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Title: Integrating Mitochondrial Aerobic Metabolism into Ecology and Evolution

Short title: Mitochondrial Metabolism in Ecology and Evolution

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46 uncoupling, reactive oxygen species, life-history trade-off

Abstract:

Biologists have long appreciated the critical role energy turnover plays in understanding variation in performance and fitness among individuals. Whole-organism metabolic studies have provided key insights into fundamental ecological and evolutionary processes. However, constraints operating at subcellular levels—such as those operating within the mitochondria—can also play important roles in optimizing metabolism over different energetic demands and time scales. Herein, we explore how mitochondrial aerobic metabolism influences different aspects of organismal performance, such as through changing ATP and reactive oxygen species production. We consider how such insights have advanced our understanding of the mechanisms underpinning key ecological and evolutionary processes, from variation in life-history traits to adaptation to changing thermal conditions, and we highlight key areas for future research.

Energy metabolism drives ecological processes

Aerobic respiration is a principal source of energy for most eukaryotes. Energy metabolism controls the amount of energy uptake from the environment and the relative allocation of this energy to life-history traits. Classical ecological approaches for quantifying energy flows have estimated metabolic rate by measuring whole-organism gas exchange [1–3], which has provided a powerful foundation for understanding variation in individual performance, population growth rate, and community structure [4]. However, respiration at the whole-organism level ultimately derives from energy flow at the subcellular level [5]. Aerobic respiration in the mitochondria is a central process by which organisms transfer chemical energy locked in substrates into the chemical bonds of adenosine triphosphate (ATP), the main form of chemical energy fueling cellular processes. The efficacy with which mitochondria execute this energy transfer can be a major determinant of organismal fitness [6–10].

Investigating variation in mitochondrial measurements provides an opportunity for integrating how mechanistic constraints and trade-offs at the cellular level may shape fundamental evolutionary and ecological patterns [5,11–15]. Such constraints and trade-offs can involve interrelationships between ATP, **reactive oxygen species (ROS; see Glossary)** production, and heat generated during cellular respiration—relationships that can be flexibly modulated in response to changing needs, but may also face physiological constraints (see below) [16,17]. Teasing apart these relationships has the potential to offer valuable insights into the nature of the physiological changes that enable organisms to adapt and to optimize function across a broad range of conditions.

Here, we suggest that characterizing energy flow via measurements of **mitochondrial aerobic metabolism** can provide new insights into differences in energy turnover and

physiological performance among and within individuals, across time, and in response to environmental change (Figure 1) [18,19]. Meeting energetic demands at the subcellular level while preventing the accumulation of potentially damaging byproducts under changing conditions requires careful orchestration of biochemical processes in the mitochondria [20]. The effectiveness of mediating such processes is driven by both genes and environments (see Online Supplementary Material Box S1), linking mitochondrial processes not only to individual fitness, but also to local adaptation (Figure 1) [21]. Our aim is to discuss how the study of mitochondria may yield new insights into evolutionary and ecological sciences. We will firstly summarize mitochondrial traits and their quantification, and then secondly discuss the implications of these processes for ecology and evolution.

Mitochondrial traits and their quantification

Mitochondria have a wide range of functions (e.g. immune signaling pathways, apoptosis, epigenetics [22–24]), but we focus predominantly on the central mitochondrial process of **oxidative phosphorylation (OXPHOS)** and on how *in vitro* mitochondrial measurements can be interpreted in relation to animal performance and fitness. OXPHOS is the specific component of mitochondrial aerobic respiration that harnesses potential energy from an electrochemical gradient to drive the phosphorylation of ADP into ATP, consuming oxygen in the process. Specifically, electrons harvested from food-derived substrates flow through the protein complexes of the **electron transport chain (ETC)** to fuel the pumping of protons from the mitochondrial matrix into the intermembrane space (Figure 2). This creates a disequilibrium in both charge and proton concentration across the inner membrane (a source of potential energy called the **protonmotive force**). Protons passing down this gradient through ATP synthase power the phosphorylation of ADP into ATP (Figure 2)

[25,26]. OXPHOS fluctuates in response to changes in substrate availability and composition, ATP demand, and environmental conditions. Flexibility in the activity of the ETC, the build-up of protonmotive force, and the movement of protons across the inner mitochondrial membrane sets the rate of ATP production and affects metabolic heat generation as well as mitochondrial ROS production (Figure 2).

Mitochondrial ROS are formed when electrons escape the ETC and react directly with oxygen, and some ROS are always generated during mitochondrial aerobic metabolism. The contribution of ROS to **oxidative stress** plays a prominent role in many ecological and evolutionary hypotheses, such as theories of aging [27–29] and of reproductive costs [30–32]. While ROS serve important signaling functions and are not inherently harmful [33], excess ROS have the potential to cause oxidative damage and lead to cellular dysfunction. Rate of mitochondrial ROS generation may increase due to altered functionality of the ETC, such as mild dysfunction from oxidative damage, mutation, replication error, or genetic incompatibilities between genes encoding subunits of OXPHOS (**mito-nuclear interactions**; Box S1) [34–36]); we can consider this structural variation as variation in mitochondrial "quality" (Figure 3). In contrast, mitochondrial ROS production also changes inherently with changing of protonmotive force (Figures 2, 3); specifically, more ROS are generated during high protonmotive force, such as may occur when demand for ATP is low (Figure 2B) [37]. This point is key because it highlights the misconception that increased metabolic rate will inherently increase rate of ROS production. Rather, ROS production increases with the build-up of protonmotive force, which can be countered either by a higher rate of ATP production (coupled with demand) [30,38,39] or a higher **proton leak**—protons flowing back to the mitochondrial matrix without passing through ATP synthase, dissipating energy as heat (Figure 2) [39].

Proton leak across the inner mitochondrial membrane can occur through two means: passive proton leak (movement of protons directly through the membrane, largely influenced by protonmotive force and membrane composition [40]) and inducible proton leak (regulated movement of protons through protein channels that span the inner mitochondrial membrane [41]). Either form of proton leak is essentially "**uncoupling**" proton movement from the phosphorylation of ADP into ATP. Proton leak decreases rate of ATP synthesis relative to mitochondrial respiration rate, but it is not necessarily a dysfunction; rather, proton leak can be beneficial for respiratory function by lowering protonmotive force and thereby reducing mitochondrial ROS production (Figure 2) [42]. Inducible proton leak can also be an important means to generate extra metabolic heat for thermoregulation in endotherms (e.g. UCP1 in the brown adipose tissue of small mammals [43]), in addition to heat already produced during ATP synthesis.

Because oxygen consumed during aerobic respiration can be uncoupled from ATP synthesis through proton leak (Figure 2), two individuals (or, more specifically, two mitochondria) with the same rate of oxygen consumption can have quite different rates of ATP synthesis. Indeed, the proportion of oxygen consumed that leads to ATP synthesis versus the proportion that leads to proton leak (e.g. as captured in measures of **mitochondrial efficiency**; Box 2, Figure 3) can vary considerably across species, individuals, and time (Box 3) [11,44]. Such variation can be due to strategic adjustment of mitochondrial processes to best respond to current conditions(e.g. increasing mitochondrial efficiency to enhance energy savings during prolonged fasting [45]; Figure 3). However, such variation may also be indicative of structural differences in, for example, the ETC—differences that could constrain ATP production capacity during times of high demand (Figure 3). Only limited

research to date has focused on the evolutionary implications of within-individual variation in mitochondrial aerobic respiration, across tissues and life stages.

Understanding the interconnections between mitochondrial oxygen consumption, ATP and ROS production, and proton leak is critical for understanding physiological trade-offs operating at the cellular level that may influence animal phenotype and performance (Box 1). The importance of these interactions has been recognized for decades, including their relevance to the evolution of body size [46], thermal biology [47], and more recently, even sexually selected displays [48]. Exploring these processes—particularly with recently developed techniques that allow for non-terminal and even repeated sampling (Box 2)—offers ecologists insights into the long-term costs and constraints of the metabolic processes that underlie life-history strategies. An important outstanding area of research with relevance to both ecological and evolutionary questions is how and why individuals differ in their mitochondrial phenotype—parameters such as the efficiency with which ATP is produced from mitochondrial aerobic metabolism, or density of mitochondria within cells (Figure 3; Box 1)—and the higher-order effects of such subcellular variation on broader aspects of organismal performance.

While a full overview of the practical and methodological considerations required to collect informative mitochondrial measures is beyond the scope of this review, we provide a brief overview of several of the most well-established methods by which mitochondrial performance can be quantified in Box 1 (though none are without limitations), and important considerations for experimental design in Box 2. In the following section, we seek to translate the broad applications of the study of mitochondrial aerobic metabolism to evolutionary and ecological processes.

Applications of mitochondrial aerobic metabolism to ecological and evolutionary research

Mitochondrial processes underlie whole-organism metabolic rate

An understanding of mitochondrial aerobic metabolism can enhance our understanding of variation in whole-organism traits. Whole-organism metabolic rates have been found to vary severalfold among species and individuals [49,50], and variation in metabolic rate can be a functional determinant of variation in fitness and fitness-related traits [51–53]. Field and laboratory studies have identified strong relationships between whole-organism metabolic rate and survival and reproductive success, but the strength and form of selection varies among species and ecological contexts [53]. Variation in mitochondrial aerobic metabolism is likely an important underlying source of the observed among-individual variation in whole-organism metabolic rate and is likely to influence the relationship between metabolic rate and fitness [44,54,55]. Almost all measurements of metabolic rate are made through the measurement of O_2 or CO_2 fluxes (e.g. the data for thousands of species summarized in [56]). Such measurements are important estimates of overall energy flow, but as described above, variation at the mitochondrial level (e.g. uncoupling) alters the proportion of that energy that ultimately results in ATP, heat, or ROS (Figures 2 and 3) [11].

Exploring variation in mitochondrial aerobic metabolism can therefore help better pinpoint the significance of variation (or lack thereof) in energy flow at the whole-organism level. For example, two animals with the same whole-organism metabolic rate can differ in energy available as ATP (e.g. if they differ in mitochondrial efficiency; Box 1); similarly, two animals that differ in whole-organism metabolic rate can have the same energy available as ATP, but different levels of heat or ROS generation (Figures 2 and 3). Differences in strategy or quality at the mitochondrial level thereby can alter the relationships between whole-

202 organism metabolic rate and physiological performance or life-history trait expression.
203 Measurements of mitochondrial aerobic metabolism (e.g. mitochondrial efficiency; Box 1, 2)
204 offer a means to further contextualize measurements of whole-organism energy flow by
205 providing information on whether that energy may be put toward ATP or instead toward
206 heat or ROS production—alternatives that have ramifications for the hypotheses we
207 formulate for how metabolic rate relates to fitness. Combining measurements of fitness with
208 measurements of mitochondrial aerobic metabolism (e.g. from repeated, non-terminal
209 sampling; Box 2) and whole-organism metabolic rate will provide an opportunity to grow our
210 understanding of the functional bases of variation.
211
212 **Can variation in mitochondrial aerobic metabolism explain life-history trade-offs?**
213 The study of metabolism has also played a central role in explaining fundamental life-history
214 trade-offs, such as that between growth rate and lifespan. Applying an understanding of
215 mitochondrial aerobic metabolism and the relationships between metabolic rate, ATP
216 production, and ROS production offers a new tool for further exploring such life-history
217 trade-offs. The "oxidative stress life-history hypothesis," for example, proposes that energy-
218 demanding phases of life that necessitate increased metabolism, such as growth or
219 reproduction, come at the cost of increased generation of mitochondrial ROS, which in turn
220 would have ultimate consequences to longevity (Figure 1) [57,58]. However, as we describe
221 above, increased metabolism does not necessarily increase the production of mitochondrial
222 ROS—and can actually have the opposite effect (Figure 2); so, it is perhaps unsurprising that
223 empirical evidence for this hypothesis is currently mixed (reviewed in [31,57,59]). More
224 comprehensive comparisons of mitochondrial and organismal respiration across species,
225 individuals, and tissues has the potential to provide new insights into longstanding questions

226 regarding major physiological and life-history patterns, such as trade-offs surrounding
227 whole-organism metabolic rate [54] or growth [60]. For example, an individual could
228 potentially maintain growth rate during times of food scarcity without altering oxygen
229 consumption by instead increasing efficiency of ATP production (Box 1)—but such increases
230 in coupling between electron flow and ATP synthesis at lower metabolic rates may come at
231 the potential cost of the increases in mitochondrial ROS production (Figures 2 and 3)
232 [17,61,62]. Mitochondrial processes can therefore provide testable predictions for how and
233 why individuals differ in performance when whole-organism measures do not fully explain
234 observed variation.

235 A second hypothesis more specifically considers how regulation of protonmotive
236 force to adjust ROS production may itself be a key to life-history trade-offs. This "uncoupling
237 to survive" hypothesis proposes that proton leak is carefully controlled to decrease
238 protonmotive force and thereby to regulate mitochondrial ROS generation (Figures 2 and 3)
239 [42,44,63,64]. Studies have tested this hypothesis by experimentally manipulating
240 uncoupling and/or by comparing uncoupling levels to life-history trait performance and
241 oxidative stress [14,65–67]. For example, the administration of an experimental
242 mitochondrial uncoupler to captive zebra finches (*Taeniopygia guttata*) caused whole-
243 organism metabolic rate to increase, likely in response to reduced ATP production efficiency
244 caused by the treatment, but did not alter oxidative stress. However, finches receiving the
245 uncoupling treatment had decreased reproductive investment, which supports the potential
246 existence of a trade-off between possible benefits of uncoupling and maximizing current
247 fitness [68]. Further studies are needed to better understand how uncoupling varies in wild
248 animals that are balancing ATP and ROS production, lifespan, and reproduction across
249 constantly varying environmental conditions.

Mitochondrial aerobic metabolism and temperature: thermoregulation, climatic adaptation, and acclimation

Uncoupling also has important ramifications for thermoregulation, as proton leak can be an important endogenous heat source. In developing chicken embryos, for example, the transition from ectothermy to endothermy is accompanied by increased mitochondrial uncoupling [69], suggesting an important role of proton leak in mechanisms of thermoregulation. In addition, a particular uncoupling protein in mammals (UCP1) has been found to drive heat production through non-shivering thermogenesis in the brown adipose tissue of many (but not all) cold-adapted mammals [43,70]. Increased proton leak may also potentially be a means to generate extra metabolic heat while decreasing ROS production [71]; for example, a study subjecting zebra finches to acute cold stress found that untreated birds had increased oxidative damage to DNA, while birds treated with an experimental mitochondrial uncoupler showed no such increases [72]. Such examples provide insight into the potential for mitochondrial measurements to provide new mechanistic explanations for the potential costs or benefits of different means of endogenous heat production.

Mitochondria are not only important to regulating internal temperature, but also to responding to changes in external temperature—on the scales of both long-term adaptation and short-term acclimation. For example, a study of wild mayfly larvae (*Baetis* and *Drunella* spp.) found that high-and low-elevation-adapted populations differed in multiple measures of mitochondrial aerobic metabolism when tested under common conditions [73]. This study demonstrates the potential for the evolution of mitochondrial phenotypes (over generations) to prevailing climatic conditions, with implications for local population adaptation. On shorter-term scales, mitochondrial aerobic metabolism also appears to be

key to organisms' response to varying temperatures, such as may occur during seasonal acclimation (e.g. [74]) or short-term thermal stress (e.g. [75]). Thermal conditions can themselves have fundamental thermodynamic effects on reaction rates in the mitochondria as cellular temperature changes, and organisms may respond by changing mitochondrial efficiency or density (potentially facing trade-offs in the process; e.g. [76,77]). Importantly, the limits of mitochondria to respond to changing conditions are expected to be a key determinant of thermal limits [73,77,78], with implications for how populations will respond to a changing climate.

Concluding remarks

There is a growing awareness among ecologists and evolutionary biologists that mitochondrial phenotype can play a central role in shaping fundamental characteristics of organisms. At the heart of this awareness is an understanding that selection acts on individual variation, and yet we know relatively little about how selection acts on mito-nuclear genotype and mitochondrial phenotype. To understand how variation in mitochondrial aerobic metabolism has contributed to shaping selection on organisms, we need to understand how mitochondrial phenotype constrains and/or enables response to environmental change. Fruitful lines of research include quantifying individual variation in mitochondria, including individual consistency in responses to changing environmental conditions, and tissue-specific responses for which fitness is dependent on managing periods of intense energy demands (e.g. rapid growth or migration). As interest in mitochondrial biology expands, there emerges an increasing need for a common foundation in the best practices for studying mitochondrial aerobic metabolism, grounded in a careful understanding of mitochondrial biology. Such a foundation will promote a more unified

298 understanding of how cellular processes may shape the evolution of life-history traits, and
299 support new research avenues that probe the broader ecological and evolutionary
300 implications (see Outstanding Questions).

Figure Captions

Figure 1. **Mitochondria link energy flow through the environment to organism life-history, impacting meta-population and community dynamics**. Environmental factors, such as temperature and water availability, affect net primary productivity (NPP) **(A)**, and thus food availability. Organisms ingest food **(B)** and liberate the organic molecules that are absorbed, transporting them to tissues and cells. Carbohydrates, fats, and proteins are each independently catabolized and the energy stored within their chemical bonds is captured to produce ATP through aerobic respiration. The final steps take place in the electron transport chain (ETC) within the inner membrane of the mitochondria. Protonmotive force across the inner mitochondrial membrane is used to fuel ATP production through ATP synthase **(C)**, but can also be dissipated through proton leak; this decreases ATP production efficiency and increases the amount of energy released as heat **(D)**. Mitochondria are also a source for reactive oxygen species (ROS), which can damage proteins, lipids, and nucleic acids, potentially causing mitochondrial and cellular dysfunction and affecting life-history traits **(E)**. Mitochondrial and nuclear genes coding for enzymes in the ETC **(F)** interact with environmental factors to play critical roles in determining the efficiency of mitochondria in producing ATP, heat, and ROS. Mitochondrial aerobic metabolism is therefore important to determining the amount of ATP available to fuel metabolic processes underlying cellular and tissue maintenance, activity, growth, and reproduction—all critical underpinnings of an organism's life-history repertoire **(G)**. Changes in life-history traits at the individual level "funnel" up to affect the dynamics of populations, and ultimately, meta-populations and communities **(H)**.

Figure 2

Simplified diagrams illustrating relationships among ATP demand, protonmotive force, mitochondrial ROS production, and proton leak. Here, we illustrate electron movement through the ETC, ATP synthesis from ADP via ATP synthase, and proton movement across the inner mitochondrial membrane through the ETC (i), ATP synthase (ii), or proton leak (iii). The top region of each graphic represents the intermembrane space, and the bottom region represents the mitochondrial matrix. The three panels represent conditions of high ATP demand **(A)**, low ATP demand **(B)**, and low ATP demand with increased inducible proton leak **(C)**. In **(A)**, high demand for ATP prevents the build-up of protonmotive force as protons are quickly passed through ATP synthase, which limits mitochondrial ROS production. In **(B)**, decreased ATP demand has increased protonmotive force because proton movement through ATP synthase has decreased; under these conditions, electrons "escape" the ETC at higher rates, forming ROS. In **(C)**, proton leak—both basal and induced (black bars across the membrane represent upregulated protein channels)—prevents the build-up of protonmotive force and thereby controls ROS generation even while demand for ATP is low, producing heat in the process. The differences between **(B)** and **(C)** are key to the concept of how the strategic adjustment of proton leak may be beneficial, but also underlie trade-offs; for example, greater proton leak in **(C)** compared to **(B)** decreases mitochondrial efficiency, but also decreases mitochondrial ROS production even when ATP demand is low.

Figure 3

Illustration of within- and among-individual variation in mitochondrial aerobic metabolism. The shading of each region represents whether oxygen is put toward ATP production (slashed region), proton leak (filled gray region), or ROS production (unshaded region). In **(A)**, the filled circles represent possible measurements of an animal at rest (i) or

349 the same animal in a state of high activity (ii), informed by data in [79]. In a highly active
350 state (ii), the high demand for ATP causes ATP synthase to rapidly use up the proton
351 gradient, so protonmotive force and rate of proton leak become relatively low. In contrast,
352 the slowing of ATP synthase activity when an active animal shifts to rest (i) causes an
353 increase in membrane potential. The open circles represent the *in vitro* measurements of
354 OXPHOS (iii) or LEAK (iv) respiration (Box 1), though these extremes would not occur *in vivo*;
355 it is worth noting that using these extremes to calculate the proportion of oxygen resulting in
356 proton leak (i.e. RCR; Box 1) would result in an misleading estimate of the actual proportions
357 of oxygen resulting in leak or ATP production at either during either high or low activity
358 [80,81]. In **(B)**, within- and among-individual variation in mitochondrial efficiency is
359 illustrated by tracing hypothetical relative proportions of different endpoints of
360 mitochondrial oxygen consumption during states of high or low demand for ATP.
361 Mitochondria with high efficiency (v) will produce more ATP at a given level of oxygen
362 consumption than those with low efficiency (vi and vii). However, higher efficiency
363 mitochondria can also generate higher levels of ROS under states of low ATP demand (v,
364 right column), so decreased efficiency may hypothetically be an adaptive "strategy" to lower
365 ROS (e.g. shift from v to vi). In contrast, lower efficiency can also be caused by decreased
366 mitochondrial "quality" (e.g. due to mild dysfunction from damage or mito-nuclear
367 incompatibility), potentially both reducing ATP production and increasing ROS generation
368 (vii). Note that the relative proportions allocated to ROS are inflated to be more visually
369 clear.

Box 1: The biological implications of methodological choices

(1) Sample type and preparation

Mitochondrial aerobic metabolism can be measured *in vitro* from isolated mitochondria, permeabilized cells/tissues, or intact cells [82]. While isolated mitochondria provide precise control of cellular conditions (i.e. substrates and ADP availability), they lack cellular context. Intact cells have a preserved cellular and mitochondrial network and are able to inform on endogenous mitochondrial aerobic metabolism, but lack control of substrates and ADP availability. Permeabilized cells/tissues share some of the advantages and drawbacks of both isolated mitochondria and intact cells [82]. Isolated mitochondria are considered a better choice for mechanistic studies, while intact cells have a greater ecological relevance [82].

(2) Mitochondrial aerobic metabolism parameters

Different mitochondrial aerobic metabolism parameters can be measured (Table I); the two main categories are respiration rates (e.g. **OXPHOS** and **LEAK respiration**) and proxies of mitochondrial efficiency (e.g. P/O, the amount of ADP used or ATP produced per atom of oxygen consumed; or, respiratory control ratio (RCR), the ratio of oxygen used for OXPHOS to oxygen used for proton leak). Although RCR (OXPHOS/LEAK respiration) is widely used, it has several mathematical and interpretation limitations; a related metric has recently been proposed to alleviate these limitations, called OXPHOS Coupling Efficiency (abbreviated here as OxCE). OxCE reflects the proportion of total mitochondrial oxygen consumption being allocated to ATP synthesis (1-LEAK/OXPHOS respiration) [80,83]. Yet, both RCR and OxCE are biased by the fact that the proton leak estimated when no ATP synthesis occurs (i.e. LEAK respiration) over-estimates the proton leak occurring when active ATP synthesis takes place (Fig. 3A). Respiration rates indicate potential metabolic capacity levels, while efficiency proxies indicate how efficient mitochondria are at producing ATP [81,82]. Both are likely to

be relevant for organismal performance, but the *in vivo* relevance of such *in vitro* measurements must be carefully considered.

		Respiration rates	RCR or OxCE	ADP/O	ATP/O ratio	
Equipment required		*Oxygraphy*	*Oxygraphy*	*Oxygraphy*	*Oxygraphy + Colorimetry*	*Oxygraphy + Fluorimetry*
Mitochondrial preparation	*Isolated mitochondria*	**Feasible**	**Feasible**	**Feasible**	**Feasible**	**Feasible**
	Permeabilized cells/tissues	**Feasible**	**Feasible**	Not feasible	Not feasible	**Feasible**
	Intact cells	**Feasible**	**Feasible**	Not feasible	Not feasible	Not feasible
Start-up effort required		Low	Low	Low	High	High
Able to measure ATP production?		No	No	No	Yes	Yes
Relative processing time per sample		Low to moderate	Low to moderate	Low to moderate	Very high	Moderate
Cost (equipment + consumables)		Low to moderate	Low to moderate	Low to moderate	Low to moderate	High
Key methodological reference		[84]	[84,85]	[84,85]	[84,85]	[86]

Table I: Logistical considerations when measuring mitochondrial aerobic metabolism parameters

(3) Normalization of mitochondrial respiration rates

Ratios like P/O, RCR, and OxCE are unitless, but mitochondrial respiration rates (e.g. OXPHOS or LEAK respiration rates) need to be normalized relative to tissue/mitochondrial content in order to be compared. Normalizing respiration rates of isolated mitochondria is usually done by expressing oxygen consumption per amount of mitochondrial protein content. Normalizing mitochondrial respiration rates from permeabilized and intact cells can either be achieved using mass of tissue or number of cells, or a proxy of mitochondrial abundance/density, and this choice has critical consequences on data interpretation [80]. For instance, two groups of animals could exhibit similar respiration rates expressed per unit of tissue, but one group may require more mitochondria to reach the same respiratory capacity; contextualizing measures in this way is important to reveal hidden complexity,

which in turn can suggest the possibility of further underlying patterns to explore (e.g. increased mitochondrial density to compensate for dysfunction, at the possible cost of increased ROS generation [87]).

Box 2: Designing experimental tests of mitochondrial aerobic metabolism

Measures of mitochondrial aerobic metabolism tend to be relatively low-throughput and most have to be measured in freshly collected biological samples (especially efficiency proxies) within a few hours of sampling ([88], but see [89,90]). Such constraints may limit the number of animals sampled and require that tissue collection be organized strategically. In addition, the sampling approach (e.g. choice of tissue, frequency of sampling), the mitochondrial properties to assess (e.g. mitochondrial respiration rates, efficiency, density; Box 1), and the experimental conditions (e.g. assay temperature [20], substrates [91]) should each be chosen with care based on the focus and constraints of a given study.

Since mitochondrial aerobic metabolism varies considerably among tissues (e.g. [17,54]), conclusions based on a single tissue should always be considered within context, and extrapolation to additional tissues without validation should be avoided. When selecting which tissue to measure, researchers should consider both the physiology that underlies the question being evaluated and the practical and ethical constraints of destructive sampling. For example, a study of ornamental red coloration in male house finches (*Haemorhous mexicanus*) measured the performance of mitochondria in the liver because the process of converting dietary yellow to red feather pigments is thought to occur specifically within the liver in that species [48]. When studies focus on small species, sample collection often must be destructive, making repeated sampling of an individual impossible. In larger animals, it may be possible to biopsy organs such as skeletal muscle [26], but the consequences of such invasive procedures should be carefully considered. Beyond ethical considerations, it is important to consider how the stressors animals experience prior to death or during sampling can impact their physiology [92].

Evaluating mitochondrial aerobic metabolism from blood cells has emerged over the last decade as a potential alternative to circumvent some of these issues. This approach using blood cells is becoming more prevalent in biomedical studies (e.g. [93]), and is starting to be implemented in ecological studies on non-model and wild animals [14,15,26,94–96]. Several studies have revealed moderate positive associations between measurements of mitochondrial aerobic metabolism in blood cells and other tissues ([26,97–99]; but see [100,101]). Additionally, a study in wild female pied flycatchers (*Ficedula hypoleuca*) during reproduction showed that mitochondrial measurements in blood cells were moderately to highly repeatable within individuals over a period of 10 days, despite those measurements being highly plastic across reproductive stage [95]. Such results suggest that measurements from blood cells have the potential to be relevant and individual-specific markers of mitochondrial parameters.

Glossary

Electron transport chain (ETC): A series of protein complexes in the inner mitochondrial membrane that transfer electrons via oxidation/reduction reactions. These reactions are coupled to the pumping of protons out of the mitochondrial matrix to create a protonmotive force.

LEAK respiration: Oxygen used to offset proton leak. Also called state 4 respiration rate.

Mitochondrial aerobic metabolism: The metabolic (anabolic and catabolic) reactions that occur within the mitochondria and that involve direct and indirect use of oxygen. This comprises activity of the electron transport chain, protonmotive force, ATP production, and ROS generation.

Mitochondrial efficiency: Efficiency of mitochondria to convert food-derived energy substrate into ATP. Efficiency is often estimated *in vitro* with P/O ratios and respiratory control ratios (RCR).

Mito-nuclear interactions: Functional associations between the products of the mitochondrial and nuclear genomes that can affect mitochondrial aerobic metabolism.

Oxidative phosphorylation (OXPHOS): Phosphorylation of ADP into ATP in the presence of oxygen. Conversion of ADP to ATP is performed by ATP synthase, and uses the energy provided by the protonmotive force.

OXPHOS respiration: Oxygen used when mitochondria are actively producing ATP. Also called state 3 respiration rate.

Oxidative stress: Imbalance between the production of pro-oxidants (such as ROS) and the ability of a biological system to neutralize them through antioxidant defenses, leading to oxidative damage to biomolecules.

Proton leak: Protons that flow back across the inner mitochondrial membrane into the matrix outside of ATP synthase. Proton leak may be passive, particularly when membrane potential is high, or inducible (and regulated through transmembrane proteins).

Protonmotive force: The potential energy stored across the inner mitochondrial membrane that is established by pumping protons from the mitochondrial membrane to the intermembrane space; the protonmotive force involves both the chemical gradient formed by the difference in proton concentration, and the electrical gradient formed by the difference in charge.

Reactive oxygen species (ROS): Derivatives of oxygen that are chemically unstable and quickly react with different kinds of biomolecules; ROS are important cellular signals, but can also cause altered function through oxidative damage.

Uncoupling: The dissociation of mitochondrial protonmotive force generation (i.e. the "uncoupling" of electron transport chain activity) from ATP synthesis.

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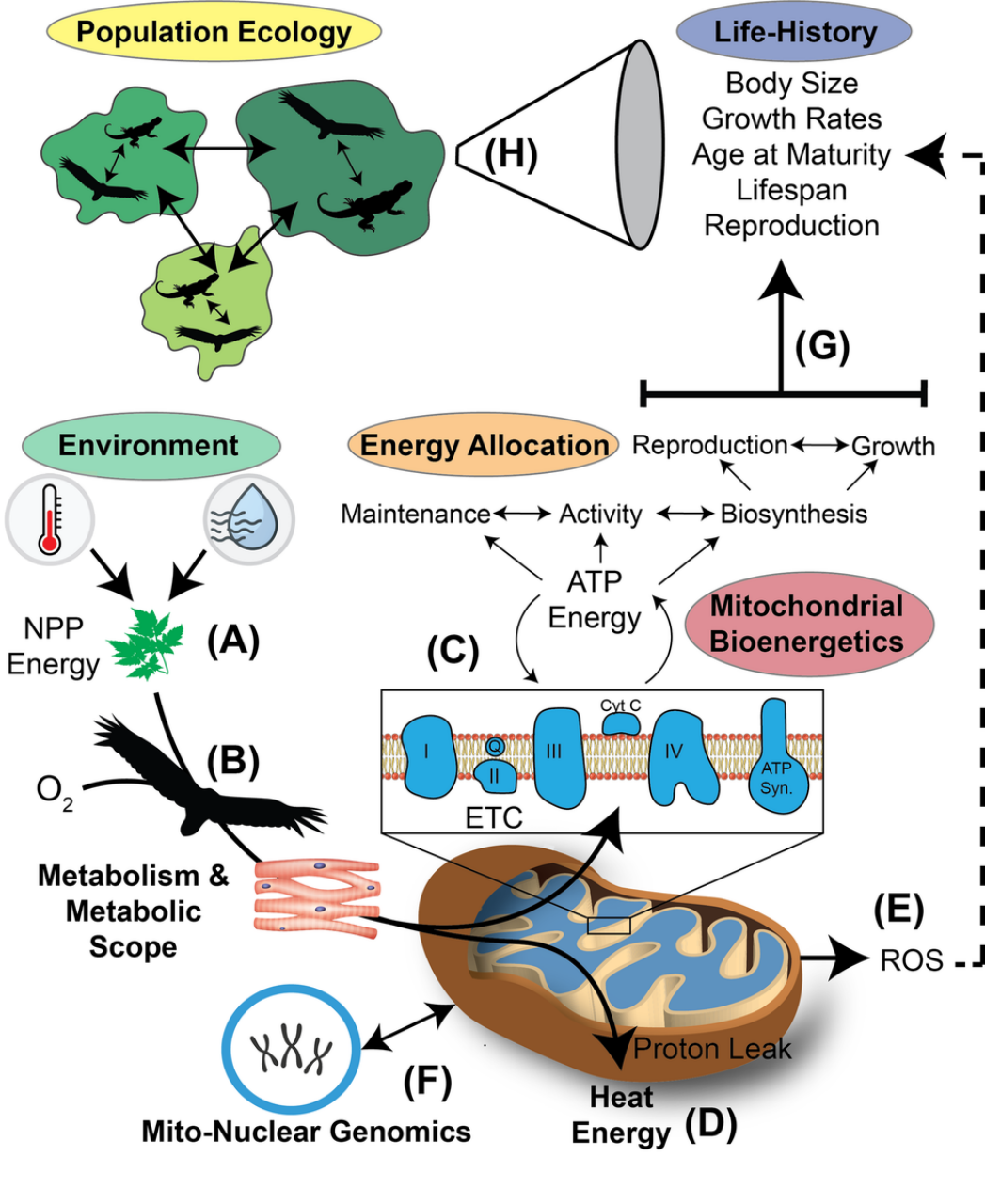
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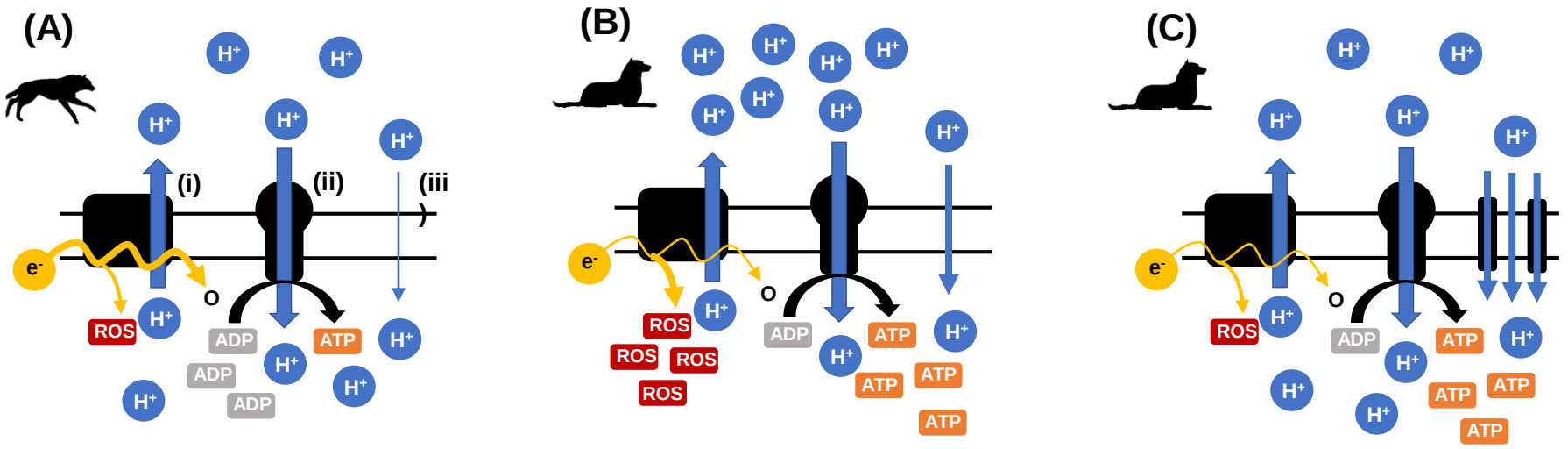
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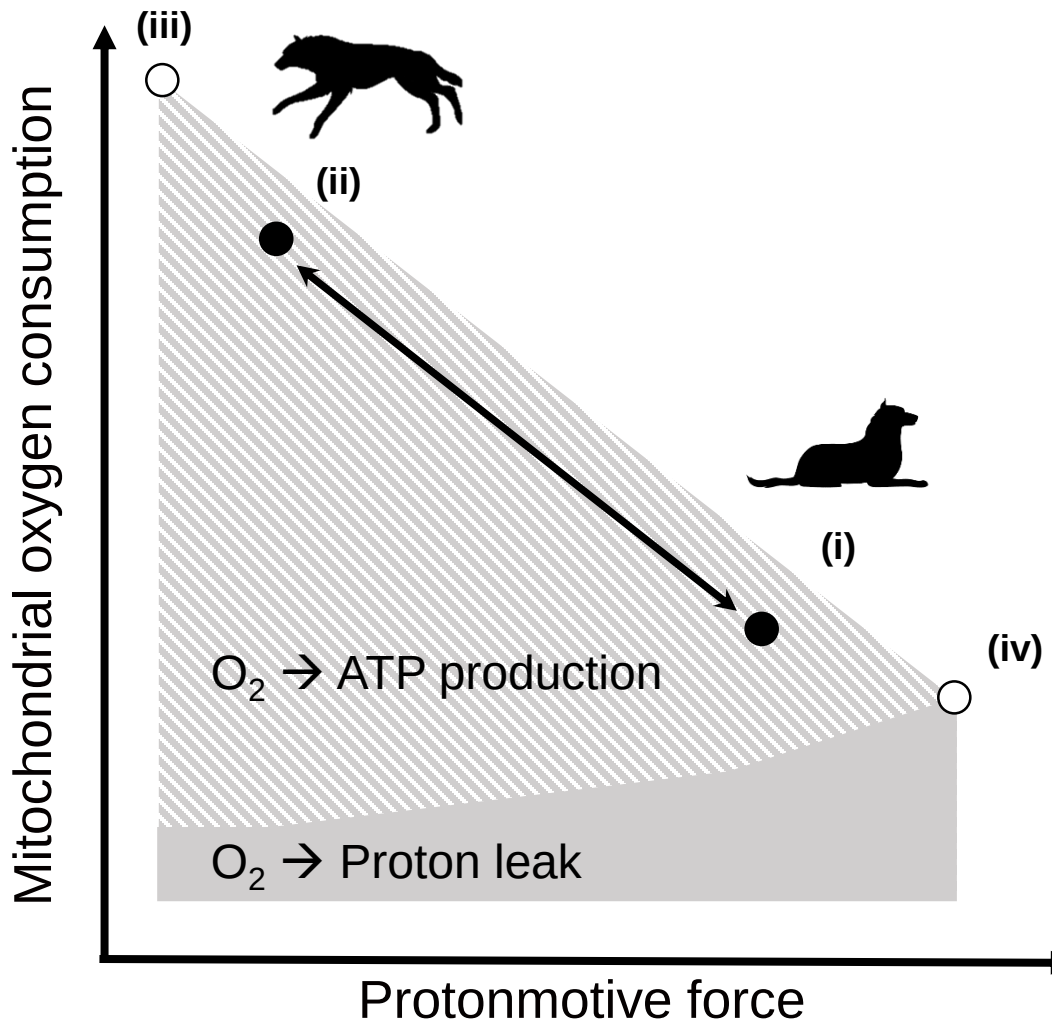

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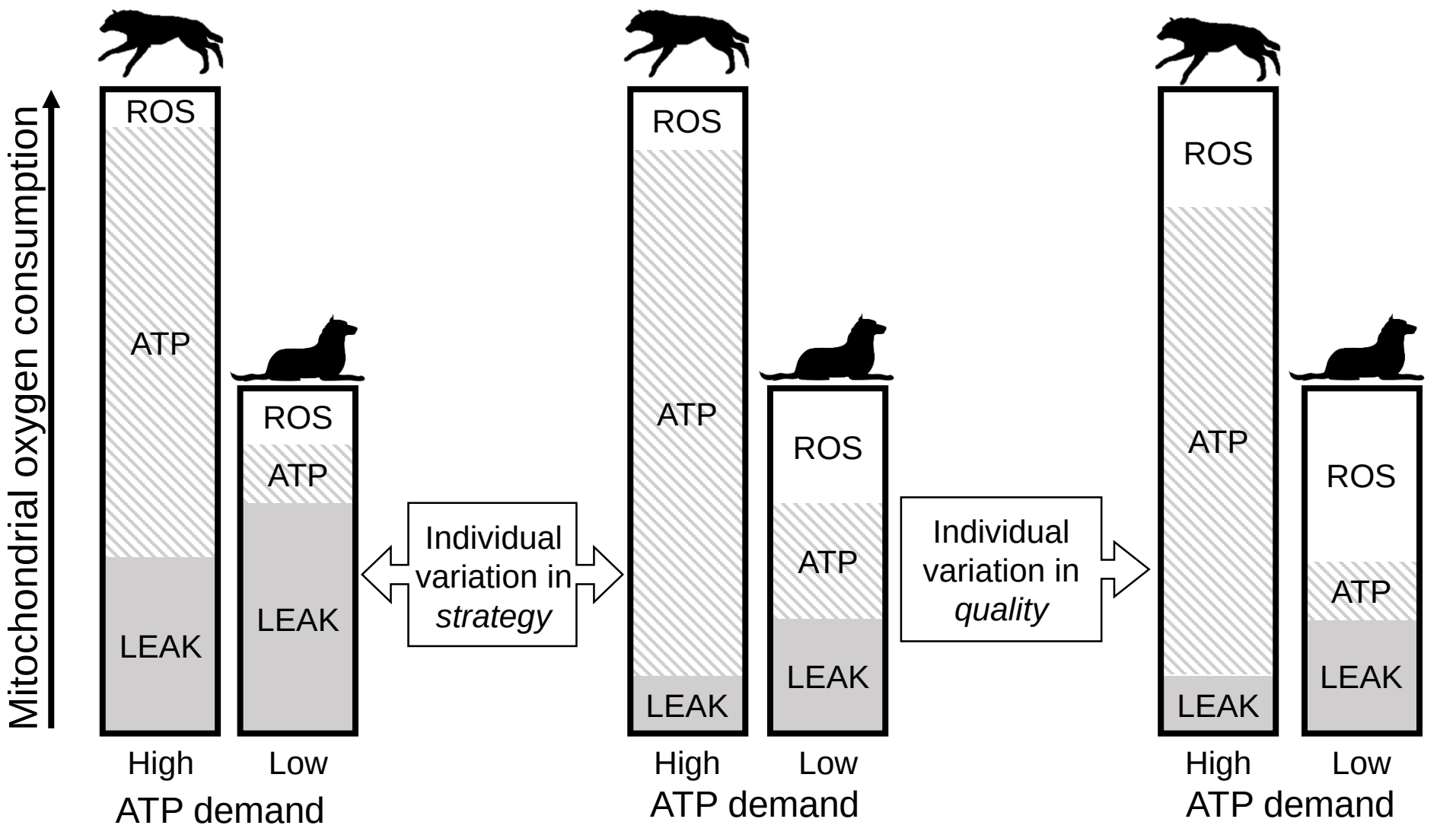

(vi) Low efficiency

(v) High efficiency

(vii) Low efficiency