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Abstract As the knowledge base and importance of mitochondrial physiology to human health expand, the necessity for harmonizing nomenclature concerning mitochondrial respiratory states and rates has become increasingly apparent. Clarity of concept and consistency of nomenclature are key trademarks of a research field. These trademarks facilitate effective transdisciplinary communication, education, and ultimately further discovery. Peter Mitchell's chemiosmotic theory establishes the mechanism of energy transformation and coupling in oxidative phosphorylation. The unifying concept of the protonmotive force provides the framework for developing a consistent theory and nomenclature for mitochondrial physiology and bioenergetics. Herein, we follow IUPAC guidelines on general terms of physical chemistry, extended by considerations on open systems and irreversible thermodynamics. We align the nomenclature and symbols of classical bioenergetics with a concept-driven constructive terminology to express the meaning of each quantity clearly and consistently. In this position statement, in the frame of COST Action MitoEAGLE, we endeavour to provide a balanced view on mitochondrial respiratory control and a critical discussion on reporting data of mitochondrial respiration in terms of metabolic flows and fluxes. Uniform standards for evaluation of respiratory states and rates will ultimately support the development of databases of mitochondrial respiratory function in species, tissues, and cells.

Keywords: Mitochondrial respiratory control, coupling control, mitochondrial preparations, protonmotive force, oxidative phosphorylation, OXPHOS, efficiency, electron transfer, ET; proton leak, LEAK, residual oxygen consumption, ROX, State 2, State 3, State 4, normalization, flow, flux, O_2

Executive summary

1. In view of the broad implications for health care, mitochondrial researchers face an increasing responsibility to disseminate their fundamental knowledge and novel discoveries to a wide range of stakeholders and scientists beyond the group of specialists. This requires implementation of a commonly accepted terminology within the discipline and standardization in the translational context. Authors, reviewers, journal editors, and lecturers are challenged to collaborate with the aim to harmonize the nomenclature in the growing field of mitochondrial physiology and bioenergetics.
2. Aerobic energy metabolism in mammalian mitochondria depends on the coupling of phosphorylation (ADP → ATP) to O_2 flux in catabolic reactions. In this process of oxidative phosphorylation, coupling is mediated by translocation of protons through respiratory proton pumps operating across the inner mitochondrial membrane and generating or utilizing the protonmotive force measured between the mitochondrial matrix and intermembrane compartment. Compartmental coupling thus distinguishes vectorial oxidative phosphorylation from fermentation as the counterpart of cellular core energy metabolism.
3. To exclude fermentation and other cytosolic interactions from exerting an effect on mitochondrial metabolism, the barrier function of the plasma membrane must be disrupted. Selective removal or permeabilization of the plasma membrane yields mitochondrial preparations—including isolated mitochondria, tissue and cellular preparations—with structural and functional integrity. Then extra-mitochondrial concentrations of fuel substrates transported into the mitochondrial matrix, ADP, ATP, inorganic phosphate, and cations including H^+ can be controlled to determine mitochondrial function under a set of conditions defined as coupling control states.

explicitly

A concept-driven terminology of bioenergetics incorporates in its terms and symbols ~~explicitly~~ information on the nature of respiratory states, that makes the technical terms readily recognized and easy to understand.

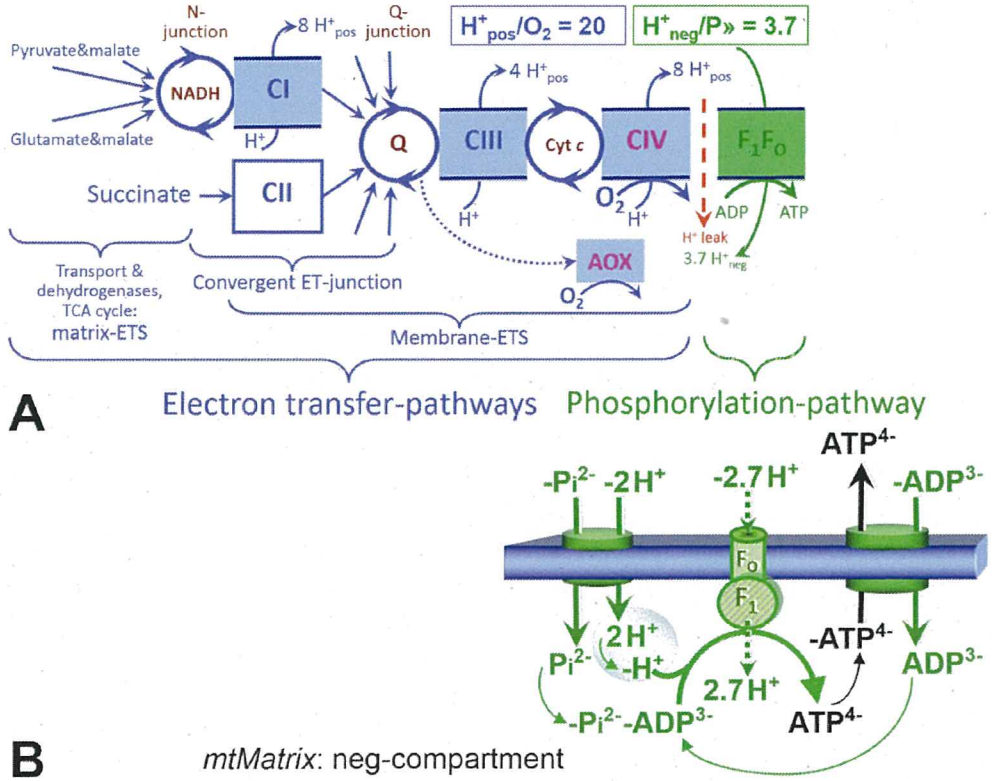

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Fig. 1. The oxidative phosphorylation (OXPHOS) system. (A) The mitochondrial electron transfer system (ETS) is fuelled by diffusion and transport of substrates across the mtOM and mtIM and consists of the matrix-ETS and membrane-ETS. ET-pathways are coupled to the phosphorylation-pathway. ET-pathways converge at the N-junction and Q-junction. Additional arrows indicate electron entry into the Q-junction through electron transferring flavoprotein, glycerophosphate dehydrogenase, dihydro-orotate dehydrogenase, choline dehydrogenase, and sulfide-ubiquinone oxidoreductase. The dotted arrow indicates the branched pathway of oxygen consumption by alternative quinol oxidase (AOX). The H^+_{pos}/O_2 ratio is the outward proton flux from the matrix space to the positively (pos) charged compartment, divided by catabolic O_2 flux in the NADH-pathway. The H^+_{neg}/P» ratio is the inward proton flux from the inter-membrane space to the negatively (neg) charged matrix space, divided by the flux of phosphorylation of ADP to ATP (Eq. 1). These are not fixed stoichiometries due to ion leaks and proton slip. **(B)** Phosphorylation-pathway catalyzed by the proton pump F_1F_O-ATPase (F-ATPase, ATP synthase), adenine nucleotide translocase, and inorganic phosphate transporter. The H^+_{neg}/P» stoichiometry is the sum of the coupling stoichiometry in the F-ATPase reaction (-2.7 H^+_{pos} from the positive intermembrane space, 2.7 H^+_{neg} to the matrix, *i.e.*, the negative compartment) and the proton balance in the translocation of ADP^{2-}, ATP^{3-} and P_i^{2-}. Modified from (A) Lemieux *et al.* (2017) and (B) Gnaiger (2014).

4. Mitochondrial coupling states are defined according to the control of respiratory oxygen flux by the protonmotive force. Capacities of oxidative phosphorylation and electron transfer capacities are measured at kinetically saturating concentrations of fuel substrates, ADP and inorganic phosphate, or at optimal uncoupler concentrations, respectively. Respiratory capacities are a measure of the upper bound of the rates of respiration, providing reference values for the diagnosis of health and disease, and for evaluation of the effects of **E**volutionary background, **A**ge, **G**ender and sex, **L**ifestyle and **E**nvironment (EAGLE).
5. Some degree of uncoupling is a characteristic of energy-transformations across membranes. Uncoupling is caused by a variety of physiological, pathological, toxicological, pharmacological and environmental conditions that exert an influence not only on the proton leak and cation cycling, but also on proton slip within the proton pumps and the structural integrity of the mitochondria. A more loosely coupled state is induced by stimulation of mitochondrial superoxide formation and the bypass of proton pumps. In addition, uncoupling by application of protonophores represents an experimental intervention for the transition from a well-coupled to the noncoupled state of mitochondrial respiration.
6. Respiratory oxygen consumption rates have to be carefully normalized to enable meta-analytic studies beyond the specific question of a particular experiment. Therefore, all raw data should be published in a supplemental table or open access data repository. Normalization of rates for the volume of the experimental chamber (the measuring system) is distinguished from normalization for (*1*) the volume or mass of the experimental sample, (*2*) the number of objects (cells, organisms), and (*3*) the concentration of mitochondrial markers in the chamber.
7. The consistent use of terms and symbols discussed in this MitoEAGLE position statement will facilitate transdisciplinary communication and support further developments of a database on bioenergetics and mitochondrial physiology. The present considerations are focused on studies with mitochondrial preparations. These will be extended in a series of reports on pathway control of mitochondrial respiration, the protonmotive force, respiratory states in intact cells, and harmonization of experimental procedures.

Box 1: In brief – Mitochondria and Bioblasts

Mitochondria are the oxygen-consuming electrochemical generators evolved from endosymbiotic bacteria (Margulis 1970; Lane 2005). They were described by Richard Altmann (1894) as 'bioblasts', which include not only the mitochondria as presently defined, but also symbiotic and free-living bacteria. The word 'mitochondria' (Greek mitos: thread; chondros: granule) was introduced by Carl Benda (1898).

Mitochondrial dysfunction is associated with a wide variety of genetic and degenerative diseases. Robust mitochondrial function is supported by physical exercise and caloric balance, and is central for sustained metabolic health throughout life. Therefore, a more consistent presentation of mitochondrial physiology will improve our understanding of the etiology of disease, the diagnostic repertoire of mitochondrial medicine, with a focus on protective medicine, lifestyle and healthy aging.

We now recognize mitochondria as dynamic organelles with a double membrane that are contained within eukaryotic cells. The mitochondrial inner membrane (mtIM) shows dynamic tubular to disk-shaped cristae that separate the mitochondrial matrix, *i.e.*, the negatively charged

internal mitochondrial compartment, and the intermembrane space; the latter being positively charged and enclosed by the mitochondrial outer membrane (mtOM). The mtIM contains the non-bilayer phospholipid cardiolipin, which is not present in any other eukaryotic cellular membrane. Cardiolipin promotes the formation of respiratory supercomplexes, which are supramolecular assemblies based upon specific, though dynamic, interactions between individual respiratory complexes (Greggio *et al.* 2017; Lenaz *et al.* 2017). Membrane fluidity exerts an influence on functional properties of proteins incorporated in the membranes (Waczulikova *et al.* 2007).

Mitochondria are the structural and functional elements of cell respiration. Cell respiration is the reduction of oxygen by electron transfer coupled to electrochemical proton translocation across the mtIM. In the process of oxidative phosphorylation (OXPHOS), the catabolic reaction of oxygen consumption is electrochemically coupled to the transformation of energy in the form of adenosine triphosphate (ATP; Mitchell 1961, 2011). Mitochondria are the powerhouses of the cell which contain the machinery of the OXPHOS-pathways, including transmembrane respiratory complexes—proton pumps with FMN, Fe-S and cytochrome *b*, *c*, aa_3 redox systems); alternative dehydrogenases and oxidases; the coenzyme ubiquinone (Q); F-ATPase or ATP synthase; the enzymes of the tricarboxylic acid cycle and fatty acid oxidation; transporters of ions, metabolites and co-factors; and mitochondrial kinases related to energy transfer pathways. The mitochondrial proteome comprises over 1,200 proteins (Calvo *et al.* 2015; 2017), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many of which are relatively well known (*e.g.*, apoptosis-regulating proteins), while others are still under investigation, or need to be identified (*e.g.*, alanine transporter).

There is a constant crosstalk between mitochondria and the other cellular components. The crosstalk between mitochondria and endoplasmic reticulum is involved in the regulation of calcium homeostasis, cell division, autophagy, differentiation, anti-viral signaling (Murley and Nunnari 2016). Cellular mitostasis is maintained through regulation at both the transcriptional and post-translational level, through cell signalling including proteostatic (*e.g.*, the ubiquitin-proteasome and autophagy-lysosome pathways), and genome stability modules throughout the cell cycle or even cell death, contributing to homeostatic regulation in response to varying energy demands and stress (Quiros *et al.* 2016). In addition to mitochondrial movement along microtubules, mitochondrial morphology can change in response to energy requirements of the cell via processes known as fusion and fission, through which mitochondria communicate within a network, and in response to intracellular stress factors causing swelling and ultimately permeability transition.

Mitochondria typically maintain several copies of their own genome known as mitochondrial DNA (mtDNA; hundred to thousands per cell; Cummins 1998), which is maternally inherited. One exception to strictly maternal inheritance in animals is found in bivalves (Breton *et al.* 2007; White *et al.* 2008). mtDNA is 16.5 kB in length, contains 13 protein-coding genes for subunits of the transmembrane respiratory Complexes CI, CIII, CIV and F-ATPase, and also encodes 22 tRNAs and the mitochondrial 16S and 12S rRNA. Additional gene content is encoded in the mitochondrial genome, *e.g.*, microRNAs, piRNA, smithRNAs, repeat associated RNA, and even additional proteins, *e.g.*, the small peptides humanin and MOTS-c (Duarte *et al.* 2014; Lee *et al.* 2015; Cobb *et al.* 2016). The mitochondrial genome is regulated and supplemented by nuclear-encoded mitochondrial targeted proteins.

Abbreviation: mt, as generally used in mtDNA. Mitochondrion is singular and mitochondria is plural.

'*For the physiologist, mitochondria afforded the first opportunity for an experimental approach to structure-function relationships, in particular those involved in active transport, vectorial metabolism, and metabolic control mechanisms on a subcellular level*' (Ernster and Schatz 1981).

1. Introduction

Mitochondria are the powerhouses of the cell with numerous physiological, molecular, and genetic functions (**Box 1**). Every study of mitochondrial health and disease is faced with **E**volution, **A**ge, **G**ender and sex, **L**ifestyle, and **E**nvironment (EAGLE) as essential background conditions intrinsic to the individual patient or subject, cohort, species, tissue and to some extent even cell line. As a large and coordinated group of laboratories and researchers, the mission of the global MitoEAGLE Network is to generate the necessary scale, type, and quality of consistent data sets and conditions to address this intrinsic complexity. Harmonization of experimental protocols and implementation of a quality control and data management system are required to interrelate results gathered across a spectrum of studies and to generate a rigorously monitored database focused on mitochondrial respiratory function. In this way, researchers within the same and across different disciplines will be positioned to compare findings across traditions and generations to an agreed upon set of clearly defined and accepted international standards.

Reliability and comparability of quantitative results depend on the accuracy of measurements under strictly-defined conditions. A conceptual framework is required to warrant meaningful interpretation and comparability of experimental outcomes carried out by research groups at different institutes. With an emphasis on quality of research, collected data can be useful far beyond the specific question of a particular experiment. Enabling meta-analytic studies is the most economic way of providing robust answers to biological questions (Cooper *et al.* 2009). Vague or ambiguous jargon can lead to confusion and may relegate valuable signals to wasteful noise. For this reason, measured values must be expressed in standard units for each parameter used to define mitochondrial respiratory function. Harmonization of nomenclature and definition of technical terms are essential to improve the awareness of the intricate meaning of current and past scientific vocabulary, for documentation and integration into databases in general, and quantitative modelling in particular (Beard 2005). The focus on coupling states and fluxes through metabolic pathways of aerobic energy transformation in mitochondrial preparations is a first step in the attempt to generate a conceptually-oriented nomenclature in bioenergetics and mitochondrial physiology. Coupling states of intact cells, the protonmotive force, and respiratory control by fuel substrates and specific inhibitors of respiratory enzymes will be reviewed in subsequent communications.

2. Oxidative phosphorylation and coupling states in mitochondrial preparations

> '*Every professional group develops its own technical jargon for talking about matters of critical concern ... People who know a word can share that idea with other members of their group, and a shared vocabulary is part of the glue that holds people together and allows them to create a shared culture*' (Miller 1991).

Mitochondrial preparations are defined as either isolated mitochondria, or tissue and cellular preparations in which the barrier function of the plasma membrane is disrupted. Since this entails the loss of cell viability, mitochondrial preparations are not studied *in vivo*. In contrast to isolated mitochondria and tissue homogenate preparations, mitochondria in permeabilized tissues and cells are *in situ* relative to the plasma membrane. The plasma membrane separates the intracellular compartment including the cytosol, nucleus, and organelles from the environment of the cell. The plasma membrane consists of a lipid bilayer, embedded proteins, and attached organic molecules that collectively control the selective permeability of ions, organic molecules, and particles across the cell boundary. The intact plasma membrane prevents the passage of many water-soluble mitochondrial substrates and inorganic ions—such as succinate, adenosine diphosphate (ADP) and inorganic phosphate (P_i),

that must be controlled at kinetically-saturating concentrations for the analysis of respiratory capacities; this limits the scope of investigations into mitochondrial respiratory function in intact cells.

The cholesterol content of the plasma membrane is high compared to mitochondrial membranes. Therefore, mild detergents—such as digitonin and saponin—can be applied to selectively permeabilize the plasma membrane by interaction with cholesterol and allow free exchange of organic molecules and inorganic ions between the cytosol and the immediate cell environment, while maintaining the integrity and localization of organelles, cytoskeleton, and the nucleus. Application of optimum concentrations of permeabilization agents (mild detergents or toxins) leads to washout of cytosolic marker enzymes—such as lactate dehydrogenase—and results in the complete loss of cell viability, tested by nuclear staining using membrane-impermeable dyes, while mitochondrial function remains intact. Respiration of isolated mitochondria remains unaltered after the addition of low concertations of digitonin or saponin. In addition to mechanical permeabilization during homogenization of tissue, permeabilization agents may be applied to ensure permeabilization of all cells. Suspensions of cells permeabilized in the respiration chamber and crude tissue homogenates contain all components of the cell at highly dilute concentrations. All mitochondria are retained in chemically-permeabilized mitochondrial preparations and crude tissue homogenates. In the preparation of isolated mitochondria, the cells or tissues are homogenized, and the mitochondria are separated from other cell fractions and purified by differential centrifugation, entailing the loss of a fraction of the total mitochondrial content. Typical mitochondrial recovery ranges from 30% to 80%. Maximization of the purity of isolated mitochondria may compromise not only the mitochondrial yield but also the structural and functional integrity. Therefore, protocols to isolate mitochondria need to be optimized according to each study. The term mitochondrial preparation does not include further fractionation of mitochondrial components, neither submitochondrial particles.

2.1. Respiratory control and coupling

Respiratory coupling control states are established in studies of mitochondrial preparations to obtain reference values for various output variables. Physiological conditions *in vivo* deviate from these experimentally obtained states. Since kinetically-saturating concentrations, *e.g.*, of ADP or oxygen (O_2; dioxygen), may not apply to physiological intracellular conditions, relevant information is obtained in studies of kinetic responses to variations in [ADP] or [O_2] in the range between kinetically-saturating concentrations and anoxia (Gnaiger 2001).

The steady-state: Mitochondria represent a thermodynamically open system in non-equilibrium states of biochemical energy transformation. State variables (protonmotive force; redox states) and metabolic *rates* (fluxes) are measured in defined mitochondrial respiratory *states*. Steady-states can be obtained only in open systems, in which changes by *internal* transformations, *e.g.*, O_2 consumption, are instantaneously compensated for by *external* fluxes, *e.g.*, O_2 supply, preventing a change of O_2 concentration in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored in closed systems satisfy the criteria of pseudo-steady states for limited periods of time, when changes in the system (concentrations of O_2, fuel substrates, ADP, P_i, H^+) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering capacity and substrates maintained at kinetically-saturating concentrations, and thus depend on the kinetics of the processes under investigation.

Specification of biochemical dose: Substrates, uncouplers, inhibitors, and other biochemical reagents are titrated to dissect mitochondrial function. Nominal concentrations of these substances are usually reported as initial amount of substance concentration [mol·L^{-1}] in

the incubation medium. When aiming at the measurement of kinetically saturated processes—such as OXPHOS-capacities, the concentrations for substrates can be chosen according to the apparent equilibrium constant, K_m'. In the case of hyperbolic kinetics, only 80% of maximum respiratory capacity is obtained at a substrate concentration of four times the K_m', whereas substrate concentrations of 5, 9, 19 and 49 times the K_m' are theoretically required for reaching 83%, 90%, 95% or 98% of the maximal rate (Gnaiger 2001). Other reagents are chosen to inhibit or alter some processes. The amount of these chemicals in an experimental incubation is selected to maximize effect, avoiding unacceptable off-target consequences that would adversely affect the data being sought. Specifying the amount of substance in an incubation as nominal concentration in the aqueous incubation medium can be ambiguous (Doskey *et al.* 2015), particularly for lipophilic substances (oligomycin; uncouplers, permeabilization agents) or cations (TPP^+; fluorescent dyes such as safranin, TMRM), which accumulate in biological membranes or in the mitochondrial matrix. For example, a dose of digitonin of 8 fmol·cell^{-1} (10 pg·cell^{-1}; 10 µg·10^{-6} cells) is optimal for permeabilization of endothelial cells, and the concentration in the incubation medium has to be adjusted according to the cell density applied (Doerrier *et al.* 2018). Generally, dose/exposure can be specified per unit of biological sample, *i.e.*, (nominal moles of xenobiotic)/(number of cells) [mol·cell^{-1}] or, as appropriate, per mass of biological sample [mol·kg^{-1}]. This approach to specification of dose/exposure provides a scalable parameter that can be used to design experiments, help interpret a wide variety of experimental results, and provide absolute information that allows researchers worldwide to make the most use of published data (Doskey *et al.* 2015).

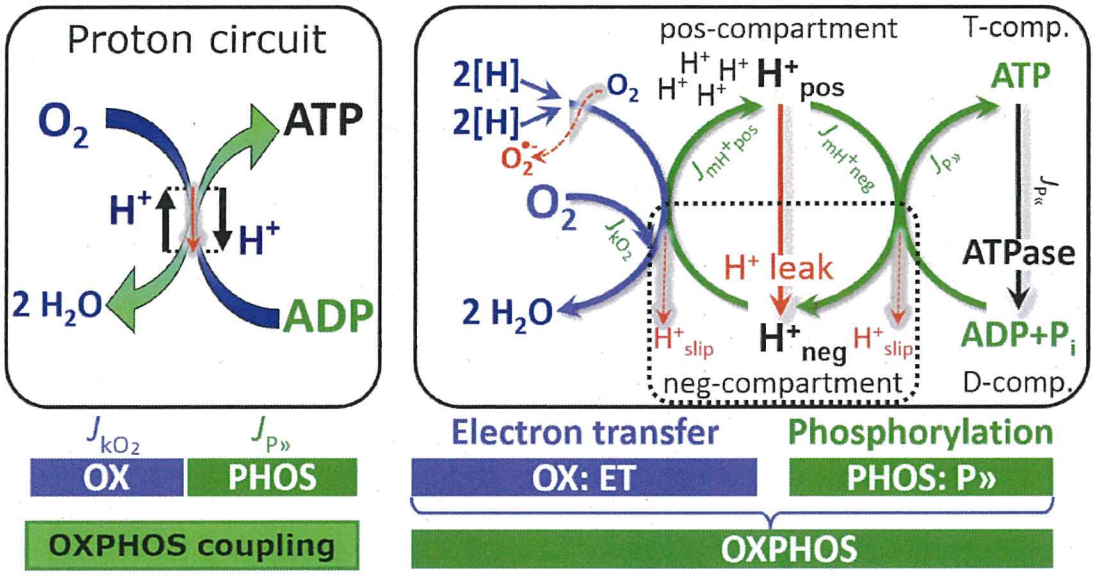


Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). 2[H] indicates the reduced hydrogen equivalents of fuel substrates of the catabolic reaction k with oxygen. O_2 flux, J_{kO_2}, through the catabolic ET-pathway, is coupled to flux through the phosphorylation-pathway of ADP to ATP, $J_{P»}$. The proton pumps of the ET-pathway drive proton flux into the positive (pos) compartment, J_{mH^+pos}, generating the output protonmotive force (motive, subscript m). F-ATPase is coupled to inward proton current into the negative (neg) compartment, J_{mH^+neg}, to phosphorylate ADP+P_i to ATP. The system defined by the boundaries (full black line) is not a black box, but is analysed as a compartmental system. The negative compartment (neg–compartment, enclosed by the dotted line) is the matrix space, separated by the mtIM from the positive compartment (pos–compartment). ADP+P_i and ATP are the substrate- and product–compartments (scalar ADP and ATP compartments, D–comp. and T–comp.), respectively. At steady-state proton turnover, $J_{\infty H^+}$, and ATP turnover, $J_{\infty P}$,

maintain concentrations constant, when $J_{mH^+\infty} = J_{mH^+pos} = J_{mH^+neg}$, and $J_{P\infty} = J_{P\gg} = J_{P\ll}$. Modified from Gnaiger (2014).

Phosphorylation, P», and P»/O₂ ratio: *Phosphorylation* in the context of OXPHOS is defined as phosphorylation of ADP by P_i to ATP. On the other hand, the term phosphorylation is used generally in many contexts, *e.g.*, protein phosphorylation. This justifies consideration of a symbol more discriminating and specific than P as used in the P/O ratio (phosphate to atomic oxygen ratio), where P indicates phosphorylation of ADP to ATP or GDP to GTP. We propose the symbol P» for the endergonic (uphill) direction of phosphorylation ADP→ATP, and likewise the symbol P« for the corresponding exergonic (downhill) hydrolysis ATP→ADP (**Fig. 2**). P» refers mainly to electrontransfer phosphorylation but may also involve substrate-level phosphorylation as part of the tricarboxylic acid (TCA) cycle (succinyl-CoA ligase) and phosphorylation of ADP catalyzed by phosphoenolpyruvate carboxykinase. Transphosphorylation is performed by adenylate kinase, creatine kinase, hexokinase and nucleoside diphosphate kinase. In isolated mammalian mitochondria, ATP production catalyzed by adenylate kinase (2 ADP ↔ ATP + AMP) proceeds without fuel substrates in the presence of ADP (Komlódi and Tretter 2017). Kinase cycles are involved in intracellular energy transfer and signal transduction for regulation of energy flux.

The P»/O_2 ratio (P»/4 e^-) is two times the 'P/O' ratio (P»/2 e^-) of classical bioenergetics. P»/O_2 is a generalized symbol, independent phosphorylation assessment by determination of P_i consumption (P_i/O_2 flux ratio), ADP depletion (ADP/O_2 flux ratio), or ATP production (ATP/O_2 flux ratio). The mechanistic P»/O_2 ratio—or P»/O_2 stoichiometry—is calculated from the proton–to–O_2 and proton–to–phosphorylation coupling stoichiometries (**Fig. 1A**),

$$P\gg/O_2 = \frac{H^+_{pos}/O_2}{H^+_{neg}/P\gg} \quad (1)$$

The H^+_{pos}/O_2 *coupling stoichiometry* (referring to the full 4 electron reduction of O_2) depends on the ET-pathway control state which defines the relative involvement of the three coupling sites (CI, CIII and CIV) in the catabolic pathway of electrons to O_2. This varies with: (*1*) a bypass of CI by single or multiple electron input into the Q-junction; and (*2*) a bypass of CIV by involvement of AOX. H^+_{pos}/O_2 is 12 in the ET-pathways involving CIII and CIV as proton pumps, increasing to 20 for the NADH-pathway (**Fig. 1A**), but a general consensus on H^+_{pos}/O_2 stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov 2015). The $H^+_{neg}/P\gg$ coupling stoichiometry (3.7; **Fig. 1A**) is the sum of 2.7 H^+_{neg} required by the F-ATPase of vertebrate and most invertebrate species (Watt *et al.* 2010) and the proton balance in the translocation of ADP, ATP and P_i (**Fig. 1B**). Taken together, the mechanistic P»/O_2 ratio is calculated at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively (Eq. 1). The corresponding classical P»/O ratios (referring to the 2 electron reduction of 0.5 O_2) are 2.7 and 1.6 (Watt *et al.* 2010), in agreement with the measured P»/O ratio for succinate of 1.58 ± 0.02 (Gnaiger *et al.* 2000).

The effective P»/O_2 flux ratio ($Y_{P\gg/O_2} = J_{P\gg}/J_{kO_2}$) is diminished relative to the mechanistic P»/O_2 ratio by intrinsic and extrinsic uncoupling and dyscoupling (**Fig. 3**). Such generalized uncoupling is different from switching to mitochondrial pathways that involve fewer than three proton pumps ('coupling sites': Complexes CI, CIII and CIV), bypassing CI through multiple electron entries into the Q-junction, or CIII and CIV through AOX (**Fig. 1**). Reprogramming of mitochondrial pathways may be considered as a switch of gears (changing the stoichiometry) rather than uncoupling (loosening the stoichiometry). In addition, $Y_{P\gg/O_2}$ depends on several experimental conditions of flux control, increasing as a hyperbolic function of [ADP] to a maximum value (Gnaiger 2001).

Control and regulation: The terms metabolic *control* and *regulation* are frequently used synonymously, but are distinguished in metabolic control analysis: 'We could understand the

regulation as the mechanism that occurs when a system maintains some variable constant over time, in spite of fluctuations in external conditions (homeostasis of the internal state). On the other hand, metabolic control is the power to change the state of the metabolism in response to an external signal' (Fell 1997). Respiratory control may be induced by experimental control signals that *exert* an influence on: (*1*) ATP demand and ADP phosphorylation-rate; (*2*) fuel substrate composition, pathway competition; (*3*) available amounts of substrates and O_2, *e.g.*, starvation and hypoxia; (*4*) the protonmotive force, redox states, flux–force relationships, coupling and efficiency; (*5*) Ca^{2+} and other ions including H^+; (*6*) inhibitors, *e.g.*, nitric oxide or intermediary metabolites such as oxaloacetate; (*7*) signalling pathways and regulatory proteins, *e.g.*, insulin resistance, transcription factor hypoxia inducible factor 1. *Mechanisms* of respiratory control and regulation include adjustments of: (*1*) enzyme activities by allosteric mechanisms and phosphorylation; (*2*) enzyme content, concentrations of cofactors and conserved moieties—such as adenylates, nicotinamide adenine dinucleotide [NAD^+/NADH], coenzyme Q, cytochrome *c*); (*3*) metabolic channeling by supercomplexes; and *(4)* mitochondrial density (enzyme concentrations and membrane area) and morphology (cristae folding, fission and fusion). Mitochondria are targeted directly by hormones, thereby affecting their energy metabolism (Lee *et al.* 2013; Gerö and Szabo 2016; Price and Dai 2016; Moreno *et al.* 2017). Evolutionary or acquired differences in the genetic and epigenetic basis of mitochondrial function (or dysfunction) between subjects and gene therapy; age; gender, biological sex, and hormone concentrations; life style including exercise and nutrition; and environmental issues including thermal, atmospheric, toxicological and pharmacological factors, exert an influence on all control mechanisms listed above. For reviews, see Brown 1992; Gnaiger 1993a, 2009; 2014; Paradies *et al.* 2014; Morrow *et al.* 2017.

Respiratory control and response: Lack of control by a metabolic pathway, *e.g.*, phosphorylation-pathway, means that there will be no response to a variable activating it, *e.g.*, [ADP]. The reverse, however, is not true as the absence of a response to [ADP] does not exclude the phosphorylation-pathway from having some degree of control. The degree of control of a component of the OXPHOS-pathway on an output variable—such as O_2 flux, will in general be different from the degree of control on other outputs—such as phosphorylation-flux or proton leak flux. Therefore, it is necessary to be specific as to which input and output are under consideration (Fell 1997).

Respiratory coupling control and ET-pathway control: Respiratory control refers to the ability of mitochondria to adjust O_2 flux in response to external control signals by engaging various mechanisms of control and regulation. Respiratory control is monitored in a mitochondrial preparation under conditions defined as respiratory states. When phosphorylation of ADP to ATP is stimulated or depressed, an increase or decrease is observed in electron flux linked to O_2 flux in respiratory coupling states of intact mitochondria ('controlled states' in the classical terminology of bioenergetics). Alternatively, coupling of electron transfer with phosphorylation is disengaged by disruption of the integrity of the mtIM or by uncouplers, functioning like a clutch in a mechanical system. The corresponding coupling control state is characterized by high levels of O_2 consumption without control by P» ('uncontrolled state').

ET-pathway control states are obtained in mitochondrial preparations by depletion of endogenous substrates and addition to the mitochondrial respiration medium of fuel substrates (CHNO; 2[H] in **Fig. 2**) and specific inhibitors, activating selected mitochondrial catabolic pathways, k (**Fig. 1**). Coupling control states and pathway control states are complementary, since mitochondrial preparations depend on an exogenous supply of pathway-specific fuel substrates and oxygen (Gnaiger 2014).

Coupling: In mitochondrial electron transfer (**Fig. 1**), vectorial transmembrane proton flux is coupled through the proton pumps CI, CIII and CIV to the catabolic flux of scalar reactions, collectively measured as O_2 flux (**Fig. 2**). Thus mitochondria are elements of energy

is a conserved quantity and

transformation. Energy cannot be lost or produced in any internal process (First Law of thermodynamics). Open and closed systems can gain or loose energy only by external fluxes—by exchange with the environment. ~~Energy is a conserved quantity~~. Therefore, energy can neither be produced by mitochondria, nor is there any internal process without energy conservation. Exergy is defined as the 'free energy' with the potential to perform work. *Coupling* is the mechanistic linkage of an exergonic process (spontaneous, negative exergy change) with an endergonic process (positive exergy change) in energy transformations which conserve part of the exergy that would be irreversible lost or dissipated in an uncoupled process.

Uncoupling: Uncoupling of mitochondrial respiration is a general term comprising diverse mechanisms. Differences of terms—uncoupled *vs.* noncoupled—are easily overlooked, although they relate to different mechanisms of uncoupling (**Fig. 3**).

1. Proton leak across the mtIM from the pos– to the neg–compartment (**Fig. 2**);
2. Cycling of other cations, strongly stimulated by permeability transition;
3. Proton slip in the proton pumps when protons are effectively not pumped (CI, CIII and CIV) or are not driving phosphorylation (F-ATPase);
4. Loss of compartmental integrity when electron transfer is acoupled;
5. Electron leak in the loosely coupled univalent reduction of O_2 to superoxide ($O_2^{\bullet-}$; superoxide anion radical).

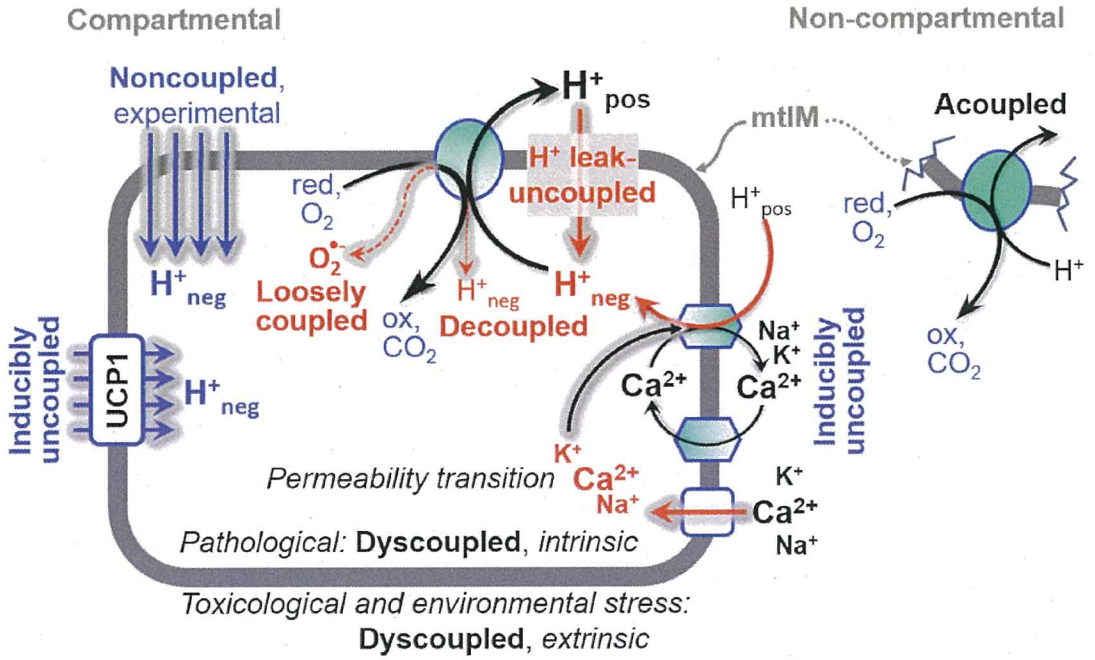
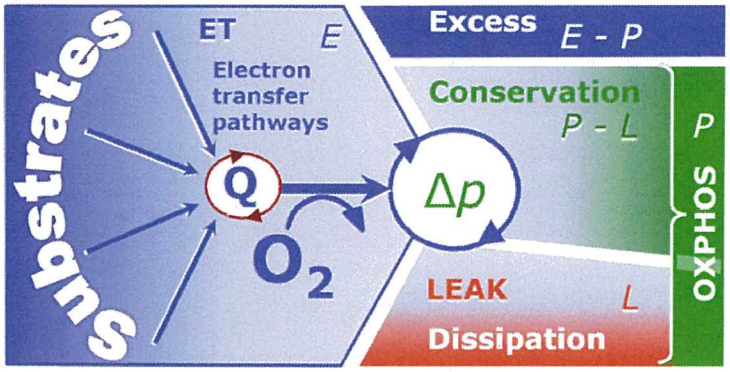


Fig 3. Mechanisms of respiratory uncoupling. An intact mitochondrial inner membrane, mtIM, is required for vectorial, compartmental coupling. 'Acoupled' respiration is the consequence of structural disruption with catalytic activity of non-compartmental mitochondrial fragments. Inducibly uncoupled (activation of UCP1) and experimentally noncoupled respiration (titration of protonophores) stimulate respiration to maximum O_2 flux. H^+ leak-uncoupled, decoupled, and loosely coupled respiration are components of intrinsic uncoupling. Pathological dysfunction may affect all types of uncoupling, including permeability transition, causing intrinsically dyscoupled respiration. Similarly, toxicological and environmental stress factors can cause extrinsically dyscoupled respiration.

2.2. Coupling states and respiratory rates

552 **Respiratory capacities in coupling control states:** To extend the classical nomenclature
553 on mitochondrial coupling states (Section 2.3) by a concept-driven terminology that
554 incorporates explicitly information on the nature of respiratory states, the terminology must be
555 general and not restricted to any particular experimental protocol or mitochondrial preparation
556 (Gnaiger 2009). We focus primarily on the conceptual 'why', along with clarification of the
557 experimental 'how'. Respiratory capacities delineate, comparable to channel capacity in
558 information theory (Schneider 2006), the upper bound of the rate of respiration measured in
559 defined coupling control states and electron transfer-pathway (ET-pathway) states (**Fig. 4**).
560
561 **Fig. 4. Four-compartment model of oxidative phosphorylation.** Respiratory
562 states (ET, OXPHOS, LEAK;
563 **Table 1**) and corresponding rates
564 (*E*, *P*, *L*) are connected by the
565 protonmotive force, Δp. ET-
566 capacity, *E*, is partitioned into (*1*)
567 dissipative LEAK-respiration, *L*,
568 when the Gibbs energy change of
569 catabolic O_2 flux is irreversibly
570 lost, (*2*) net OXPHOS-capacity, *P*-*L*,
571 with partial conservation of the
572 capacity to perform work,
573 and (*3*) the excess capacity, *E*-*P*.
574 Modified from Gnaiger (2014).



575 **Table 1. Coupling states and residual oxygen consumption in mitochondrial**
576 **preparations in relation to respiration- and phosphorylation-flux, J_{kO_2} and $J_{P»}$,**
577 **and protonmotive force, Δp.** Coupling states are established at kinetically-saturating
578 concentrations of fuel substrates and O_2.

State	J_{kO_2}	$J_{P»}$	Δp	Inducing factors	Limiting factors
LEAK	*L*; low, cation leak-dependent respiration	0	max.	proton leak, slip, and cation cycling	$J_{P»} = 0$: (*1*) without ADP, L_N; (*2*) max. ATP/ADP ratio, L_T; or (*3*) inhibition of the phosphorylation-pathway, L_{Omy}
OXPHOS	*P*; high, ADP-stimulated respiration	max.	high	kinetically-saturating [ADP] and [P_i]	$J_{P»}$ by phosphorylation-pathway; or J_{kO_2} by ET-capacity
ET	*E*; max., noncoupled respiration	0	low	optimal external uncoupler concentration for max. $J_{O_2,E}$	J_{kO_2} by ET-capacity
ROX	*Rox*; min., residual O_2 consumption	0	0	$J_{O_2,Rox}$ in non-ET-pathway oxidation reactions	full inhibition of ET-pathway; or absence of fuel substrates

579
580 To provide a diagnostic reference for respiratory capacities of core energy metabolism,
581 the capacity of *oxidative phosphorylation*, OXPHOS, is measured at kinetically-saturating
582 concentrations of ADP and P_i. The *oxidative* ET-capacity reveals the limitation of OXPHOS-

583 capacity mediated by the *phosphorylation*-pathway. The ET- and phosphorylation-pathways
584 comprise coupled segments of the OXPHOS-system. ET-capacity is measured as noncoupled
585 respiration by application of *external uncouplers*. The contribution of *intrinsically uncoupled*
586 O_2 consumption is studied in the absence of ADP—by not stimulating phosphorylation, or by
587 inhibition of the phosphorylation-pathway. The corresponding states are collectively classified
588 as LEAK-states, when O_2 consumption compensates mainly for ion leaks, including the proton
589 leak. Defined coupling states are induced by: (*1*) adding cation chelators such as EGTA, binding
590 free Ca^{2+} and thus limiting cation cycling; (*2*) adding ADP and P_i; (*3*) inhibiting the
591 phosphorylation-pathway; and (*4*) uncoupler titrations, while maintaining a defined ET-
592 pathway state with constant fuel substrates and inhibitors of specific branches of the ET-
593 pathway (**Fig. 1**).
594 The three coupling states, ET, LEAK and OXPHOS, are shown schematically with the
595 corresponding respiratory rates, abbreviated as *E*, *L* and *P*, respectively (**Fig. 4**). We distinguish
596 metabolic *pathways* from metabolic *states* and the corresponding metabolic *rates*; for example:
597 ET-pathways (**Fig. 4**), ET-state (**Fig. 5C**), and ET-capacity, *E*, respectively (**Table 1**). The
598 protonmotive force is *high* in the OXPHOS-state when it drives phosphorylation, *maximum* in
599 the LEAK-state of coupled mitochondria, driven by LEAK-respiration at a minimum back flux
600 of cations to the matrix side, and *very low* in the ET-state when uncouplers short-circuit the
601 proton cycle (**Table 1**).
602 *E* may exceed or be equal to *P*. $E > P$ is observed in many types of mitochondria, varying
603 between species, tissues and cell types (Gnaiger 2009). *E-P* is the excess ET-capacity pushing
604 the phosphorylation-flux (**Fig. 1B**) to the limit of its *capacity of utilizing* the protonmotive force.
605 In addition, the magnitude of *E-P* depends on the tightness of respiratory coupling or degree of
606 uncoupling, since an increase of *L* causes *P* to increase towards the limit of *E*. The *excess E-P*
607 capacity, *E-P*, therefore, provides a sensitive diagnostic indicator of specific injuries of the
608 phosphorylation-pathway, under conditions when *E* remains constant but *P* declines relative to
609 controls (**Fig. 4**). Substrate cocktails supporting simultaneous convergent electron transfer to
610 the Q-junction for reconstitution of TCA cycle function establish pathway control states with
611 high ET-capacity, and consequently increase the sensitivity of the *E-P* assay.
612 *E* cannot theoretically be lower than *P*. $E < P$ must be discounted as an artefact, which
613 may be caused experimentally by: (*1*) loss of oxidative capacity during the time course of the
614 respirometric assay, since *E* is measured subsequently to *P*; (*2*) using insuffient uncoupler
615 concentrations; (*3*) using high uncoupler concentrations which inhibit ET (Gnaiger 2008); (*4*)
616 high oligomycin concentrations applied for measurement of *L* before titrations of uncoupler,
617 when oligomycin exerts an inhibitory effect on *E*. On the other hand, the excess ET-capacity is
618 overestimated if non-saturating [ADP] or [P_i] are used. See State 3 in the next section.
619 The net OXPHOS-capacity is calculated by subtracting *L* from *P* (**Fig. 4**). Then the net
620 P»/O_2 equals P»/(*P-L*), wherein the dissipative LEAK component in the OXPHOS-state may
621 be overestimated. This can be avoided by measuring LEAK-respiration in a state when the
622 protonmotive force is adjusted to its slightly lower value in the OXPHOS-state—by titration of
623 an ET inhibitor (Divakaruni and Brand 2011). Any turnover-dependent components of proton
624 leak and slip, however, are underestimated under these conditions (Garlid *et al.* 1993). In
625 general, it is inappropriate to use the term *ATP production* or *ATP turnover* for the difference
626 of O_2 flux measured in states *P* and *L*. The difference *P-L* is the upper limit of the part of
627 OXPHOS-capacity that is freely available for ATP production (corrected for LEAK-
628 respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry
629 (**Fig. 4**).
630 **LEAK-state** (**Fig. 5A**): The LEAK-state is defined as a state of mitochondrial respiration
631 when O_2 flux mainly compensates for ion leaks in the absence of ATP synthesis, at kinetically-
632 saturating concentrations of O_2 and respiratory fuel substrates. LEAK-respiration is measured
633 to obtain an estimate of *intrinsic uncoupling* without addition of an experimental uncoupler: (*1*)

in the absence of adenylates; (*2*) after depletion of ADP at a maximum ATP/ADP ratio; or (*3*) after inhibition of the phosphorylation-pathway by inhibitors of F-ATPase—such as oligomycin, or of adenine nucleotide translocase—such as carboxyatractyloside. Adjustment of the nominal concentration of these inhibitors to the density of biological sample applied can minimize or avoid inhibitory side-effects exerted on ET-capacity or even some dyscoupling.

Proton leak and uncoupled respiration: Proton leak is a leak current of protons. The intrinsic proton leak is the *uncoupled* process in which protons diffuse across the mtIM in the dissipative direction of the downhill protonmotive force without coupling to phosphorylation (**Fig. 5A**). The proton leak flux depends non-linearly on the protonmotive force (Garlid *et al.* 1989; Divakaruni and Brand 2011), it is a property of the mtIM and may be enhanced due to possible contaminations by free fatty acids. Inducible uncoupling mediated by uncoupling protein 1 (UCP1) is physiologically controlled, *e.g.*, in brown adipose tissue. UCP1 is a member of the mitochondrial carrier family which is involved in the translocation of protons across the mtIM (Klingenberg 2017). Consequently, the short-circuit diminishes the protonmotive force and stimulates electron transfer to O_2 and heat dissipation without phosphorylation of ADP.

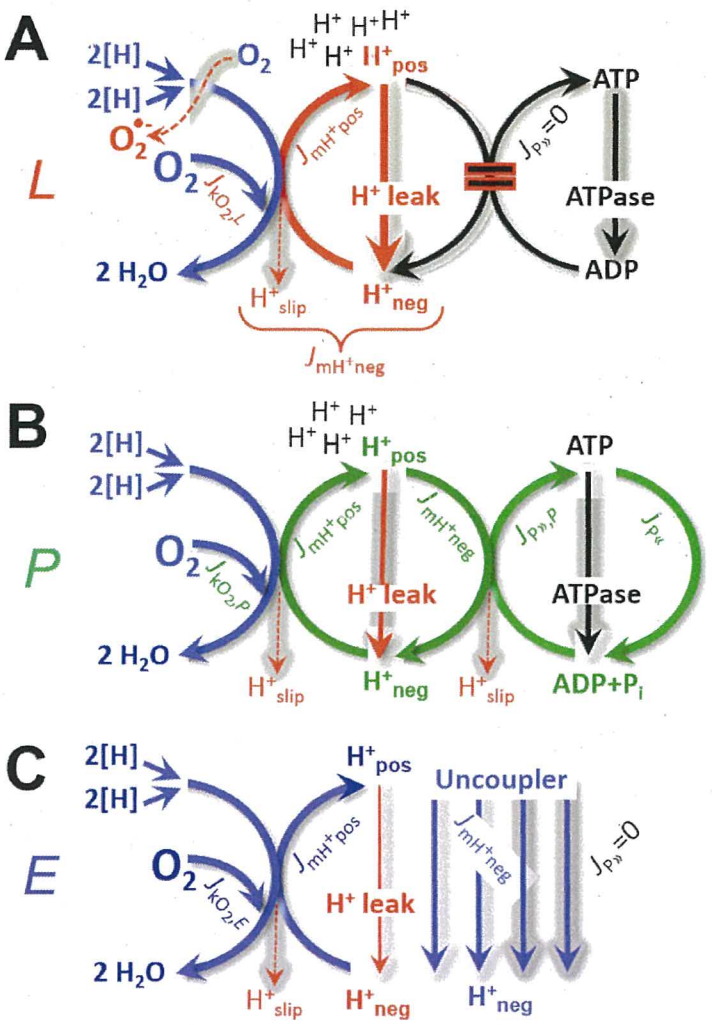


Fig. 5. Respiratory coupling states. A: LEAK-state and rate, *L*: Phosphorylation is arrested, $J_{P\text{»}} = 0$, and catabolic O_2 flux, $J_{kO_2,L}$, is controlled mainly by the proton leak, $J_{mH^+neg,L}$, at maximum protonmotive force (**Fig. 3**). **B: OXPHOS-state and rate, *P*:** Phosphorylation, $J_{P\text{»}}$, is stimulated by kinetically-saturating [ADP] and [P_i], and is supported by a high protonmotive force. O_2 flux, $J_{kO_2,P}$, is well-coupled at a $P\text{»}/O_2$ ratio of $J_{P\text{»},P}/J_{O_2,P}$. **C: ET-state and rate, *E*:** Noncoupled respiration, $J_{kO2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{P\text{»}} = 0$. See also **Fig. 2**.

Cation cycling: There can be other cation contributors to leak current including calcium and probably magnesium. Calcium current is balanced by mitochondrial Na^+/Ca^{2+} exchange, which is balanced by Na^+/H^+ or K^+/H^+ exchanges. This is another effective uncoupling mechanism different from proton leak.

Table 2. Terms on respiratory coupling and uncoupling.

Term		J_{kO_2}	P»/O_2	Note
acoupled			0	electron transfer in mitochondrial fragments without vectorial proton translocation (**Fig. 3**)
intrinsic, no protonophore added	uncoupled	*L*	0	non-phosphorylating LEAK-respiration (**Fig. 5A**)
	proton leak-uncoupled		0	component of *L*, H^+ diffusion across the mtIM (**Fig. 3**)
	decoupled		0	component of *L*, proton slip (**Fig. 3**)
	loosely coupled		0	component of *L*, lower coupling due to superoxide formation and bypass of proton pumps (**Fig. 3**)
	dyscoupled		0	pathologically, toxicologically, environmentally increased uncoupling, mitochondrial dysfunction
	inducibly uncoupled		0	by UCP1 or cation (*e.g.*, Ca^{2+}) cycling (**Fig. 3**)
noncoupled		*E*	0	non-phosphorylating respiration stimulated to maximum flux at optimum exogenous uncoupler concentration (**Fig. 5C**)
well-coupled		*P*	high	phosphorylating respiration with an intrinsic LEAK component (**Fig. 5B**)
fully coupled		*P* – *L*	max.	OXPHOS-capacity corrected for LEAK-respiration (**Fig. 4**)

Proton slip and decoupled respiration: Proton slip is the *decoupled* process in which protons are only partially translocated by a proton pump of the ET-pathways and slip back to the original compartment. The proton leak is the dominant contributor to the overall leak current in mammalian mitochondria incubated under physiological conditions at 37 °C, whereas proton slip is increased at lower experimental temperature (Canton *et al.* 1995). Proton slip can also happen in association with the F-ATPase, in which the proton slips downhill across the pump to the matrix without contributing to ATP synthesis. In each case, proton slip is a property of the proton pump and increases with the pump turnover rate.

Electron leak and loosely coupled respiration: Superoxide production by the ETS leads to a bypass of proton pumps and correspondingly lower P»/O_2 ratio. This depends on the actual site of electron leak and the scavenging of hydrogen peroxide by cytochrome *c*, whereby electrons may re-enter the ETS with proton translocation by CIV.

Loss of compartmental integrity and acoupled respiration: Electron transfer and catabolic O_2 flux proceed without compartmental proton translocation in disrupted mitochondrial fragments. Such fragments form during mitochondrial isolation, and may not fully fuse to re-establish structurally intact mitochondria. Loss of mtIM integrity, therefore, is the cause of acoupled respiration, which is a nonvectorial dissipative process without control by the protonmotive force.

Dyscoupled respiration: Mitochondrial injuries may lead to *dyscoupling* as a pathological or toxicological cause of *uncoupled* respiration. Dyscoupling may involve any type of uncoupling mechanism, *e.g.*, opening the permeability transition pore. Dyscoupled respiration is distinguished from the experimentally induced *noncoupled* respiration in the ET-state (**Fig. 3**).

OXPHOS-state (**Fig. 5B**): The OXPHOS-state is defined as the respiratory state with kinetically-saturating concentrations of O_2, respiratory and phosphorylation substrates, and

absence of exogenous uncoupler, which provides an estimate of the maximal respiratory capacity in the OXPHOS-state for any given ET-pathway state. Respiratory capacities at kinetically-saturating substrate concentrations provide reference values or upper limits of performance, aiming at the generation of data sets for comparative purposes. Physiological activities and effects of substrate kinetics can be evaluated relative to the OXPHOS-capacity.

As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria (Gnaiger 2001; Puchowicz *et al.* 2004); greater ADP concentration is required, particularly in permeabilized muscle fibres and cardiomyocytes, to overcome limitations by intracellular diffusion and by the reduced conductance of the mtOM (Jepihhina *et al.* 2011, Illaste *et al.* 2012, Simson *et al.* 2016), either through interaction with tubulin (Rostovtseva *et al.* 2008) or other intracellular structures (Birkedal *et al.* 2014). In permeabilized muscle fibre bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks *et al.* 1998), consistent with experimental evidence that >90% saturation is reached only at >5 mM ADP (Pesta and Gnaiger 2012). Similar ADP concentrations are also required for accurate determination of OXPHOS-capacity in human clinical cancer samples and permeabilized cells (Klepinin *et al.* 2016; Koit *et al.* 2017). Whereas 2.5 to 5 mM ADP is sufficient to obtain the actual OXPHOS-capacity in many types of permeabilized tissue and cell preparations, experimental validation is required in each specific case.

Electron transfer-state (Fig. 5C): The ET-state is defined as the *noncoupled* state with kinetically-saturating concentrations of O_2, respiratory substrate and optimum *exogenous* uncoupler concentration for maximum O_2 flux, as an estimate of ET-capacity. Inhibition of respiration is observed at higher than optimum uncoupler concentrations. As a consequence of the nearly collapsed protonmotive force, the driving force is insufficient for phosphorylation, and $J_{P\text{»}} = 0$.

ROX state and *Rox*: Besides the three fundamental coupling states of mitochondrial preparations, the state of residual O_2 consumption, ROX, is relevant to assess respiratory function. ROX is not a coupling state. The rate of residual oxygen consumption, *Rox*, is defined as O_2 consumption due to oxidative side reactions remaining after inhibition of ET—with rotenone, malonic acid and antimycin A. Cyanide and azide inhibit CIV and several peroxidases involved in *Rox*. ROX represents a baseline that is used to correct mitochondrial respiration in defined coupling states. *Rox* is not necessarily equivalent to non-mitochondrial respiration, considering O_2-consuming reactions in mitochondria not related to ET—such as O_2 consumption in reactions catalyzed by monoamine oxidases (type A and B), monooxygenases (cytochrome P450 monooxygenases), dioxygenase (sulfur dioxygenase and trimethyllysine dioxygenase), and several hydoxylases. Mitochondrial preparations, especially those obtained from liver, may be contaminated by peroxisomes. This fact makes the exact determination of mitochondrial O_2 consumption and mitochondria-associated generation of reactive oxygen species complicated (Schönfeld *et al.* 2009). The dependence of ROX-linked O_2 consumption needs to be studied in detail together with non-ET enzyme activities, availability of specific substrates, O_2 concentration, and electron leakage leading to the formation of reactive oxygen species.

2.3. Classical terminology for isolated mitochondria

> *'When a code is familiar enough, it ceases appearing like a code; one forgets that there is a decoding mechanism. The message is identical with its meaning'* (Hofstadter 1979).

Chance and Williams (1955; 1956) introduced five classical states of mitochondrial respiration and cytochrome redox states. **Table 3** shows a protocol with isolated mitochondria in a closed respirometric chamber, defining a sequence of respiratory states. States and rates are not specifically distinguished in this nomenclature.

Table 3. Metabolic states of mitochondria (Chance and Williams, 1956; Table V).

State	[O_2]	ADP level	Substrate level	Respiration rate	Rate-limiting substance
1	>0	low	low	slow	ADP
2	>0	high	~0	slow	substrate
3	>0	high	high	fast	respiratory chain
4	>0	low	high	slow	ADP
5	0	high	high	0	oxygen

State 1 is obtained after addition of isolated mitochondria to air-saturated isoosmotic/isotonic respiration medium containing P_i, but no fuel substrates and no adenylates, *i.e.*, AMP, ADP, ATP.

State 2 is induced by addition of a 'high' concentration of ADP (typically 100 to 300 μM), which stimulates respiration transiently on the basis of endogenous fuel substrates and phosphorylates only a small portion of the added ADP. State 2 is then obtained at a low respiratory activity limited by exhausted endogenous fuel substrate availability (**Table 3**). If addition of specific inhibitors of respiratory complexes—such as rotenone—does not cause a further decline of O_2 flux, State 2 is equivalent to the ROX state (See below.). If inhibition is observed, undefined endogenous fuel substrates are a confounding factor of pathway control, contributing to the effect of subsequently externally added substrates and inhibitors. In contrast to the original protocol, an alternative sequence of titration steps is frequently applied, in which the alternative 'State 2' has an entirely different meaning, when this second state is induced by addition of fuel substrate without ADP (LEAK-state; in contrast to State 2 defined in **Table 1** as a ROX state), followed by addition of ADP.

State 3 is the state stimulated by addition of fuel substrates while the ADP concentration is still high (**Table 3**) and supports coupled energy transformation through oxidative phosphorylation. 'High ADP' is a concentration of ADP specifically selected to allow the measurement of State 3 to State 4 transitions of isolated mitochondria in a closed respirometric chamber. Repeated ADP titration re-establishes State 3 at 'high ADP'. Starting at O_2 concentrations near air-saturation (ca. 200 μM O_2 at sea level and 37 °C), the total ADP concentration added must be low enough (typically 100 to 300 μM) to allow phosphorylation to ATP at a coupled O_2 flux that does not lead to O_2 depletion during the transition to State 4. In contrast, kinetically-saturating ADP concentrations usually are 10-fold higher than 'high ADP', *e.g.*, 2.5 mM in isolated mitochondria. The abbreviation State 3u is occasionally used in bioenergetics, to indicate the state of respiration after titration of an uncoupler, without sufficient emphasis on the fundamental difference between OXPHOS-capacity (*well-coupled* with an *endogenous* uncoupled component) and ET-capacity (*noncoupled*).

State 4 is a LEAK-state that is obtained only if the mitochondrial preparation is intact and well-coupled. Depletion of ADP by phosphorylation to ATP leads to a decline of O_2 flux in the transition from State 3 to State 4. Under these conditions of State 4, a maximum protonmotive force and high ATP/ADP ratio are maintained. For calculation of $P\gg/O_2$ ratios the gradual decline of $Y_{P\gg/O_2}$ towards diminishing [ADP] at State 4 must be taken into account (Gnaiger 2001). State 4 respiration, L_T (**Table 1**), reflects intrinsic proton leak and intrinsic ATP hydrolysis activity. O_2 flux in State 4 is an overestimation of LEAK-respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, $J_{P\ll}$, which stimulates respiration coupled to phosphorylation, $J_{P\gg} > 0$. This can be tested by inhibition of the phosphorylation-pathway using oligomycin, ensuring that $J_{P\gg} = 0$ (State 4o). Alternatively, sequential ADP titrations re-establish State 3, followed by State 3 to State 4 transitions while

sufficient O_2 is available. Anoxia may be reached, however, before exhaustion of ADP (State 5).

State 5 is the state after exhaustion of O_2 in a closed respirometric chamber. Diffusion of O_2 from the surroundings into the aqueous solution may be a confounding factor preventing complete anoxia (Gnaiger 2001). Chance and Williams (1955) provide an alternative definition of State 5, which gives it the different meaning of ROX versus anoxia: 'State 5 may be obtained by antimycin A treatment or by anaerobiosis'.

In **Table 3**, only States 3 and 4 (and 'State 2' in the alternative protocol: addition of fuel substrates without ADP; not included in the table) are coupling control states, with the restriction that O_2 flux in State 3 may be limited kinetically by non-saturating ADP concentrations (**Table 1**).

3. Normalization: fluxes and flows

3.1. Normalization: system or sample

The term *rate* is not sufficiently defined to be useful for reporting data (**Fig. 6**). The inconsistency of the meanings of rate becomes fully apparent when considering Galileo Galilei's famous principle, that 'bodies of different weight all fall at the same rate (have a constant acceleration)' (Coopersmith 2010).

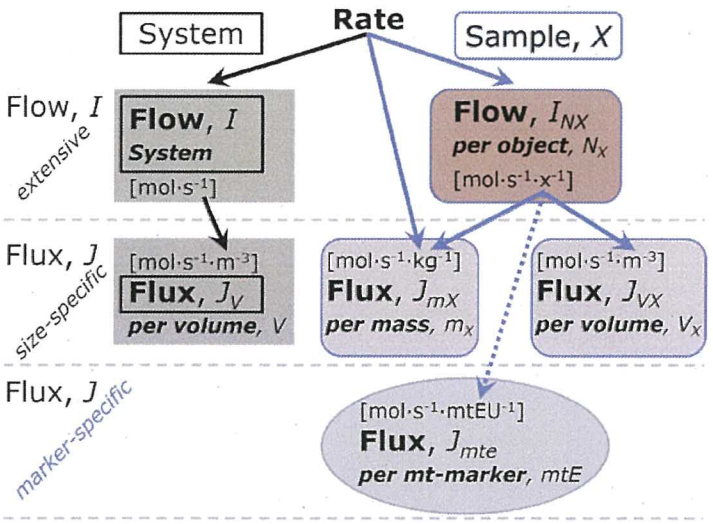


Fig. 6. Different meanings of *rate* may lead to confusion, if the normalization is not sufficiently specified. Results are frequently expressed as mass-specific *flux*, J_{mX}, per mg protein, dry or wet weight (mass). Cell volume, V_{cell}, may be used for normalization (volume-specific flux, J_{Vcell}), which must be clearly distinguished from flow per cell, I_{Ncell}, or flux, J_V, expressed for methodological reasons per volume of the measurement system. For details see **Table 4**.

Flow per system, *I*: In a generalization of electrical terms, flow as an extensive quantity (I; per system) is distinguished from flux as a size-specific quantity (J; per system size) (**Fig. 6**). Electric current is flow, I_{el} [$A \equiv C \cdot s^{-1}$] per system (extensive quantity). When dividing this extensive quantity by system size (cross-sectional area of a 'wire'), a size-specific quantity is obtained, which is flux (current density), $\boldsymbol{J}_{el}$ [$A \cdot m^{-2} = C \cdot s^{-1} \cdot m^{-2}$].

Extensive quantities: An extensive quantity increases proportionally with system size. The magnitude of an extensive quantity is completely additive for non-interacting subsystems—such as mass or flow expressed per defined system. The magnitude of these quantities depends on the extent or size of the system (Cohen *et al.* 2008).

Size-specific quantities: 'The adjective *specific* before the name of an extensive quantity is often used to mean *divided by mass*' (Cohen *et al.* 2008). In this system-paradigm, mass-specific flux is flow divided by mass of the *system* (the total mass of everything within the measuring chamber or reactor). A mass-specific quantity is independent of the extent of non-interacting homogenous subsystems. Tissue-specific quantities (related to the *sample* in

contrast to the *system*) are of fundamental interest in comparative mitochondrial physiology, where *specific* refers to the *type of the sample* rather than *mass of the system*. The term *specific*, therefore, must be clarified; *sample*-specific, *e.g.*, muscle mass-specific normalization, is distinguished from *system*-specific quantities (mass or volume; **Fig. 6**).

Box 2: Metabolic fluxes and flows: vectorial and scalar

Fluxes are *vectors*, if they have *spatial* geometric direction in addition to magnitude. Electric charge per unit time is electric flow or current, $I_{el} = dQ_{el} \cdot dt^{-1}$ [A]. When expressed per unit cross-sectional area, A [m^2], a vector flux is obtained, which is current density or surface-density of flow) perpendicular to the direction of flux, $\boldsymbol{J}_{el} = I_{el} \cdot A^{-1}$ [$A \cdot m^{-2}$] (Cohen et al. 2008). For all transformations *flows*, I_{tr}, are defined as extensive quantities. Vector and scalar *fluxes* are obtained as $\boldsymbol{J}_{tr} = I_{tr} \cdot A^{-1}$ [$mol \cdot s^{-1} \cdot m^{-2}$] and $J_{tr} = I_{tr} \cdot V^{-1}$ [$mol \cdot s^{-1} \cdot m^{-3}$], expressing flux as an area-specific vector or volume-specific vectorial or scalar quantity, respectively (Gnaiger 1993b).

We suggest to define: (*1*) *vectoral* fluxes, which are translocations as functions of *gradients* with direction in geometric space in continuous systems; (*2*) *vectorial* fluxes, which describe translocations in discontinuous systems and are restricted to information on *compartmental differences* (**Fig. 2**, transmembrane proton flux); and (*3*) *scalar* fluxes, which are transformations in a *homogenous* system (**Fig. 2**, catabolic O_2 flux, J_{kO_2}).

Vectorial transmembrane proton fluxes, J_{mH^+pos} and J_{mH^+neg}, are analyzed in a heterogenous compartmental system as a quantity with *directional* but not *spatial* information. Translocation of protons across the mtIM has a defined direction, either from the negative compartment (matrix space; negative, neg–compartment) to the positive compartment (inter-membrane space; positive, pos–compartment) or *vice versa* (**Fig. 2**). The arrows defining the direction of the translocation between the two compartments may point upwards or downwards, right or left, without any implication that these are actual directions in space. The pos–compartment is neither above nor below the neg–compartment in a spatial sense, but can be visualized arbitrarily in a figure in the upper position (**Fig. 2**). In general, the *compartmental direction* of vectorial translocation from the neg–compartment to the pos–compartment is defined by assigning the initial and final state as *ergodynamic compartments*, $H^+_{neg} \rightarrow H^+_{pos}$ or $0 = -1\ H^+_{neg} + 1\ H^+_{pos}$, related to work (erg = work) that must be performed to lift the proton from a lower to a higher electrochemical potential or from the lower to the higher ergodynamic compartment (Gnaiger 1993b).

In analogy to *vectorial* translocation, the direction of a *scalar* chemical reaction, $A \rightarrow B$ or $0 = -1\ A + 1\ B$, is defined by assigning substrates and products, A and B, as ergodynamic compartments. O_2 is defined as a substrate in respiratory O_2 consumption, which together with the fuel substrates comprises the substrate compartment of the catabolic reaction (**Fig. 2**). Volume-specific scalar O_2 flux is coupled to vectorial translocation, yielding the H^+_{pos}/O_2 ratio (**Fig. 1**).

3.2. Normalization for system-size: flux per chamber volume

System-specific flux, J_{V,O_2}: The experimental system (experimental chamber) is part of the measurement apparatus, separated from the environment as an isolated, closed, open, isothermal or non-isothermal system (**Table 4**). On another level, we distinguish between (*1*) the *system* with volume V and mass m defined by the system boundaries, and (*2*) the *sample* or *objects* with volume V_X and mass m_X which are enclosed in the experimental chamber (**Fig. 6**). Metabolic O_2 flow per object, $I_{O_2/X}$, increases as the mass of the object is increased. Sample mass-specific O_2 flux, $J_{O_2/mX}$ should be independent of the mass of the sample studied in the instrument chamber, but system volume-specific O_2 flux, J_{V,O_2} (per volume of the instrument

that

chamber), should increase in direct proportion to the mass of the sample in the chamber. Whereas J_{V,O_2} depends on mass-concentration of the sample in the chamber, it should be independent of the chamber (system) volume at constant sample mass. There are practical limitations to increase the mass-concentration of the sample in the chamber, when one is concerned about crowding effects and instrumental time resolution.

When the reactor volume does not change during the reaction, which is typical for liquid phase reactions, the volume-specific *flux of a chemical reaction* r is the time derivative of the advancement of the reaction per unit volume, $J_{V,rB} = d_r\xi_B/dt \cdot V^{-1}$ [(mol·s^{-1})·L^{-1}]. The *rate of concentration change* is dc_B/dt [(mol·L^{-1})·s^{-1}], where concentration is $c_B = n_B/V$. There is a difference between (*1*) J_{V,rO_2} [mol·s^{-1}·L^{-1}] and (*2*) rate of concentration change [mol·L^{-1}·s^{-1}]. These merge to a single expression only in closed systems. In open systems, external fluxes (such as O_2 supply) are distinguished from internal transformations (catabolic flux, O_2 consumption). In a closed system, external flows of all substances are zero and O_2 consumption (internal flow of catabolic reactions k), I_{kO_2} [pmol·s^{-1}], causes a decline of the amount of O_2 in the system, n_{O_2} [nmol]. Normalization of these quantities for the volume of the system, V [L ≡ dm^3], yields volume-specific O_2 flux, $J_{V,kO_2} = I_{kO_2}/V$ [nmol·s^{-1}·L^{-1}], and O_2 concentration, [O_2] or $c_{O_2} = n_{O_2}/V$ [μmol·L^{-1} = μM = nmol·mL^{-1}]. Instrumental background O_2 flux is due to external flux into a non-ideal closed respirometer; then total volume-specific flux has to be corrected for instrumental background O_2 flux—O_2 diffusion into or out of the instrumental chamber. J_{V,kO_2} is relevant mainly for methodological reasons and should be compared with the accuracy of instrumental resolution of background-corrected flux, *e.g.*, ±1 nmol·s^{-1}·L^{-1} (Gnaiger 2001). 'Metabolic' or catabolic indicates O_2 flux, J_{kO_2}, corrected for: (*1*) instrumental background O_2 flux; (*2*) chemical background O_2 flux due to autoxidation of chemical components added to the incubation medium; and (*3*) *Rox* for O_2-consuming side reactions unrelated to the catabolic pathway k.

3.3. Normalization: per sample

The challenges of measuring mitochondrial respiratory flux are matched by those of normalization. Application of common and defined units is required for direct transfer of reported results into a database. The second [s] is the *SI* unit for the base quantity *time*. It is also the standard time-unit used in solution chemical kinetics. A rate may be considered as the numerator and normalization as the complementary denominator, which are tightly linked in reporting the measurements in a format commensurate with the requirements of a database. Normalization (**Table 4**) is guided by physicochemical principles, methodological considerations, and conceptual strategies (**Fig. 7**).

Sample concentration, C_{mX}: Normalization for sample concentration is required to report respiratory data. Considering a tissue or cells as the sample, X, the sample mass is m_X [mg], which is frequently measured as wet or dry weight, W_w or W_d [mg], or as amount of tissue or cell protein, $m_{Protein}$. In the case of permeabilized tissues, cells, and homogenates, the sample concentration, $C_{mX} = m_X/V$ [g·L^{-1} = mg·mL^{-1}], is the mass of the subsample of tissue that is transferred into the instrument chamber.

Mass-specific flux, $J_{O_2/mX}$: Mass-specific flux is obtained by expressing respiration per mass of sample, m_X [mg]. X is the type of sample—isolated mitochondria, tissue homogenate, permeabilized fibres or cells. Volume-specific flux is divided by mass concentration of X, $J_{O_2/mX} = J_{V,O_2}/C_{mX}$; or flow per cell is divided by mass per cell, $J_{O_2/mcell} = I_{O_2/cell}/M_{cell}$. If mass-specific O_2 flux is constant and independent of sample size (expressed as mass), then there is no interaction between the subsystems. A 1.5 mg and a 3.0 mg muscle sample respires at identical mass-specific flux. Mass-specific O_2 flux, however, may change with the mass of a tissue sample, cells or isolated mitochondria in the measuring chamber, in which the nature of the interaction becomes an issue. Therefore, cell density must be optimized, particularly in

experiments carried out in wells, considering the confluency of the cell monolayer or clumps of cells (Salabei *et al.* 2014).

Number concentration, C_{NX}: C_{NX} is the experimental *number concentration* of sample X. In the case of cells or animals, *e.g.*, nematodes, $C_{NX} = N_X/V$ [x·L^{-1}], where N_X is the number of cells or organisms in the chamber (**Table 4**).

Table 4. Sample concentrations and normalization of flux.

Expression	Symbol	Definition	Unit	Notes
Sample				
identity of sample	X	object: cell, tissue, animal, patient		
number of sample entities X	N_X	number of objects	x	
mass of sample X	m_X		kg	1
mass of object X	M_X	$M_X = m_X \cdot N_X^{-1}$	kg·x^{-1}	1
Mitochondria				
Mitochondria	mt	X = mt		
amount of mt-elements	mtE	quantity of mt-marker	mtEU	
Concentrations				
object number concentration	C_{NX}	$C_{NX} = N_X \cdot V^{-1}$	x·m^{-3}	2
sample mass concentration	C_{mX}	$C_{mX} = m_X \cdot V^{-1}$	kg·m^{-3}	
mitochondrial concentration	C_{mtE}	$C_{mtE} = mtE \cdot V^{-1}$	mtEU·m^{-3}	3
specific mitochondrial density	D_{mtE}	$D_{mtE} = mtE \cdot m_X^{-1}$	mtEU·kg^{-1}	4
mitochondrial content, mtE per object X	mtE_X	$mtE_X = mtE \cdot N_X^{-1}$	mtEU·x^{-1}	5
O_2 flow and flux				6
flow, system	I_{O_2}	internal flow	mol·s^{-1}	7
volume-specific flux	J_{V,O_2}	$J_{V,O_2} = I_{O_2} \cdot V^{-1}$	mol·s^{-1}·m^{-3}	8
flow per object X	$I_{O_2/X}$	$I_{O_2/X} = J_{V,O_2} \cdot C_{NX}^{-1}$	mol·s^{-1}·x^{-1}	9
mass-specific flux	$J_{O_2/mX}$	$J_{O_2/mX} = J_{V,O_2} \cdot C_{mX}^{-1}$	mol·s^{-1}·kg^{-1}	
mitochondria-specific flux	$J_{O_2/mtE}$	$J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$	mol·s^{-1}·mtEU^{-1}	10

1 The *SI* prefix k is used for the SI base unit of mass (kg = 1,000 g). In praxis, various *SI* prefixes are used for convenience, to make numbers easily readable, *e.g.,* 1 mg tissue, cell or mitochondrial mass instead of 0.000001 kg.

2 In case sample X = cells, the object number concentration is $C_{Ncell} = N_{cell} \cdot V^{-1}$, and volume may be expressed in [dm^3 ≡ L] or [cm^3 = mL]. See **Table 5** for different object types.

3 mt-concentration is an experimental variable, dependent on sample concentration: (*1*) $C_{mtE} = mtE \cdot V^{-1}$; (*2*) $C_{mtE} = mtE_X \cdot C_{NX}$; (*3*) $C_{mtE} = C_{mX} \cdot D_{mtE}$.

4 If the amount of mitochondria, mtE, is expressed as mitochondrial mass, then D_{mtE} is the mass fraction of mitochondria in the sample. If mtE is expressed as mitochondrial volume, V_{mt}, and the mass of sample, m_X, is replaced by volume of sample, V_X, then D_{mtE} is the volume fraction of mitochondria in the sample.

5 $mtE_X = mtE \cdot N_X^{-1} = C_{mtE} \cdot C_{NX}^{-1}$.

6 O_2 can be replaced by other chemicals B to study different reactions, *e.g.,* ATP, H_2O_2, or compartmental translocations, *e.g.,* Ca^{2+}.

7 I_{O_2} and V are defined per instrument chamber as a system of constant volume (and constant temperature), which may be closed or open. I_{O_2} is abbreviated for I_{rO_2}, *i.e.*, the metabolic or internal O_2 flow of the chemical reaction r in which O_2 is consumed, hence the negative stoichiometric number, $\nu_{O_2} = -1$. $I_{rO_2} = d_r n_{O_2}/dt \cdot \nu_{O_2}^{-1}$. If r includes all chemical reactions in which O_2 participates, then $d_r n_{O_2} = dn_{O_2} - d_e n_{O_2}$, where dn_{O_2} is the change in the amount of O_2 in the instrument chamber and $d_e n_{O_2}$

is the amount of O_2 added externally to the system. At steady state, by definition $dn_{O_2} = 0$, hence $d_r n_{O_2} = -d_e n_{O_2}$.

8 J_{V,O_2} is an experimental variable, expressed per volume of the instrument chamber.

9 $I_{O_2/X}$ is a physiological variable, depending on the size of entity X.

10 There are many ways to normalize for a mitochondrial marker, that are used in different experimental approaches: (*1*) $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$; (*2*) $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mX}^{-1} \cdot D_{mtE}^{-1} = J_{O_2/mX} \cdot D_{mtE}^{-1}$; (*3*) $J_{O_2/mtE} = J_{V,O_2} \cdot C_{NX}^{-1} \cdot mtE_X^{-1} = I_{O_2/X} \cdot mtE_X^{-1}$; (*4*) $J_{O_2/mtE} = I_{O_2} \cdot mtE^{-1}$. The mt-elemental unit [mtEU] varies between different mt-markers.

Table 5. Sample types, *X*, abbreviations, and quantification.

Identity of sample mitochondrial preparation	*X* mt-prep	N_X [x]	Mass[a] [kg]	Volume [m^3]	mt-Marker [mtEU]
isolated mitochondria	imt		m_{mt}	V_{mt}	mtE
tissue homogenate	thom		m_{thom}		mtE_{thom}
permeabilized tissue	pti		m_{pti}		mtE_{pti}
permeabilized fibre	pfi		m_{pfi}		mtE_{pfi}
permeabilized cell	pce	N_{pce}	M_{pce}	V_{pce}	mtE_{pce}
intact cell	ce	N_{ce}	M_{ce}	V_{ce}	mtE_{ce}
Organism	org	N_{org}	M_{org}	V_{org}	

[a] Instead of mass, frequently the wet weight or dry weight is stated, W_w or W_d. m_X is mass of the sample [kg], M_X is mass of the object [kg·x^{-1}].

Flow per object, $I_{O_2/X}$: A special case of normalization is encountered in respiratory studies with permeabilized (or intact) cells. If respiration is expressed per cell, the O_2 flow per measurement system is replaced by the O_2 flow per cell, $I_{O_2/cell}$ (**Table 4**). O_2 flow can be calculated from volume-specific O_2 flux, J_{V,O_2} [nmol·s^{-1}·L^{-1}] (per V of the measurement chamber [L]), divided by the number concentration of cells, $C_{Nce} = N_{ce}/V$ [cell·L^{-1}], where N_{ce} is the number of cells in the chamber. Cellular O_2 flow can be compared between cells of identical size. To take into account changes and differences in cell size, normalization is required to obtain cell size-specific or mitochondrial marker-specific O_2 flux (Renner *et al.* 2003).

The complexity changes when the sample is a whole organism studied as an experimental model. The scaling law in respiratory physiology reveals a strong interaction of O_2 flow and individual body mass of an organism, since *basal* metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific O_2 flux, $\dot{V}_{O2max}$ or $\dot{V}_{O2peak}$, is approximately constant across a large range of individual body mass (Weibel and Hoppeler 2005), with individuals, breeds, and species deviating substantially from this relationship. $\dot{V}_{O2peak}$ of human endurance athletes is 60 to 80 mL O_2·min^{-1}·kg^{-1} body mass, converted to $J_{O_2peak/M}$ of 45 to 60 nmol·s^{-1}·g^{-1} (Gnaiger 2014; **Table 6**).

3.4. Normalization for mitochondrial content

Tissues can contain multiple cell populations that may have distinct mitochondrial subtypes. Mitochondria undergo dynamic fission and fusion cycles, and can exist in multiple stages and sizes ~~which~~ that may be altered by a range of factors. The isolation of mitochondria (often achieved through differential centrifugation) can therefore yield a subsample of the mitochondrial types present in a tissue, depending on isolation protocols utilized (*e.g.*, centrifugation speed). This possible bias should be taken into account when planning experiments using isolated mitochondria. Different sizes of mitochondria are enriched at specific centrifugation speeds, which is used for isolation of mitochondrial subpopulations.

Part of the mitochondrial content of a tissue is lost during preparation of isolated mitochondria. The fraction of mitochondria in the isolate is expressed as mitochondrial recovery. At a high mitochondrial recovery the sample of isolated mitochondria is more

representative of the total mitochondrial population than in preparations characterized by low recovery. Determination of the mitochondrial recovery and yield is based on measurement of the concentration of a mitochondrial marker in the tissue homogenate, $C_{mtE,thom}$, which simultaneously provides information on the specific mitochondrial density in the sample.

Normalization is a problematic subject; it is essential to consider the question of the study. If the study aims at comparing tissue performance—such as the effects of a treatment on a specific tissue, then normalization can be successful, using tissue mass or protein content, for example. However, if the aim is to find differences on mitochondrial function independent of mitochondrial density (**Table 4**), then normalization to a mitochondrial marker is imperative (**Fig. 7**). One cannot assume that quantitative changes in various markers—such as mitochondrial proteins—necessarily occur in parallel with one another. It should be established that the marker chosen is not selectively altered by the performed treatment. In conclusion, the normalization must reflect the question under investigation to reach a satisfying answer. On the other hand, the goal of comparing results across projects and institutions requires standardization on normalization for entry into a databank.

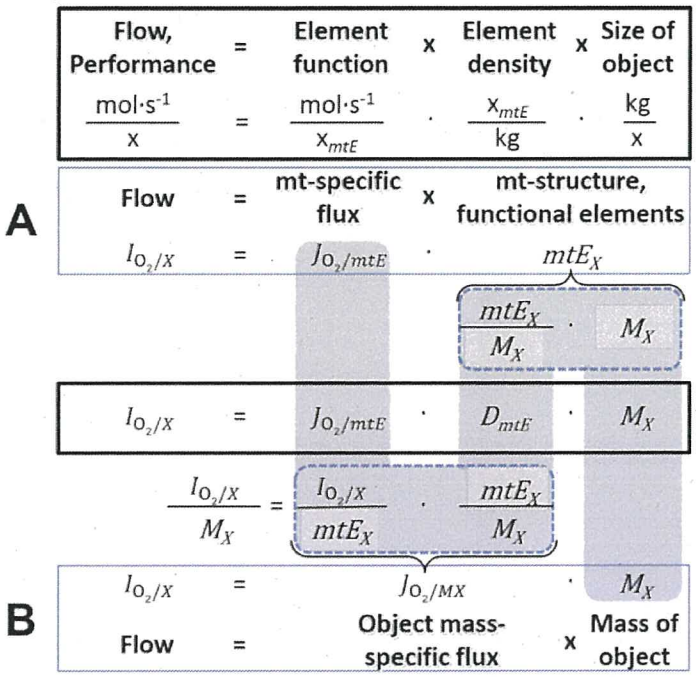


Fig. 7. Structure-function analysis of performance of an organism, organ or tissue, or a cell (sample entity, *X*). O_2 flow, $I_{O_2/X}$, is the product of performance per functional element (element function, mitochondria-specific flux), element density (mitochondrial density, D_{mtE}), and size of entity *X* (mass, M_X). (**A**) Structured analysis: performance is the product of mitochondrial *function* (mt-specific flux) and *structure* (functional elements; D_{mtE} times mass of *X*). (**B**) Unstructured analysis: performance is the product of *entity mass-specific flux*, $J_{O_2/MX} = I_{O_2/X}/M_X = I_{O_2}/m_X$ [mol·s^{-1}·kg^{-1}] and *size of entity*, expressed as mass of *X*; $M_X = m_X \cdot N_X^{-1}$ [kg·x^{-1}]. See **Table 4** for further explanation of quantities and units. Modified from Gnaiger (2014).

Mitochondrial concentration, C_{mtE}, and mitochondrial markers: Mitochondrial organelles comprise a dynamic cellular reticulum in various states of fusion and fission. Hence, the definition of an "amount" of mitochondria is often misconceived: mitochondria cannot be counted reliably as a number of occurring elements. Therefore, quantification of the "amount" of mitochondria depends on the measurement of chosen mitochondrial markers. 'Mitochondria are the structural and functional elemental units of cell respiration' (Gnaiger 2014). The