance in natural populations (but see [6,61–63]). However, correlations between metabolic rate and Darwinian fitness can disguise multiple independent factors that contribute to both metabolic rate and fitness. For instance, they do not reveal whether it is the cumulative energy demand, and/or the efficiency of ATP production, that are under selection. In addition, the organism may change its behaviour or physiology to optimise the amount of energy available to enhance fitness outcomes in a given set of circumstances.

Compensatory responses

There is increasing evidence that rates of energy metabolism can change within individuals in response to short-term shifts in environmental pressures. For example, across a wide diversity of both endothermic and ectothermic taxa, mass-independent BMR and SMR

are known to change as a function of food availability.[64] Low food availability can also trigger torpor use in heterothermic endotherms, reducing the energetic costs of thermoregulation by regulating body temperature below normothermic levels.[65] These within-individual shifts in metabolic rate are a result of mitochondrial plasticity, whereby the mitochondrial activity and number changes as a function of environmental conditions.[4] For instance, at the onset of physical exercise, there is a redistribution of blood flow from inactive tissues (e.g., digestive organs) to active ones (e.g., skeletal muscle[66]). This can alter the relative contributions of different tissues to whole-animal metabolic rate as whilst mitochondrial activity or efficiency are sometimes correlated between tissues within the same individual,[67] this is not always the case.[68] It is also important to recognise that mitochondria in different tissues of the same individual may respond differently to environmental changes, at least based on the relatively small number of studies that have investigated this issue.[69,70]

While previously assumed to be neutral, there is increasing evidence that allelic variation in the mitochondrial and nuclear genomes that influences mitochondrial function can give rise to bioenergetic adaptations at the population- and species-level, and even at higher taxonomic levels.[71] For example, complex I subunits (ND4, ND1, ND3) involved in mitochondrial oxidative phosphorylation are under both positive and purifying selection in Atlantic salmon *Salmo salar*, with selection for increased aerobic capacity in lower-temperature waters.[72] Furthermore, nearly a quarter of all mitochondrial-encoded genes were found to be subject to positive selection in bats, and a greater proportion of the nuclear encoded genes that are associated with oxidative phosphorylation were under positive selection than were the non-respiratory nuclear genes,[73] highlighting the importance of mitochondrial and mitonuclear adaptations in the evolution of species with an energetically demanding lifestyle.

From respiration to power generation

A major goal of whole-organism respiration measurements is to estimate how the energy provided by nutrients to different tissues (heart, muscle, etc.) is used to generate work and power (chemical or mechanical). This transduction from energy to power depends on mitochondrial phenotype and efficiency, which (as mentioned above) can vary according to species, tissues and environmental conditions. However, it is also affected by the food substrate that is used by the mitochondria, which varies across the animal kingdom. Most studies of mitochondrial bioenergetics use common metabolites provided by carbohydrates (pyruvate, malate and succinate), proteins (glutamate) or lipids (fatty acids), but underestimate the importance of alternative substrates that can also be important (e.g., proline in some invertebrates[74,75]). These different classes of substrate generate different amounts of ATP per unit of oxygen consumed (the ATP/O ratio, sometimes referred to as P/O), ranging from approximately 2.5 ATP/O for glucose to 3.5 for palmitate. ATP/O, a measure of mitochondrial efficiency, can also depend on the intensity of mitochondrial respiration and the quality of mitochondria, which in turn will hinge on factors such as mitochondrial morphology, membrane composition and organisation, and the content and state of different enzymes.[12,76,77] The results of these differences will also translate into variation in rates of ROS production or proton leakage from the inner membrane, both of which lead to variation in the efficiency of oxidative phosphorylation.[12] High rates of ROS generation in the absence of sufficient antioxidant and repair capacity lead to oxidative stress, which can disrupt mitochondrial components and further magnify dysfunction and loss of efficiency.[54]

Given these complexities, simply measuring mitochondrial content and mitochondrial respiration rates with standard substrates at maximal capacity is insufficient to allow a proper assessment of the ability to perform work per unit of time, or the rate of ATP synthesis. Information on mitochondrial substrate utilisation and efficiency of oxidative phosphorylation are also required, which will partly depend on the proportion of mitochondrial capacity that is being used in situ. Furthermore, depending on physiological and environmental conditions, the limits of aerobic capacity may only be reached at the expense of oxidative damage accumulation. Therefore, the true cost of living needs to be measured in terms not only of energy expenditure, but also of the resulting oxidative stress that is incurred.

PROGRESS WILL DEPEND ON APPROPRIATE ADOPTION OF NEW METHODS

Techniques for measuring metabolic rate need to be continually developed to enable us to record the most robust measurements and to answer new questions. To this end, existing technologies can be adjusted to measure additional metabolic parameters that shed new light on organisms' capacity to cope with their environment. For example, a modified static respirometry chamber, with a built-in device to induce swimming, was used to determine a fish's hypoxic performance curve and estimate the proportion of the AAS an individual can reach depending on the ambient oxygen availability.[78] Alternatively, existing techniques can be combined to address novel questions: the combination of cardiac loggers and accelerometers revealed that the sudden dives by adult narwhals *Monodon monoceros* caused by anthropogenic noise had twice the metabolic cost of routine dives of equivalent duration and depth.[79] New technology is emerging to facilitate measurement of metabolic rates in novel contexts (summarised in Table 2), particularly in more ecologically relevant conditions or across the animalt's ontogeny, and so potentially enhancing our understanding of the factors that affect Darwinian fitness. For instance, it has long been considered almost impossible to measure the FMR of wild fish.[34] Recent advances have shown, however, that this can be estimated from the isotopic composition of carbon in their otoliths ($\delta^{13}C_{oto}$)[35] or from high resolution acoustic telemetry.[80] Moreover, tissue biopsies are being developed to estimate mitochondrial respiration, with the potential to be used in longitudinal studies tracking animal metabolic performance, for example, in response to environmental challenges.[67,81,82] While some of these new techniques need further validation and calibration, they open promising new avenues for investigating metabolic rate.

TABLE 2 New technical developments at the cellular and whole-animal levels that are opening areas for research in the field of animal metabolic physiology, with examples of the taxa in which they have been used to date

Broad categories	Specific techniques	Description	Taxa	Lab, field	Whole-animal, cellular	References
Mitochondrial aerobic metabolism: Quantifies the efficiency of conversion of food resources into energy (reviewed in ref.[54])	Mitochondrial oxygen consumption	Aerobic respiration in mitochondria associated with ATP production (which can also be measured)	Birds, mammals, fish, insects	Both	Cellular	[103]
	31-Phosphorus magnetic resonance spectroscopy	Measures mitochondrial oxidative phosphorylation capacity in muscle tissue	Mammals	Lab	Cellular	[104,105]
	Electrochemical multi-sensors	Multi-sensor device measuring oxygen and hydrogen peroxide in mitochondria	Mammals	Lab	Cellular	[106]
	Tissue biopsy	Non-lethal tissue sampling to assess mitochondrial metabolism	Fish	Both	Cellular	[82]
Respirometry: Measures the rate gas exchange as a proxy for aerobic metabolic rate (reviewed in ref.[107])	Intermittent-flow respirometry	Rate of O_2 uptake measured by intermittently flushing in a gas-impermeable respirometry chamber connected to an O_2 sensor	Aquatic ectotherms	Both	Whole-animal	[108]
	Open-flow respirometry	Gas concentrations (e.g., carbon dioxide, water vapor) measured in air continuously flowing before and after a sealed chamber	Birds, mammals, insects	Lab	Whole-animal	[109]
	Colorimetric microrespirometer	Digital environmental pressure sensor inside respirometry chamber controlled by microcontroller	Insects	Lab	Whole-animal	[110]
Biologgers: Data storage tags that are attached directly to animals to collect information about the animal and/or the animal's environment (reviewed in ref.[111])	Temperature logger	Internal body temperature as a proxy for torpor and associated metabolic rate	Mammals, amphibians	Field	Whole-animal	[112,113]
	Cardiac logger	Heart rate frequency recorded as proxy for metabolic rate	Mammals, birds, fish	Both	Whole-animal	[114]
	Accelerometry	Bio-logging sensors measuring high-resolution, tri-axial acceleration data for the study of animal movement	Mammals, birds, fish	Both	Whole-animal	[90]
	Aquatic telemetry	Extrapolation from movements and swimming measurements	Mammals, fish	Field	Whole-animal	[80,95]
Field metabolic rate: Holistic measurement of metabolic rate in a particular ecological context (reviewed in refs.[35, 88])	Doubly labelled water method	Turnover of hydrogen and oxygen in body water as a proxy for carbon dioxide production	Terrestrial species	Both	Whole-animal	[115]
	Otolith microchemistry	Isotopic composition of carbon in fish otoliths as a proxy for field metabolic rate	Fish	Field	Whole-animal	[35]

However, all methods force us to make assumptions about how representative or relevant measurements will be of real world situations. For example, measurements of mitochondrial function using in vitro assays are often made under non-limiting conditions (such as maximal uncoupling respiration) and yet are used to infer changes in mitochondrial function when the body is at rest.[83] Similarly, measurements of the oxygen consumption of confined animals are usually taken at a single constant temperature, despite the fact that organisms will face variable temperatures under natural conditions, with likely strong effects on energy requirements.[84,85] Measuring the oxygen consumption of wild animals is usually done after they have been subjected to stress, either due to capture, the attachment of measurement equipment or from injections.[86] Similarly, when taking measurements in humans, we need to consider how well their behaviour in a laboratory setting reflects that in 'real life'.

The doubly labelled water (DLW) method is a relatively effective method for measuring total energy expenditure in free living individuals, at least in terrestrial species, and is often considered to be the 'gold standard'. However, it does assume that the subject behaves normally for the 24–48 h after having been injected with the sample of DLW (an assumption that is rarely tested and may not be true[87]) and has limitations such as high costs, low temporal resolution and a time-consuming procedure to analyse the sample.[88] Individuals also typically have to be recaptured for final blood sampling, although single sampling methods (e.g., using faeces rather than blood for the final sample) can be used to reduce disturbance.[89] Alternative approaches are to measure heart rate[88] or use accelerometry,[90] both of which can provide estimates of metabolic rate over much finer time periods whilst being less direct measures of metabolism where conversion equations for a particular species of interest are often not available.[91]

Overall, researchers need to be aware of the limitations of metabolic measurements, relating to measurement type, environmental/laboratory scenarios and statistical adjustments, and state these explicitly in their reports. They need to be aware of the risk of measurement error (especially evident when measuring minimal metabolism, when 'impossible' records stand out, but more hidden in other measurements). It is also important to consider whether and how to correct for differences in body composition, so as to avoid confounding factors due to inconsistencies in tissue mass.[56] For example, calorie restriction appeared to cause cellular-level metabolic suppression in mice if analyses of resting metabolic rate took account of changes in fat and lean mass.[92] However, more refined models based on changes in individual organ sizes found that the reduction in resting metabolic rate was explained fully by changes in organ size, so that there was no evidence for metabolic suppression at the level of the cells.[92] One way to better recognise the limitations inherent in our measurements would be to set up standardised 'reference states' of animals and experimental conditions under which metabolic rates are measured. This would provide baseline data against which to interpret data from new studies. There have been some attempts to standardise measurement settings of energy expenditure in humans, with attention to details such as the time of day and duration of measurements, lighting levels, conditions under which the subjects spent the previous night, and their activity and posture at the time of measurement,[93] but this approach needs to be expanded further to cover more species and methods. Such standardisation could lead to a more coherent interpretation of metabolic rate measurements and a better understanding of real-world effects on energy expenditure.

Metabolic rate is flexible in response to the variable environments that most organisms inhabit over both daily and seasonal timeframes. The need to incorporate realistic conditions into measurements of metabolic rate is therefore an important consideration. Indeed, given rates of global environmental change, accounting for varying conditions within our measurements may be particularly timely.[76,94] While technical limitations have hitherto precluded our ability to obtain whole-animal and subcellular measurements under non-standardised (i.e., non-laboratory) conditions, it is now becoming possible to take measurements under ecologically relevant scenarios, including those from swimming fish,[95] hovering hummingbirds,[96] diving mammals[79] and hibernating wild lemurs.[97] Within a subcellular context, the largest limitations to measurements of energy metabolism are the invasiveness of procedures (but see ref.[67]), and the fact that measurements are taken either when mitochondria are functioning at their highest rate, or when they are not producing ATP at all. Whilst measurements of subcellular metabolism may provide important information on the mitochondrial capacity of ATP production per amount of oxygen consumed (thereby revealing the effectiveness of cellular respiration[12,54]) values may often be unrepresentative of natural functioning.

CONCLUSIONS: WHERE SHOULD THE FOCUS OF FUTURE RESEARCH LIE?

Technical advances are making it increasingly possible to obtain measures of metabolic rates that are high-throughput, taken under natural field conditions, and across scales of biological organisation (Table 2). However, the greatest gains in knowledge will be achieved if the field is also open to incorporating ideas, expertise and methods from other disciplines, such as genomics and quantitative genetics. For instance, selection studies have yet to show whether metabolic rate itself is under selection, or evolves through, for example, a genetic correlation with (an)other trait(s) under selection, possibly body size[98] and/or growth.[99,100] With increased accessibility of genetic analyses and tools (online databases, gene ontologies, etc.), efforts should be made to untangle these links between metabolic rate and other key traits such as body size/growth/body composition. Systems biology or bioenergetics modelling may help, for instance by revealing where relationships among traits are constrained due to the laws of physics.

We suggest a list of unanswered (but answerable) questions in Table 3. Where possible, the following principles in approach should be adopted. Future work should include *longitudinal studies* of both whole-animal and tissue-level metabolic rates in both animals and humans, throughout all life stages.[101] Although challenging for immature individuals of many species, these investigations should consider the role of *sex differences* in metabolic rate variation and evolvability, espe-

TABLE 3 Questions relating to metabolic rate (MR) variation that can now be addressed, given recent technical and statistical developments, along with appropriate potential study systems and references to the application of each approach

Question	Method	Description	Ideal study system	References
1. How can we better standardise measurements?	All	Provide reference conditions for measurements that include previous nutritional state and activity of animal, full description of environmental conditions of measurements (time of day, temperature, light and noise levels…) and appropriate baseline against which other states are measured	Any	[93]
2. How reproducible and repeatable are measures of MR at whole-animal and cellular levels?	Respirometry (whole-animal and tissue/mitochondrial level)	Assess whether measurements from MR, mitochondrial function and associated traits are robustly reproducible, and repeatable in the same individual over time	Species that are studied by multiple laboratories with the same equipment and where results are openly shared (data transparency)	[82]
3. To what extent does cellular metabolism correlate with or determine whole-organism MR?	Combination of molecular, cellular and whole-animal techniques	Compare metabolic scaling at the cellular and whole-animal levels Determine contributions of respiration in different tissues to whole-animal MR Determine effects of manipulations of mitochondrial function on whole-animal MR	Species where biochemical methods are robust and well-established, and where whole-animal respirometry and omics can be performed	[116]
4. Do mitochondrial traits (e.g., efficiency of ATP production) constrain whole-animal performance?	Measures of mitochondrial function and whole-animal traits, for example, behaviour, digestion, growth, locomotion	Correlate measures of mitochondrial respiration, ATP production and ROS production with whole-animal performance, ideally using longitudinal measurements and/or after manipulation of mitochondrial function	Species in which traits can be measured repeatedly within individuals	[82]
5. Do MR measurements made in a laboratory setting have any relevance or correlation with those measured in free-living animals?	Whole-animal respirometry in lab matched with related measurements of MR in field	Establish the extent to which lab and field measures of MR are correlated (and for which taxa and under what conditions the correlation is strongest)	Species in which MR can be measured in both lab and field	[50]
6. Is the body size allometry of MR the same at cellular and whole-animal levels?	Respirometry (whole-animal and tissue/mitochondrial level)	Measurements across the life course in a cohort where growth rate has been manipulated to generate significant variation in size-at-age; analysis to focus on the link between cellular and whole-animal MR	Ectothermic species that can be maintained throughout life in the lab and in which growth rate and size at sexual maturity can be manipulated	[117]

(Continues)

TABLE 3 (Continued)

Question	Method	Description	Ideal study system	References
7. Why do these allometric relationships become shallower as animals get older?	Respirometry (whole-animal and tissue/mitochondrial level)	Measurements across the life course	Ectothermic species that can be maintained throughout life in the lab	[117]
8. To what extent is variation in MR sex-specific?	Separation of the two sexes in the experimental design	Determine the extent and the basis for selection on MR among sexes	Species where selective breeding based on MR in each sex is feasible, or analysis of correlational data separating by sex	[118]
9. To what extent is MR (reversibly) plastic, does this change over ontogeny, and does this plasticity trade off against other traits?	Whole-animal respirometry plus measurements of related traits	Test for extent to which MR traits change in response to environmental conditions (e.g., food availability, temperature), with same tests run at different ontogenetic stages. Test for trade-offs between metabolic plasticity and other traits, for example, immunocompetence, locomotor performance	Species which can be monitored over time (in either lab or field) in changing environments and in which measures of MR and other relevant traits can be taken across ontogeny	[94]
10. How can we disentangle genetic from plastic sources of variation in metabolic rates?	Breeding design (laboratory)	Estimate the additive genetic variance and heritability (e.g., parent-offspring regression) of MR	Species amenable to paired breeding designs such as via in vitro crosses, for example, zebrafish	[10]
11. Is the variation in MR present from birth, or does it arise from developmental and environmental influences?	Pedigree or genomic relatedness matrix (laboratory or field)	Measure MR in individuals of known parentage or extent of shared genome, so as to estimate heritability	Species where families/relatedness can be tracked (pedigree), for example, Soay sheep, red deer, fairy wren, or with small population size (SNP data for genomic relatedness matrix)	[119]
	Artificial selection experiment (laboratory)	Use selection lines for metabolic traits (genotype or phenotype, for example, mtDNA haplotypes)	Species amenable to long-term rearing in the laboratory (e.g., mice, *Drosophila*)	[120]
	Reciprocal transplant and common garden studies (in field or semi-natural conditions, for example, greenhouse, mesocosm)	Test for evidence of adaptive divergence in MR through measures of individual MR and fitness	Species with known variation in MR among populations that experience contrasting environmental conditions, for example, food availability, temperature, predation intensity	[121]
12. To what extent is selection on metabolic rates context-dependent?	Multivariate selection analysis (longitudinal, field)	Measure MRs (e.g., BMR, MMR), correlated traits (e.g., growth, longevity) and lifetime reproductive output. Estimate the strength, form (linear, nonlinear), and direction of multivariate selection (i.e., slope and curvature of fitness function with phenotypic trait) across multiple environments	Species in which measures of MR can be taken across ontogeny and with feasible measures of lifetime reproductive output	[16]

cially considering that the ATP-generating machinery, mitochondria, is maternally inherited. Studies should also include different *environmental circumstances*, reflecting the natural variation, and ideally include *experiments conducted in the wild as well as the laboratory*. Studies should include *all ontogenetic stages*, and use a *standardised method* to avoid confounding effects.[102]

The future for studies into the causes and consequences of variation in metabolic rate is bright: we need to seize the opportunities that are opening for us.

AUTHOR CONTRIBUTIONS

All authors were involved in discussing the ideas presented in this paper. All wrote sections of the manuscript, which was compiled and edited by Neil B. Metcalfe and Pat Monaghan. All authors contributed to final paper revisions.

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CONFLICT OF INTEREST STATEMENT

The authors do not have a conflict of interest.

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