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Mitochondrial respiratory states and rates: Building blocks of mitochondrial physiology
Part 1.

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This manuscript on ‘Mitochondrial respiratory states and rates’ is a position statement in the frame of COST Action CA15203 MitoEAGLE. The list of co-authors evolved beyond phase 1 (phase 1 versions 1-44) in the bottom-up spirit of COST.

This is an open invitation to scientists and students to join as co-authors, to provide a balanced view on mitochondrial respiratory control, a fundamental introductory presentation of the concept of the protonmotive force, and a consensus statement on reporting data of mitochondrial respiration in terms of metabolic flows and fluxes.

Phase 2: MitoEAGLE preprint ‘The protonmotive force and respiratory control’ (Versions 01 – 21): We continue to invite comments and suggestions, particularly if you are an early career investigator adding an open future-oriented perspective, or an established scientist providing a balanced historical basis. Your critical input into the quality of the manuscript will be most welcome, improving our aims to be educational, general, consensus-oriented, and practically helpful for students working in mitochondrial respiratory physiology.

2017-11-11: Print version for MiP2017 and MitoEAGLE workshop in Hradec Kralove:
» http://www.mitoeagle.org/index.php/MiP2017_Hradec_Kralove_CZ

Phase 3: Discussion of manuscript submission to a preprint server, such as BioRxiv; invite further opinion leaders: To join as a co-author, please feel free to focus on a particular section in terms of direct input and references, contributing to the scope of the manuscript from the perspective of your expertise. Your comments will be largely posted on the discussion page of the MitoEAGLE preprint website.

If you prefer to submit comments in the format of a referee’s evaluation rather than a contribution as a co-author, I will be glad to distribute your views to the updated list of co-authors for a balanced response. We would ask for your consent on this open bottom-up policy.

Phase 4: Journal submission. We plan a series of follow-up reports by the expanding MitoEAGLE Network, to increase the scope of recommendations on harmonization and facilitate global communication and collaboration. Further discussions: MitoEAGLE Working Group Meetings, various conferences (EBEC 2018 in Budapest).

» http://www.mitoglobal.org/index.php/EBEC2018_Budapest_HU

I thank you in advance for your feedback.
With best wishes,
Erich Gnaiger

Chair Mitochondrial Physiology Society - http://www.mitophysiology.org
Chair COST Action MitoEAGLE - http://www.mitoeagle.org
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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 link between vectorial and scalar 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

Executive summary

In preparation.
**Box 1: In brief – Mitochondria and Bioblasts**

**Mitochondria** are the oxygen-consuming electrochemical generators which evolved from endosymbiotic bacteria (Margulis 1970; Lane 2005). They were described by Richard Altman (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 is an important parameter influencing 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 consumption of oxygen by electron transfer coupled to electrochemical proton translocation across the mtIM. In the process of oxidative phosphorylation (OXPHOS), the reduction of \( O_2 \) 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 (i.e., proton pumps with FMN, Fe-S and cytochrome \( b, c, a_a \) redox systems); alternative dehydrogenases and oxidases; the coenzyme ubiquinone (Q); F-ATPase or ATP synthase; the enzymes of the tricarboxylic acid cycle and the fatty acid oxidation enzymes; 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, maintaining cellular mitostasis through regulation at both the transcriptional and post-translational level, and 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 the 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 (hundred to thousands per cell; Cummins 1998), which is maternally inherited (White et al. 2008) and...
known as mitochondrial DNA (mtDNA). One exception to strictly maternal inheritance in animals is found in bivalves (Breton et al. 2007). mtDNA is 16.5 kB in length, contains 13 protein-coding genes for subunits of the transmembrane respiratory Complexes I, II, and E. The mitochondrial genome also encodes 22 tRNAs and the mitochondrial 16S and 12S rRNAs. Additional gene content is encoded in the mitochondrial genome, e.g. microRNAs, piRNA, smithRNAs, repeat associated RNA, and even additional proteins (Duarte et al. 2014; Lee et al. 2015; Cobb et al. 2016). The mitochondrial genome is both 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 function and disease is faced with an evolutionary, age, gender, and lifestyle 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 highly 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 standardized units for each parameter used to define mitochondrial respiratory function. Standardization 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 harmonized and 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. The plasma membrane separates the cytosol, nucleus, and organelles (the intracellular compartment) 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, therefore, prevents the passage of many water-soluble mitochondrial substrates, such as succinate or adenosine diphosphate (ADP), that are required for the analysis of respiratory capacity at kinetically-saturating concentrations, thus limiting 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 cytosolic components with ions and organic molecules of 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 the complete loss of cell viability, tested by nuclear staining and washout of cytosolic marker enzymes such as lactate dehydrogenase, while mitochondrial function remains intact. The respiration rate of isolated mitochondria remains unaltered after the addition of low concentrations 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 diluted 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 mitochondria. 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 for isolation of mitochondria need to be optimized according to the relevant questions addressed in a study. The term mitochondrial preparation does not include further fractionation of mitochondrial components, as well as submitochondrial particles.

2.1. Three coupling states of mitochondrial preparations and residual oxygen consumption

Respiratory capacities in coupling control states: To extend the classical nomenclature on mitochondrial coupling states (Section 2.3) by a concept-driven terminology that incorporates explicitly information on the nature of respiratory states, the terminology must be general and not restricted to any particular experimental protocol or mitochondrial preparation (Gnaiger 2009). We focus primarily on the conceptual ‘why’, along with clarification of the experimental ‘how’. In the following section, the concept-driven terminology is explained and coupling states are defined. We define respiratory capacities, comparable to channel capacity in information theory (Schneider 2006), as the upper bound of the rate of respiration measured in defined coupling control states and electron transfer-pathway (ET-pathway) states.
Fig. 1. The oxidative phosphorylation (OXPHOS) system. (A) The mitochondrial electron transfer system (ETS) is fueled by diffusion and transport of substrates across the mtOM and mtIM and consists of the matrix-ETS and membrane-ETS. Electron transfer (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 $H^{+}_{\text{pos}}/O_2$ 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 $H^{+}_{\text{neg}}/P_{\text{pos}}$ 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). Due to ion leaks and proton slip these are not fixed stoichiometries. (B) Phosphorylation-pathway catalyzed by the proton pump F$_1$F$_0$-ATPase, adenine nucleotide translocase, and inorganic phosphate transporter. The $H^{+}_{\text{neg}}/P_{\text{pos}}$ stoichiometry is the sum of the coupling stoichiometry in the F-ATPase reaction (-2.7 $H^{+}_{\text{pos}}$ from the positive intermembrane space, 2.7 $H^{+}_{\text{neg}}$ to the matrix, i.e., the negative compartment) and the proton balance in the translocation of ADP$^2$, ATP$^3$- and P$^2$. Modified from (A) Lemieux et al. (2017) and (B) Gnaiger (2014).

To provide a diagnostic reference for respiratory capacities of core energy metabolism, the capacity of oxidative phosphorylation, OXPHOS, is measured at kinetically-saturating concentrations of ADP and inorganic phosphate, P$_i$. The oxidative ET-capacity reveals the limitation of OXPHOS-capacity mediated by the phosphorylation-pathway. The ET- and phosphorylation-pathways comprise coupled segments of the OXPHOS-system. ET-capacity is measured as noncoupled respiration by application of external uncouplers. The contribution of intrinsically uncoupled oxygen consumption is most easily studied in the absence of ADP, i.e., by not stimulating phosphorylation, or by inhibition of the phosphorylation-pathway. The corresponding states are collectively classified as LEAK-states, when oxygen consumption...
compensates mainly for ion leaks including the proton leak (Table 1). Defined coupling states are induced by: (1) adding cation chelators such as EGTA, binding free Ca$^{2+}$ and thus limiting cation cycling; (2) adding ADP and P$_i$; (3) inhibiting the phosphorylation-pathway; and (4) uncoupler titrations, while maintaining a defined ET-pathway state with constant fuel substrates and inhibitors of specific branches of the ET-pathway (Fig. 1).

Table 1. Coupling states and residual oxygen consumption in mitochondrial preparations in relation to respiration- and phosphorylation-rate, $J_{kO_2}$ and $J_{p\text{to}}$, and protonmotive force, pmf. Coupling states are established at kinetically-saturating concentrations of fuel substrates and $O_2$.

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

**Kinetic control:** Coupling control states are established in the study 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, may not apply to physiological intracellular conditions, relevant information is obtained in studies of kinetic responses to conditions intermediate between the LEAK state at zero [ADP] and the OXPHOS-state at saturating [ADP], or of respiratory capacities in the range between kinetically-saturating [O$_2$] 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. Strictly, 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, such that oxygen concentration does not change 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 kinetically-saturating concentrations of substrates to be maintained, 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 in light of 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 process. The amount of these chemicals in an experimental incubation is selected to maximize effect, yet not lead to 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 when lipophilic substances (oligomycin; uncouplers, permeabilization agents) or cations (TPP\(^+\); fluorescent dyes such as safranin, TMRM) are applied which accumulate in biological membranes or the mitochondrial matrix. For example, a dose of digitonin of 8 fmol·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).

**Phosphorylation, \(P^\rightarrow\):** Phosphorylation in the context of OXPHOS is defined as phosphorylation of ADP by \(P\) to ATP. On the other hand, the term phosphorylation is used generally in many different 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; \(O = 0.5\) O\(_2\)), where \(P\) indicates phosphorylation of ADP to ATP or GDP to GTP. We propose the symbol \(P^\rightarrow\) for the endergonic (uphill) direction of phosphorylation ADP→ATP, and likewise the symbol \(P^\leftarrow\) for the corresponding exergonic (downhill) hydrolysis ATP→ADP (Fig. 2). \(P^\rightarrow\) refers mainly to electrontransfer phosphorylation but may also involve substrate-level phosphorylation as part of the tricarboxylic acid 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.

**Coupling:** In mitochondrial electron transfer (Fig. 1), vectorial transmembrane proton flux is coupled through the proton pumps CI, CII and CIV to the catabolic flux of scalar reactions, collectively measured as oxygen flux (Fig. 2). Thus mitochondria are important sites of energy transformation. Energy cannot be lost or produced in any internal process (First Law of thermodynamics). Open and closed systems can gain or lose energy only by external fluxes, i.e., by exchange with the environment. Energy is a fundamentally 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.
Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, $J_{\text{Ko}_2}$, through the catabolic ET-pathway, k, is coupled to flux through the phosphorylation-pathway of ADP to ATP, $J_{\text{P»}}$. The proton pumps of the ET-pathway drive proton flux into the positive (pos) compartment, $J_{\text{mH+pos}}$, which generates the output protonmotive force. F-ATPase is coupled to inward proton current into the negative (neg) compartment, $J_{\text{mH+neg}}$, to phosphorylate ADP+$P_i$ to ATP. 2[H] indicates the reduced hydrogen equivalents of fuel substrates of the catabolic reaction k with oxygen. Fluxes are expressed per volume, $V$ [m$^3$], of the system. 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-compartment (scalar ADP and ATP compartments, D-comp. and T-comp.), respectively. At steady-state proton turnover, $J_{\text{P∞}}$, and ATP turnover, $J_{\text{P»}}$, maintain concentrations constant, when $J_{\text{mH+∞}} = J_{\text{mH+pos}}$, and $J_{\text{P∞}} = J_{\text{P»}}$. Modified from Gnaiger (2014).

Uncoupling: Uncoupling is a general term comprising diverse mechanisms. Small differences of terms, e.g., uncoupled vs. noncoupled, are easily overlooked, although they relate to different mechanisms of uncoupling (Fig. 3). An attempt at rigorous definition is required for clarification of concepts (Table 2).

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 oxygen (O$_2$; dioxygen) to superoxide anion radical (O$_2^•$).
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 oxygen flux of ET-capacity. Uncoupled, decoupled, and loosely coupled respiration are components of intrinsic LEAK respiration. 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.

LEAK-state (Fig. 4): The LEAK-state is defined as a state of mitochondrial respiration when O₂ flux mainly compensates for ion leaks in the absence of ATP synthesis, at kinetically-saturating concentrations of O₂ and respiratory fuel substrates. LEAK-respiration is measured 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. It is important to consider adjustment of the nominal concentration of these inhibitors to the density of biological sample applied, to minimize or avoid inhibitory side-effects exerted on ET-capacity or even some dyscoupling.

Fig. 4. LEAK-state: Phosphorylation is arrested, \( J_{P_0} = 0 \), and catabolic oxygen flux, \( J_{kO_2} \), is controlled mainly by the proton leak, \( J_{mH^+} \), at maximum protonmotive force. See also Fig. 2 and 3.
Table 2. Distinction of terms related to coupling and uncoupling (Fig. 3).

<table>
<thead>
<tr>
<th>Term</th>
<th>Respiration</th>
<th>P/O₂</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>coupled</td>
<td>0</td>
<td></td>
<td>electron transfer in mitochondrial fragments without vectorial proton translocation</td>
</tr>
<tr>
<td>uncoupled</td>
<td>L</td>
<td>0</td>
<td>non-phosphorylating intrinsic LEAK-respiration, without added protonophore</td>
</tr>
<tr>
<td>uncoupled</td>
<td>0</td>
<td></td>
<td>component of LEAK-respiration, uncoupled sui generis, ion diffusion across the mtIM</td>
</tr>
<tr>
<td>decoupled</td>
<td>0</td>
<td></td>
<td>component of LEAK-respiration, proton slip</td>
</tr>
<tr>
<td>loosely coupled</td>
<td>0</td>
<td></td>
<td>component of LEAK-respiration, lower coupling due to superoxide anion radical formation and bypass of proton pumps</td>
</tr>
<tr>
<td>dyscoupled</td>
<td>0</td>
<td></td>
<td>pathologically, toxicologically, environmentally increased uncoupling, mitochondrial dysfunction</td>
</tr>
<tr>
<td>inducibly uncoupled</td>
<td>E</td>
<td>0</td>
<td>by UCP1 or cation (e.g. Ca²⁺) cycling</td>
</tr>
<tr>
<td>noncoupled</td>
<td>E</td>
<td>0</td>
<td>non-phosphorylating respiration stimulated to maximum flux at optimum exogenous uncoupler concentration (Fig. 6)</td>
</tr>
<tr>
<td>well-coupled</td>
<td>P</td>
<td>high</td>
<td>phosphorylating respiration with an intrinsic LEAK component (Fig. 5)</td>
</tr>
<tr>
<td>fully coupled</td>
<td>P – L</td>
<td>max.</td>
<td>OXPHOS-capacity corrected for LEAK-respiration (Fig. 7)</td>
</tr>
</tbody>
</table>

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. 4). The proton leak flux depends non-linearly on the protonmotive force (Garlid et al. 1989; Divakaruni and Brand 2011), 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). As a consequence of this effective short-circuit, the protonmotive force diminishes, resulting in stimulation of electron transfer to O₂ and heat dissipation without phosphorylation of ADP.

Cation cycling: There can be other cation contributors to leak current including calcium and probably magnesium. Calcium current is balanced by mitochondrial Na⁺/Ca²⁺ exchange, which is balanced by Na⁺/H⁺ exchange or K⁺/H⁺ exchange. This is another effective uncoupling mechanism different from proton leak.

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 case 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 turnover rate of the pump.
**Electron leak and loosely coupled respiration:** Superoxide anion radical production by the ETS leads to a bypass of proton pumps and correspondingly lower P/O ratio, which 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 O2 consumption 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. 5):**

The OXPHOS-state is defined as the respiratory state with kinetically-saturating concentrations of O2, 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.

---

**Fig. 5. OXPHOS-state:** Phosphorylation, $J_{P\text{ha}}$, is stimulated by kinetically-saturating [ADP] and inorganic phosphate, $[P_i]$, and is supported by a high protonmotive force. O2 flux, $J_{O2,P}$, is well-coupled at a P/O ratio of $J_{P\text{ha}}/J_{O2,P}$. See also Fig. 2.
Electron transfer-state (Fig. 6): 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 $J_{P} = 0$.

Besides the three fundamental coupling states of mitochondrial preparations, the following respiratory state also is relevant to assess respiratory function:

ROX state and Rox: The rate of residual oxygen consumption, $Rox$, is defined as $O_2$ consumption due to oxidative side reactions remaining after inhibition of ET, e.g., with rotenone, malonic acid and antimycin A. Cyanide and azide not only inhibit CIV but several peroxidases which should be involved in $Rox$. ROX is not a coupling state. 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 oxygen-consuming reactions in mitochondria not related to ET, such as oxygen consumption in reactions catalyzed by monoamine oxidases (type A and B), monoxygenases (cytochrome P450 monoxygenases), dioxygenase (sulfur dioxygenase and trimethyllysine dioxygenase), several hydroxylases, and more. Mitochondrial preparations, especially those obtained from liver, may be contaminated by peroxisomes. This fact makes the exact determination of mitochondrial oxygen consumption and mitochondria-associated generation of reactive oxygen species complicated (Schönfeld et al. 2009). The dependence of ROX-linked oxygen consumption needs to be studied in detail with respect to non-ET enzyme activities, availability of specific substrates, oxygen concentration, and electron leakage leading to the formation of reactive oxygen species.

2.2. Coupling states and respiratory rates

As an improvement of previous terminologies, we distinguish metabolic pathways from metabolic states and the corresponding metabolic rates; for example: ET-pathways (Fig. 7), ET-state (Fig. 6), and ET-capacity, $E$, respectively (Table 1). The protonmotive force is high in the OXPHOS-state when it drives phosphorylation, maximum in the LEAK-state of coupled mitochondria, driven by LEAK-respiration at a minimum back flux of cations to the matrix side, and very low in the ET-state when uncouplers short-circuit the proton cycle (Table 1).

The three coupling states, ET, LEAK and OXPHOS, are shown schematically with the corresponding respiratory rates, abbreviated as $E$, $L$ and $P$, respectively (Fig. 7).
Fig. 7. Four-compartment model of oxidative phosphorylation. Respiratory states (ET, OXPHOS, LEAK) and corresponding rates \(E/P/L\) are connected by the protonmotive force, pmf. Electron transfer-capacity, \(E\), is partitioned into (1) dissipative LEAK-respiration, \(L\), when the Gibbs energy change of catabolic \(O_2\) consumption is irreversibly lost, (2) net OXPHOS-capacity, \(P/L\), with partial conservation of the capacity to perform work, and (3) the excess capacity, \(E-P\). Modified from Gnaiger (2014).

\(E\) may exceed or be equal to \(P\). \(E>P\) is observed in many types of mitochondria, varying between species, tissues and cell types (Gnaiger 2009). \(E-P\) is the excess ET-capacity pushing the phosphorylation-flux (Fig. 1B) to the limit of its capacity of utilizing the protonmotive force. In addition, the magnitude of \(E-P\) depends on the tightness of coupling or degree of uncoupling, since an increase of \(L\) causes \(P\) to increase towards the limit of \(E\). The excess \(E-P\) capacity, \(E-P\), therefore, provides a sensitive diagnostic indicator of specific injuries of the phosphorylation-pathway, under conditions when \(E\) remains constant but \(P\) declines relative to controls (Fig. 7). Substrate cocktails supporting simultaneous convergent electron transfer to the Q-junction for reconstitution of tricarboxylic acid cycle (TCA cycle or Krebs cycle) function establish pathway control states with high ET-capacity, and consequently increase the sensitivity of the \(E-P\) assay.

\(E\) cannot theoretically be lower than \(P\). \(E<P\) must be discounted as an artefact, which may be caused experimentally by: (1) loss of oxidative capacity during the time course of the respirometric assay, since \(E\) is measured subsequently to \(P\); (2) using insufficient uncoupler concentrations; (3) using high uncoupler concentrations which inhibit ET (Gnaiger 2008); (4) high oligomycin concentrations applied for measurement of \(L\) before titrations of uncoupler, when oligomycin exerts an inhibitory effect on \(E\). On the other hand, the excess ET-capacity is overestimated if non-saturating [ADP] or [\(P_i\)] are used. See State 3 in the next section.

**\(P_o/O_2\) ratio:** The \(P_o/O_2\) ratio \((P_o/4\ \text{e}^-)\) is two times the ‘\(P/O\’\) ratio \((P_o/2\ \text{e}^-)\) of classical bioenergetics. \(P_o/O_2\) is a generalized symbol, independent of measurement of phosphorylation by determination of \(P_i\) consumption \((P_o/O_2\ \text{flux ratio})\), ADP depletion \((\text{ADP}/O_2\ \text{flux ratio})\), or ATP production \((\text{ATP}/O_2\ \text{flux ratio})\).

The mechanistic \(P_o/O_2\) ratio, which may be referred to also as \(P_o/O_2\) stoichiometry, is calculated from the proton-to-oxygen and proton-to-phosphorylation coupling stoichiometries (Fig. 1A),

\[
P_o/O_2 = \frac{H_{\text{pos}}^{+}/O_2}{H_{\text{neg}}^{+}/P_o} \tag{1}
\]

The \(H_{\text{pos}}^{+}/O_2\) 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. \(H_{\text{pos}}^{+}/O_2\) 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 \(H_{\text{pos}}^{+}/O_2\) stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov...
The H$^-$/P$^+$ coupling stoichiometry (3.7; Fig. 1A) is the sum of 2.7 H$^-$/P$^+$ 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$^\circ$/O$_2$ ratio is calculated at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively (Eq. 1). The corresponding classical P$^\circ$/O ratios (referring to the 2 electron reduction of 0.5 O$_2$) are 2.7 and 1.6 (Watt et al. 2010), in direct agreement with the measured P$^\circ$/O ratio for succinate of 1.58 ± 0.02 (Gnaiger et al. 2000).

The effective P$^\circ$/O$_2$ flux ratio ($Y_{P^\circ/O_2} = J_{P^\circ}/J_{O_2}$) is diminished relative to the mechanistic P$^\circ$/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, $Y_{P^\circ/O_2}$ depends on several experimental conditions of flux control, increasing as a hyperbolic function of [ADP] to a maximum value (Gnaiger 2001).

The net OXPHOS-capacity is calculated by subtracting $L$ from $P$ (Fig. 7). Then the net $P^\circ$/O$_2$ equals $P^\circ$/(P-L), wherein the dissipative LEAK component in the OXPHOS-state may be overestimated. This can be avoided by measuring LEAK-respiration in a state when the protonmotive force is adjusted to its slightly lower value in the OXPHOS-state, e.g., by titration of an ET inhibitor (Divakaruni and Brand 2011). Any turnover-dependent components of proton leak and slip, however, are underestimated under these conditions (Garlid et al. 1993). In general, it is inappropriate to use the term ATP production or ATP turnover for the difference of oxygen consumption measured in states $P$ and $L$. The difference $P-L$ is the upper limit of the part of OXPHOS-capacity that is freely available for ATP production (corrected for LEAK-respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry (Fig. 7).

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 oxygen, e.g., starvation and hypoxia; (3) the protonmotive force, redox states, flux-force relationships, coupling and efficiency; (4) Ca$^{2+}$ and other ions including H$^+$; (5) inhibitors, e.g., nitric oxide or intermediary metabolites, such as oxaloacetate; (6) signalling pathways and regulatory proteins, e.g. insulin resistance, transcription factor HIF-1 or inhibitory 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). (5) 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, does mean that there will be no response to a variable activating it, e.g. [ADP]. However, the reverse 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 oxygen 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:** Respiratory control refers to the ability of mitochondria to adjust oxygen consumption 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 oxygen consumption 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 oxygen consumption without control by phosphorylation (‘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]) and specific inhibitors, activating selected mitochondrial catabolic pathways, k (Fig. 1 and 2). 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).

### 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>
<thead>
<tr>
<th>State</th>
<th>[O₂]</th>
<th>ADP level</th>
<th>Substrate Level</th>
<th>Respiration rate</th>
<th>Rate-limiting substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;0</td>
<td>low</td>
<td>low</td>
<td>slow</td>
<td>ADP</td>
</tr>
<tr>
<td>2</td>
<td>&gt;0</td>
<td>high</td>
<td>~0</td>
<td>slow</td>
<td>substrate</td>
</tr>
<tr>
<td>3</td>
<td>&gt;0</td>
<td>high</td>
<td>high</td>
<td>fast</td>
<td>respiratory chain</td>
</tr>
<tr>
<td>4</td>
<td>&gt;0</td>
<td>low</td>
<td>high</td>
<td>slow</td>
<td>ADP</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>high</td>
<td>high</td>
<td>0</td>
<td>oxygen</td>
</tr>
</tbody>
</table>

**State 1** is obtained after addition of isolated mitochondria to air-saturated isoosmotic/isotonic respiration medium containing inorganic phosphate, 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 oxygen consumption, State 2 is equivalent to the state of residual oxygen consumption, ROX (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 oxygen concentrations near air-saturation (ca. 200 μM O₂ 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 rate of oxygen consumption that does not lead to oxygen depletion during the transition to State 4. In contrast, kinetically-saturating ADP concentrations usually are an order of magnitude 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 in the rate of oxygen consumption in the transition from State 3 to State 4. Under these conditions of State 4, a maximum protonotive force and high ATP/ADP ratio are maintained. For calculation of P/O ratios the gradual decline of Y₉ₒₐₓ toward 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. Oxygen consumption in State 4 is an overestimation of LEAK-respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, Jₚₒ, which stimulates respiration coupled to phosphorylation, Jₚₒ > 0. This can be tested by inhibition of the phosphorylation-pathway using oligomycin, ensuring that Jₚₒ = 0 (State 4o). Alternatively, sequential ADP titrations re-establish State 3, followed by State 3 to State 4 transitions while sufficient oxygen is available. However, anoxia may be reached before exhaustion of ADP (State 5).

State 5 is the state after exhaustion of oxygen in a closed respirometric chamber. Diffusion of oxygen 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 O₂ 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 a database (Fig. 8). The inconsistency of the meanings of rate becomes fully apparent when considering Galilei’s famous principle, that ‘bodies of different weight all fall at the same rate (have a constant acceleration)’ (Coopersmith 2010).

Fig. 8. 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, $J_{vc}$), which must be clearly distinguished from flux per cell, $I_{scell}$, 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 (per system) is distinguished from flux as a size-specific quantity (per system size) (Fig. 8). Electric current is flow, $I_{el}$ [A $\equiv$ C 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), $J_{el}$ [A m$^{-2}$ $\equiv$ C s$^{-1}$ 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 general system-paradigm, mass-specific flux is flow divided by mass of the system (the total mass of everything within the measuring chamber). 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 further clarified, such that sample-specific, e.g., muscle mass-specific normalization is distinguished from system-specific (mass or volume) quantities (Fig. 8).

Box 2: Metabolic fluxes and flows: vectorial and scalar

Fluxes are vectors, if they have spatial direction in addition to magnitude. A vector flux (surface-density of flow) is expressed per unit cross-sectional area, $A$ [m$^{-2}$], perpendicular to the direction of flux. Flows are defined as extensive quantities of the system, as vector or scalar flow, $I$ or $I$ [mol s$^{-1}$], respectively, then the corresponding vector and scalar fluxes are $J = I A^{-1}$ [mol s$^{-1}$ m$^{-2}$] and $J = I V^{-1}$ [mol s$^{-1}$ m$^{-3}$], respectively, expressing flux as an area-specific vector...
or volume-specific scalar quantity. We suggest to define: (1) vectorial fluxes, which analyze
translocations in continuous systems as functions of gradients; (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\textsubscript{2} flux, \(J_{\text{KO}}\) [mol\textsuperscript{-1}\textsuperscript{-1}\textsuperscript{-1}]).

Vectorial transmembrane proton fluxes, \(J_{\text{mtIM}}^{+}\text{pos}\) and \(J_{\text{mtIM}}^{+}\text{neg}\), 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
declared by assigning the initial and final state as ergodynamic compartments, \(H^{+}\text{neg} \to H^{+}\text{pos}\) or
\(0 = -1 H^{+}\text{neg} + 1 H^{+}\text{pos}\), 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 direct analogy to vectorial translocation, the direction of a scalar chemical reaction, \(A
\to B\) or \(0 = -1 A + 1 B\), is defined by assigning substrates and products, \(A\) and \(B\), as ergodynamic
compartments. \(O\text{\textsubscript{2}}\) is defined as a substrate in respiratory \(O\text{\textsubscript{2}}\) consumption, which together with
the fuel substrates comprises the substrate compartment of the catabolic reaction (Fig. 2).
Volume-specific scalar \(O\text{\textsubscript{2}}\) flux is coupled to vectorial translocation, yielding the \(H^{+}\text{pos}/O\text{\textsubscript{2}}\) ratio
(Fig. 1).

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

System-specific flux, \(J\): The experimental system (the experimental chamber) is part of
the measurement apparatus, separated from the environment as an isolated, closed, open,
isothermal or non-isothermal system (Table 4). It is important to distinguish between (1) the
system with volume \(V\) and mass \(m\) defined by the system boundaries, and (2) in the experimental
chamber enclosed sample or objects with volume \(V\text{\textsubscript{X}}\) and mass \(m\text{\textsubscript{X}}\) (Fig. 8). Metabolic \(O\text{\textsubscript{2}}\) flow
per object, \(J_{\text{VO}}\), increases as the mass of the object is increased. Object mass-specific \(O\text{\textsubscript{2}}\) flux,
\(J_{\text{VO}}\text{\textsubscript{m}}\) should be independent of the mass of the object studied in the instrument chamber, but
system volume-specific \(O\text{\textsubscript{2}}\) flux, \(J_{\text{VO}}\text{\textsubscript{v}}\) (per volume of the instrument chamber), should increase
in direct proportion to the mass of the object in the chamber. \(J_{\text{VO}}\text{\textsubscript{v}}\) depends on mass-
centration of the sample in the chamber, but should be independent of the chamber (system)
volume. There are practical limitations to increasing 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, \(J_{r} = \frac{dc_{r}}{dr} \text{[mol}\textsuperscript{-1}\textsuperscript{-1}\textsuperscript{-1}]\). The rate of
concentration change is \(dc_{r}/dr\) [mol\textsuperscript{-1}\textsuperscript{-1}\textsuperscript{-1}], where concentration is \(c_{r} = m_{r}/V\). It is important
to make the fundamental distinction between (1) \(J_{r}\text{\textsubscript{o}}\text{\textsubscript{m}}\) [mol\textsuperscript{-1}\textsuperscript{-1}\textsuperscript{-1}] and (2) rate of concentration
change [mol\textsuperscript{-1}\textsuperscript{-1}\textsuperscript{-1}]. These merge to a single expression only in closed systems. In open
systems, external fluxes (such as \(O\text{\textsubscript{2}}\) supply) are distinguished from internal transformations
(metabolic flux, \(O\text{\textsubscript{2}}\) consumption). In a closed system, external flows of all substances are zero
and \(O\text{\textsubscript{2}}\) consumption (internal flow of catabolic reactions \(k\)), \(I_{\text{KO}}\) [pmol\textsuperscript{-1}\textsuperscript{-1}1], causes a decline of
the amount of \(O\text{\textsubscript{2}}\) in the system, \(n_{\text{O}}\) [nmol]. Normalization of these quantities for the volume of
the system, \(V\) [L \(\equiv dm^{3}\)], yields volume-specific \(O\text{\textsubscript{2}}\) flux, \(J_{\text{VKO}} = I_{\text{KO}}/V\) [pmol\textsuperscript{-1}\textsuperscript{-1}\textsuperscript{-1}], and \(O\text{\textsubscript{2}}\)
concentration, $[O_2]$ or $c_{O_2} = n_{O_2}/V$ [$\mu$mol·L$^{-1}$ = $\mu$M = nmol·mL$^{-1}$]. Instrumental background $O_2$ flux is due to external flux into a non-ideal closed respirometer, such that total volume-specific flux has to be corrected for instrumental background $O_2$ flux, i.e., $O_2$ diffusion into or out of the instrumental chamber. $J_{f \text{,kO}_2}$ 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, $J_{\text{AO}_2}$, 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 generally defined units is required for direct transfer of reported results into a database. The second [s] is the SI unit for the base quantity $\text{time}$. 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 (Fig. 9), and conceptual strategies (Fig. 10).

### Table 4. Sample concentrations and normalization of flux.

<table>
<thead>
<tr>
<th>Expression</th>
<th>Symbol</th>
<th>Definition</th>
<th>Unit</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>identity of sample</td>
<td>$X$</td>
<td>object: cell, tissue, animal, patient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>number of sample entities $X$</td>
<td>$N_X$</td>
<td>number of objects</td>
<td>$x$</td>
<td></td>
</tr>
<tr>
<td>mass of sample $X$</td>
<td>$m_X$</td>
<td></td>
<td>$kg$</td>
<td>1</td>
</tr>
<tr>
<td>mass of object $X$</td>
<td>$M_X$</td>
<td>$M_X = m_X N_X^{-1}$</td>
<td>$kg\cdot x^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>$mt$</td>
<td>$X = mt$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amount of mt-elements</td>
<td>$mtE$</td>
<td>quantity of mt-marker</td>
<td>$mtEU$</td>
<td></td>
</tr>
<tr>
<td><strong>Concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>object number concentration</td>
<td>$C_{NX}$</td>
<td>$C_{NX} = N_X V^{-1}$</td>
<td>$x\cdot m^{-3}$</td>
<td>2</td>
</tr>
<tr>
<td>sample mass concentration</td>
<td>$C_{mX}$</td>
<td>$C_{mX} = m_X V^{-1}$</td>
<td>$kg\cdot m^{-3}$</td>
<td>3</td>
</tr>
<tr>
<td>mitochondrial concentration</td>
<td>$C_{mtE}$</td>
<td>$C_{mtE} = mtE V^{-1}$</td>
<td>$mtEU\cdot m^{-3}$</td>
<td>4</td>
</tr>
<tr>
<td>specific mitochondrial density</td>
<td>$D_{mtE}$</td>
<td>$D_{mtE} = mtE m_X^{-1}$</td>
<td>$mtEU\cdot kg^{-1}$</td>
<td>5</td>
</tr>
<tr>
<td>mitochondrial content, $mtE$ per object $X$</td>
<td>$mtE_X$</td>
<td>$mtE_X = mtE N_X^{-1}$</td>
<td>$mtEU\cdot x^{-1}$</td>
<td></td>
</tr>
<tr>
<td><strong>O_2 flow and flux</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flow, system</td>
<td>$I_{O_2}$</td>
<td>internal flow</td>
<td>mol·s$^{-1}$</td>
<td>7</td>
</tr>
<tr>
<td>volume-specific flux</td>
<td>$J_{f\text{,O}_2}$</td>
<td>$J_{f\text{,O}<em>2} = I</em>{O_2} V^{-1}$</td>
<td>mol·s$^{-1}$·m$^{-3}$</td>
<td>8</td>
</tr>
<tr>
<td>flow per object $X$</td>
<td>$I_{X\text{,O}_2}$</td>
<td>$I_{X\text{,O}<em>2} = J</em>{f\text{,O}<em>2} C</em>{NX}$</td>
<td>mol·s$^{-1}$·x$^{-1}$</td>
<td>9</td>
</tr>
<tr>
<td>mass-specific flux</td>
<td>$J_{m\text{,X,O}_2}$</td>
<td>$J_{m\text{,X,O}<em>2} = J</em>{f\text{,O}<em>2} C</em>{mX}$</td>
<td>mol·s$^{-1}$·kg$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>mitochondria-specific flux</td>
<td>$J_{mtE\text{,X,O}_2}$</td>
<td>$J_{mtE\text{,X,O}<em>2} = J</em>{f\text{,O}<em>2} C</em>{mtE}$</td>
<td>mol·s$^{-1}$·mtEU$^{-1}$</td>
<td>10</td>
</tr>
</tbody>
</table>
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.

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

$C_{\text{mit}}$ is an experimental variable, dependent on sample concentration: (1) $C_{\text{mit}} = m_{\text{E}} V^1$; (2) $C_{\text{mit}} = m_{\text{E}} N_{\text{mit}} / V^1$; (3) $C_{\text{mit}} = m_{\text{E}} D_{\text{mit}}$.

If the amount of mitochondria, $m_{\text{E}}$, is expressed as mitochondrial mass, then $D_{\text{mit}}$ is the mass fraction of mitochondria in the sample. If $m_{\text{E}}$ is expressed as mitochondrial volume, $V_{\text{mit}}$, and the mass of sample, $m_X$, is replaced by volume of sample, $V_X$, then $D_{\text{mit}}$ is the volume fraction of mitochondria in the sample.

$5 \times m_{\text{E}} = m_{\text{E}} N_{\text{mit}} / V^1 = C_{\text{mit}} / N_{\text{mit}}$.

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

$I_{\text{O}}$ and $V$ are defined per instrument chamber as a system of constant volume (and constant temperature), which may be closed or open. $I_{\text{O}}$ is abbreviated for $I_{\text{O}}$, 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, $v_{O_2} = -1$. $I_{\text{O}} = d_{\text{O}} / d_{\text{O}} - 1$. If $r$ includes all chemical reactions in which $O_2$ participates, then $d_{\text{O}} = d_{\text{O}} - d_{\text{O}}$, where $d_{\text{O}}$ is the change in the amount of $O_2$ in the instrument chamber and $d_{\text{O}}$ is the amount of $O_2$ added externally to the system. At steady state, by definition $d_{\text{O}} = 0$, hence $d_{\text{O}} = d_{\text{O}}$

$J_{\text{cell}}$ is an experimental variable, expressed per volume of the instrument chamber.

$I_{\text{cell}}$ is a physiological variable, depending on the size of entity $X$.

There are many ways to normalize for a mitochondrial marker, that are used in different experimental approaches: (1) $J_{\text{cell}} = J_{\text{cell}} / C_{\text{mit}}$; (2) $J_{\text{cell}} = J_{\text{cell}} / C_{\text{mit}} - 1 = J_{\text{cell}} / D_{\text{mit}}$; (3) $J_{\text{cell}} = J_{\text{cell}} / C_{\text{mit}}$; (4) $J_{\text{cell}} = J_{\text{cell}} / I_{\text{cell}}$. The mt- elemental unit [mtEU] varies between different mt-markers.

### Table 5. Sample types, $X$, abbreviations, and quantification.

<table>
<thead>
<tr>
<th>Identity of sample</th>
<th>$X$</th>
<th>$N_X$</th>
<th>Mass$^a$</th>
<th>Volume</th>
<th>mt-Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>mitochondrial preparation</td>
<td>mtprep</td>
<td>[x]</td>
<td>$m_{\text{mt}}$</td>
<td>$V_{\text{mt}}$</td>
<td>$m_{\text{E}}$</td>
</tr>
<tr>
<td>tissue homogenate</td>
<td>thom</td>
<td></td>
<td>$m_{\text{thom}}$</td>
<td>$m_{\text{E}}$</td>
<td></td>
</tr>
<tr>
<td>permeabilized tissue</td>
<td>pti</td>
<td></td>
<td>$m_{\text{pti}}$</td>
<td>$m_{\text{E}}$</td>
<td></td>
</tr>
<tr>
<td>permeabilized fibre</td>
<td>pfi</td>
<td></td>
<td>$m_{\text{pti}}$</td>
<td>$m_{\text{E}}$</td>
<td></td>
</tr>
<tr>
<td>permeabilized cell</td>
<td>pce</td>
<td>$N_{\text{pce}}$</td>
<td>$M_{\text{pce}}$</td>
<td>$V_{\text{pce}}$</td>
<td>$m_{\text{E}}$</td>
</tr>
<tr>
<td>intact cell</td>
<td>ce</td>
<td>$N_{\text{ce}}$</td>
<td>$M_{\text{ce}}$</td>
<td>$V_{\text{ce}}$</td>
<td>$m_{\text{E}}$</td>
</tr>
<tr>
<td>Organism</td>
<td>org</td>
<td>$N_{\text{org}}$</td>
<td>$M_{\text{org}}$</td>
<td>$V_{\text{org}}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 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$^{-1}$].

### Sample concentration, $C_{mX}$:

Normalization for sample concentration is required for reporting respiratory data. Consider a tissue or cells as the sample, $X$, and the sample mass, $m_X$ [mg] from which a mitochondrial preparation is obtained. $m_X$ is frequently measured as wet or dry weight, $W_w$ or $W_d$ [mg], or as amount of tissue or cell protein, $m_{\text{protein}}$. In the case of permeabilized tissues, cells, and homogenates, the sample concentration, $C_{mX} = m_X / V [\text{mg·mL}^{-1}]$, is simply the mass of the subsample of tissue that is transferred into the instrument chamber.

### Mass-specific flux, $J_{mX}$:

Mass-specific flux is obtained by expressing respiration per mass of sample, $m_X$ [mg]. $X$ is the type of sample, e.g., tissue homogenate, permeabilized fibres or cells. Volume-specific flux is divided by mass concentration of $X$, $J_{mX} = J_{mX} / C_{mX}$, or flow per cell is divided by mass per cell, $J_{\text{cell}} = I_{\text{cell}} / M_{\text{cell}}$. 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 respirates 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 case the nature of the interaction becomes an issue. Optimization of cell density and arrangement is generally important and particularly in experiments carried out in wells, considering the confluence of the cell monolayer or clumps of cells (Salabei et al. 2014).

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

**Flow per object, \( I_{X,O} \):** 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, \( I_{cell,O} \) (Table 4). O\(_2\) flow can be calculated from volume-specific O\(_2\) flux, \( J_{O_2} [\text{nmol} \cdot \text{s}^{-1} \cdot \text{L}^{-1}] \) (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, further 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 well-established scaling law in respiratory physiology reveals a strong interaction of O\(_2\) consumption 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, \( \dot{V}_{O_2\text{max}} \) or \( \dot{V}_{O_2\text{peak}} \), is approximately constant across a large range of individual body mass (Weibel and Hoppeler 2005), with individuals, breeds, and certain species deviating substantially from this general relationship. \( \dot{V}_{O_2\text{peak}} \) of human endurance athletes is 60 to 80 mL O\(_2\)-min\(^{-1}\)-kg\(^{-1}\) body mass, converted to \( J_{M,O_2\text{peak}} \) 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 which may have distinct mitochondrial subtypes. Mitochondria undergo dynamic fission and fusion cycles, and can exist in multiple stages and sizes which 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, dependent on isolation protocols utilized (e.g. centrifugation speed). This possible artefact should be taken into account when planning experiments using isolated mitochondria. The tendency for mitochondria of specific sizes to be enriched at different centrifugation speeds also has the potential to allow the isolation of specific mitochondrial subpopulations and therefore the analysis of mitochondria from multiple cell lineages within a single tissue.

Part of the mitochondria from the tissue is lost during preparation of isolated mitochondria. The fraction of mitochondria obtained is expressed as mitochondrial recovery (Fig. 9). 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 (Fig. 9).

Normalization is a problematic subject and it is essential to consider the question of the study. If the study aims to compare tissue performance, such as the effects of a certain treatment on a specific tissue, then normalization can be successful, using tissue mass or protein content, for example. If the aim, however, is to find differences of mitochondrial function independent of mitochondrial density (Table 4), then normalization to a mitochondrial marker is imperative (Fig. 10). However, one cannot assume that quantitative changes in various markers such as mitochondrial proteins necessarily occur in parallel with one another. It is important to first
establish 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 some standardization on normalization for entry into a databank.

Fig. 9. Normalization of volume-specific flux of isolated mitochondria and tissue homogenate. A: Recovery, \( Y_{mtE} \), in preparation of isolated mitochondria. \( V_{thom,1} \) and \( V_{stock,1} \) are the volumes transferred from the total volume, \( V_{thom} \) and \( V_{stock} \), respectively. \( mtE_{thom,1} \) is the amount of mitochondrial elements in volume \( V_{thom,1} \) used for isolation. B: Homogenate, \( V_{thom,1} \) is transferred directly into the respirometer chamber. See Table 4 for further symbols.

**Mitochondrial concentration, \( C_{mtE} \), and mitochondrial markers:** It is important that mitochondrial concentration in the tissue and the measurement chamber be quantified, as a physiological output that is the result of mitochondrial biogenesis and degradation, and as a quantity for normalization in functional analyses. 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...
measurement of chosen mitochondrial markers. ‘Mitochondria are the structural and functional elemental units of cell respiration’ (Gnaiger 2014). The quantity of a mitochondrial marker can be considered to reflect the amount of mitochondrial elements, mtE, expressed in various mitochondrial elemental units [mtEU] specific for each measured mt-marker (Table 4). However, since mitochondrial quality changes under certain stimuli, particularly in mitochondrial dysfunction and after exercise training (Pesta et al. 2011; Campos et al. 2017), some markers can vary while other markers are unchanged: (1) Mitochondrial volume and membrane area are structural markers, whereas mitochondrial protein mass is frequently used as a marker for isolated mitochondria. (2) Molecular and enzymatic mitochondrial markers (amounts or activities) can be selected as matrix markers, e.g., citrate synthase activity, mtDNA; mtIM-markers, e.g., cytochrome c oxidase activity, aa3 content, cardiolipin, or mtOM-markers, e.g., TOM20. (3) Extending the measurement of mitochondrial marker enzyme activity to mitochondrial pathway capacity, ET- or OXPHOS-capacity can be considered as an integrative functional mitochondrial marker.

**Fig. 10.** Structure-function analysis of performance of an organism, organ or tissue, or a cell (sample entity, X). O2 flow, \( I_{X,O_2} \), 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, \( J_{mX,O_2} \), and size of entity, expressed as mass of \( X \); \( M_X = m_X N_X^{-1} \) [kg\( \cdot \)x\(^{-1}\)]. See Table 4 for further explanation of quantities and units. Modified from Gnaiger (2014).

Depending on the type of mitochondrial marker, the mitochondrial elements, \( mtE \), are expressed in marker-specific units. It is recommended to distinguish experimental mitochondrial concentration, \( C_{mtE} = mtE/V \) and physiological mitochondrial density, \( D_{mtE} = mtE/m_X \). Then mitochondrial density is the amount of mitochondrial elements per mass of tissue, which is a biological variable (Fig. 10). The experimental variable is mitochondrial density multiplied by sample mass concentration in the measuring chamber, \( C_{mtE} = D_{mtE} C_{mX} \), or mitochondrial content multiplied by sample number concentration, \( C_{mtE} = mtE X \cdot C_{NX} \) (Table 4).
Mitochondria-specific flux, \( J_{mtE,O2} \): Volume-specific metabolic O\(_2\) flux depends on: (1) the sample concentration in the volume of the instrument chamber, \( C_{mX} \), or \( C_{NX} \); (2) the mitochondrial density in the sample, \( D_{mtE} = mtE/mX \) or \( mtE/NX \); and (3) the specific mitochondrial activity or performance per elemental mitochondrial unit, \( J_{mtE,O2} = J_{V,O2}/C_{mtE} \) [mol\( \cdot \)s\(^{-1}\)\( \cdot \)mtEU\(^{-1}\)] (Table 4). Obviously, the numerical results for \( J_{mtE,O2} \) vary according to the type of mitochondrial marker chosen for measurement of \( mtE \) and \( C_{mtE} = mtE/V \) [mtEU\( \cdot \)m\(^{-3}\)].

3.5. Evaluation of mitochondrial markers

Different methods are implicated in quantification of mitochondrial markers and have different strengths. Some problems are common for all mitochondrial markers, \( mtE \): (1) Accuracy of measurement is crucial, since even a highly accurate and reproducible measurement of O\(_2\) flux results in an inaccurate and noisy expression normalized for a biased and noisy measurement of a mitochondrial marker. This problem is acute in mitochondrial respiration because the denominators used (the mitochondrial markers) are often very small moieties whose accurate and precise determination is difficult. This problem can be avoided when O\(_2\) fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for flux in a defined respiratory reference state, which is used as an internal marker and yields flux control ratios, FCRs (Fig. 8). FCRs are independent of any externally measured markers and, therefore, are statistically very robust, considering the limitations of ratios in general (Jasienski and Bazzaz 1999). FCRs indicate qualitative changes of mitochondrial respiratory control, with highest quantitative resolution, separating the effect of mitochondrial density or concentration on \( J_{mX,O2} \) and \( J_{X,O2} \) from that of function per elemental mitochondrial marker, \( J_{mtE,O2} \) (Pesta et al. 2011; Gnaiger 2014). (2) If mitochondrial quality does not change and only the amount of mitochondria varies as a determinant of mass-specific flux, any marker is equally qualified in principle; then in practice selection of the optimum marker depends only on the accuracy and precision of measurement of the mitochondrial marker. (3) If mitochondrial flux control ratios change, then there may not be any best mitochondrial marker. In general, measurement of multiple mitochondrial markers enables a comparison and evaluation of normalization for a variety of mitochondrial markers. Particularly during postnatal development, the activity of marker enzymes, such as cytochrome c oxidase and citrate synthase, follows different time courses (Drahota et al. 2004). Evaluation of mitochondrial markers in healthy controls is insufficient for providing guidelines for application in the diagnosis of pathological states and specific treatments.

In line with the concept of the respiratory control ratio (Chance and Williams 1955a), the most readily used normalization is that of flux control ratios and flux control factors (Gnaiger 2014). Selection of the state of maximum flux in a protocol as the reference state has the advantages of: (1) internal normalization; (2) statistical linearization of the response in the range of 0 to 1; and (3) consideration of maximum flux for integrating a very large number of elemental steps in the OXPHOS- or ET-pathways. This reduces the risk of selecting a functional marker that is specifically altered by the treatment or pathology, yet increases the chance that the highly integrative pathway is disproportionately affected, e.g. the OXPHOS- rather than ET-pathway in case of an enzymatic defect in the phosphorylation-pathway. In this case, additional information can be obtained by reporting flux control ratios based on a reference state which indicates stable tissue-mass specific flux. Stereological determination of mitochondrial content via two-dimensional transmission electron microscopy can have limitations due to the dynamics of mitochondrial size (Meinild Lundby et al. 2017). Accurate determination of three-dimensional volume by two-dimensional microscopy can be both time consuming and statistically challenging (Larsen et al. 2012).

The validity of using mitochondrial marker enzymes (citrate synthase activity, Complex I–IV amount or activity) for normalization of flux is limited in part by the same factors that
apply to flux control ratios. Strong correlations between various mitochondrial markers and
citrate synthase activity (Reichmann et al. 1985; Boushel et al. 2007; Mogensen et al. 2007)
are expected in a specific tissue of healthy subjects and in disease states not specifically
targeting citrate synthase. Citrate synthase activity is acutely modifiable by exercise
(Tonkonogi et al. 1997; Leek et al. 2001). Evaluation of mitochondrial markers related to a
selected age and sex cohort cannot be extrapolated to provide recommendations for
normalization in respirometric diagnosis of disease, in different states of development and
ageing, different cell types, tissues, and species. mtDNA normalised to nDNA via qPCR is
related to functional mitochondrial markers including OXPHOS- and ET-capacity in some
cases (Puntschart et al. 1995; Wang et al. 1999; Menshikova et al. 2006; Boushel et al. 2007),
but lack of such correlations have been reported (Menshikova et al. 2005; Schultz and Wiesner
2000; Pesta et al. 2011). Several studies indicate a strong correlation between cardiolipin
content and increase in mitochondrial function with exercise (Menshikova et al. 2005;
Menshikova et al. 2007; Larsen et al. 2012; Faber et al. 2014), but its use as a general
mitochondrial biomarker in disease remains questionable.

3.6. Conversion: units

Many different units have been used to report the rate of oxygen consumption, OCR
(Table 6). SI base units provide the common reference for introducing the theoretical principles
(Fig. 8), and are used with appropriately chosen SI prefixes to express numerical data in the
most practical format, with an effort towards unification within specific areas of application
(Table 7). For studies of cells, we recommend that respiration be expressed, as far as possible,
as: (1) $O_2$ flux normalized for a mitochondrial marker, for separation of the effects of
mitochondrial quality and content on cell respiration (this includes FCRs as a normalization for
a functional mitochondrial marker); (2) $O_2$ flux in units of cell volume or mass, for comparison
of respiration of cells with different cell size (Renner et al. 2003) and with studies on tissue
preparations, and (3) $O_2$ flow in units of attomole ($10^{-18}$ mol) of $O_2$ consumed in a second by
each cell [amol·s$^{-1}$·cell$^{-1}$], numerically equivalent to [pmol·s$^{-1}$·10$^{-6}$ cells]. This convention
allows information to be easily used when designing experiments in which oxygen consumption
must be considered. For example, to estimate the volume-specific $O_2$ flux in an instrument
chamber that would be expected at a particular cell number concentration, one simply needs to
multiply the flow per cell by the number of cells per volume of interest. This provides the
amount of $O_2$ [mol] consumed per time [s$^{-1}$] per unit volume [L$^{-1}$]. At an $O_2$ flow of 100
amol·s$^{-1}$·cell$^{-1}$ and a cell density of $10^9$ cells·L$^{-1}$ ($10^6$ cells·mL$^{-1}$), the volume-specific $O_2$ flux is
100 nmol·s$^{-1}$·L$^{-1}$ (100 pmol·s$^{-1}$·mL$^{-1}$).

Although volume is expressed as m$^3$ using the SI base unit, the litre [dm$^3$] is the basic unit
of volume for concentration and is used for most solution chemical kinetics. If one multiplies
$I_{cell,O_2}$ by $C_{Ncell}$, then the result will not only be the amount of $O_2$ [mol] consumed per time [s$^{-1}$]
in one litre [L$^{-1}$], but also the change in the concentration of oxygen per second (for any volume
of an ideally closed system). This is ideal for kinetic modeling as it blends with chemical rate
equations where concentrations are typically expressed in mol·L$^{-1}$ (Wagner et al. 2011). In
studies of multinuclear cells, such as differentiated skeletal muscle cells, it is easy to determine
the number of nuclei but not the total number of cells. A generalized concept, therefore, is
obtained by substituting cells by nuclei as the sample entity. This does not hold, however, for
enucleated platelets.
Table 6. Conversion of various units used in respirometry and ergometry. E is the number of electrons or reducing equivalents. $z_B$ is the charge number of entity B.

<table>
<thead>
<tr>
<th>1 Unit</th>
<th>$x$</th>
<th>Multiplication factor</th>
<th>SI-unit</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng.atom O·s$^{-1}$</td>
<td>(2 e$^-$)</td>
<td>0.5</td>
<td>nmol O·s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>ng.atom O·min$^{-1}$</td>
<td>(2 e$^-$)</td>
<td>8.33</td>
<td>pmol O·s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>natom O·min$^{-1}$</td>
<td>(2 e$^-$)</td>
<td>8.33</td>
<td>pmol O·s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>nmol O$_2$·min$^{-1}$</td>
<td>(4 e$^-$)</td>
<td>16.67</td>
<td>pmol O$_2$·s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>nmol O$_2$·h$^{-1}$</td>
<td>(4 e$^-$)</td>
<td>0.2778</td>
<td>pmol O$_2$·s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>mL O$_2$·min$^{-1}$ at STPD$^a$</td>
<td></td>
<td>0.744</td>
<td>µmol O$_2$·s$^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td>W = J/s at -470 kJ/mol O$_2$</td>
<td></td>
<td>-2.128</td>
<td>µmol O$_2$·s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>mA = mC·s$^{-1}$</td>
<td>($z_{H^+} = 1$)</td>
<td>10.36</td>
<td>nmol H$^+$·s$^{-1}$</td>
<td>2</td>
</tr>
<tr>
<td>mA = mC·s$^{-1}$</td>
<td>($z_O = 4$)</td>
<td>2.59</td>
<td>nmol O$_2$·s$^{-1}$</td>
<td>2</td>
</tr>
<tr>
<td>nmol H$^+$·s$^{-1}$</td>
<td>($z_{H^+} = 1$)</td>
<td>0.09649</td>
<td>mA</td>
<td>3</td>
</tr>
<tr>
<td>nmol O$_2$·s$^{-1}$</td>
<td>($z_O = 4$)</td>
<td>0.38594</td>
<td>mA</td>
<td>3</td>
</tr>
</tbody>
</table>

1 At standard temperature and pressure dry (STPD: 0 °C = 273.15 K and 1 atm = 101.325 kPa = 760 mmHg), the molar volume of an ideal gas, $V_m$, and $V_{m,O_2}$ is 22.414 and 22.392 L·mol$^{-1}$ respectively. Rounded to three decimal places, both values yield the conversion factor of 0.744. For comparison at NTPD (20 °C), $V_{m,O_2}$ is 24.038 L·mol$^{-1}$. Note that the SI standard pressure is 100 kPa.

2 The multiplication factor is $10^6/(z_B F)$.

3 The multiplication factor is $z_B F/10^6$.

Table 7. Conversion of units with preservation of numerical values.

<table>
<thead>
<tr>
<th>Name</th>
<th>Frequently used unit</th>
<th>Equivalent unit</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>volume-specific flux, $J_{V,O_2}$</td>
<td>pmol·s$^{-1}$·mL$^{-1}$</td>
<td>nmol·s$^{-1}$·L$^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td>cell-specific flow, $I_{O_2}$</td>
<td>pmol·s$^{-1}$·10$^9$ cells</td>
<td>amol·s$^{-1}$·cell$^{-1}$</td>
<td>2</td>
</tr>
<tr>
<td>cell number concentration, $C_{Nce}$</td>
<td>pmol·s$^{-1}$·10$^9$ cells</td>
<td>zmol·s$^{-1}$·cell$^{-1}$</td>
<td>3</td>
</tr>
<tr>
<td>mitochondrial protein concentration, $C_{mtE}$</td>
<td>10$^6$ cells·mL$^{-1}$</td>
<td>10$^9$ cells·L$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>mass-specific flux, $J_{m,O_2}$</td>
<td>0.1 mg·mL$^{-1}$</td>
<td>0.1 g·L$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>catabolic power, $P_k$</td>
<td>pmol·s$^{-1}$·mg$^{-1}$</td>
<td>nmol·s$^{-1}$·g$^{-1}$</td>
<td>4</td>
</tr>
<tr>
<td>volume</td>
<td>µW·10$^{-6}$ cells</td>
<td>pW·cell$^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td>amount of substance concentration</td>
<td>1,000 L</td>
<td>m$^3$ (1,000 kg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>dm$^3$ (kg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mL</td>
<td>cm$^3$ (g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>µL</td>
<td>mm$^3$ (mg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fL</td>
<td>µm$^3$ (pg)</td>
<td>5</td>
</tr>
</tbody>
</table>

1 pmol: picomole = 10$^{-12}$ mol
2 amol: attomole = 10$^{-18}$ mol
3 zmol: zeptomole = 10$^{-21}$ mol
4 nmol: nanomole = 10$^{-9}$ mol
5 fL: femtolitre = 10$^{-15}$ L
ET-capacity in various human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 amol·s⁻¹·cell⁻¹, measured in intact cells in the noncoupled state (see Gnaiger 2014). At 100 amol·s⁻¹·cell⁻¹ corrected for Rox, the current across the mitochondrial membranes, \( I_{ET} \), approximates 193 pA·cell⁻¹ or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W. Modelling approaches illustrate the link between protonmotive force and currents (Willis et al. 2016).

We consider isolated mitochondria as powerhouses and proton pumps as molecular machines to relate experimental results to energy metabolism of the intact cell. The cellular \( P»/O_2 \) based on oxidation of glycogen is increased by the glycolytic (fermentative) substrate-level phosphorylation of 3 P»/Glyc, i.e., 0.5 mol P» for each mol O₂ consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial \( P»/O_2 \) ratio of 5.4 yields a bioenergetic cell physiological \( P»/O_2 \) ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either by the malate-aspartate shuttle or by the glycerophosphate shuttle resulting in different theoretical yields of ATP generated by mitochondria, the energetic cost of which potentially must be taken into account. Considering also substrate-level phosphorylation in the TCA cycle, this high \( P»/O_2 \) ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993a).

4. Conclusions

MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects linked to genetic variation, age-related health risks, sex-specific mitochondrial performance, lifestyle with its effects on degenerative diseases, and thermal and chemical environment. The present recommendations on coupling control states and rates, linked to the concept of the protonmotive force, are focused on studies with mitochondrial preparations. These will be extended in a series of reports on pathway control of mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental procedures.

The optimal choice for expressing mitochondrial and cell respiration (Box 3) as O₂ flow per biological system, and normalization for specific tissue-markers (volume, mass, protein) and mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes, respiratory reference state) is guided by the scientific question under study. Interpretation of the obtained data depends critically on appropriate normalization, and therefore reporting rates merely as nmol·s⁻¹ is discouraged, since it restricts the analysis to intra-experimental comparison of relative (qualitative) differences. Expressing O₂ consumption per cell may not be possible when dealing with tissues. For studies with mitochondrial preparations, we recommend that normalizations be provided as far as possible: (1) on a per cell basis as O₂ flow (a biophysical normalization); (2) per g cell or tissue protein, or per cell or tissue mass as mass-specific O₂ flux (a cellular normalization); and (3) per mitochondrial marker as mt-specific flux (a mitochondrial normalization). With information on cell size and the use of multiple normalizations, maximum potential information is available (Renner et al. 2003; Wagner et al. 2011; Gnaiger 2014).

When using isolated mitochondria, mitochondrial protein is a frequently applied mitochondrial marker, the use of which is basically restricted to isolated mitochondria. The mitochondrial recovery and yield, and experimental criteria for evaluation of purity versus integrity should be reported. Mitochondrial markers, such as citrate synthase activity as an enzymatic matrix marker, provide a link to the tissue of origin on the basis of calculating the
Table 8. Terms, symbols, and units.

<table>
<thead>
<tr>
<th>Term</th>
<th>Symbol</th>
<th>SI unit</th>
<th>Links and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>alternative quinol oxidase</td>
<td>AOX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amount of substance B</td>
<td>( n_B )</td>
<td>[mol]</td>
<td></td>
</tr>
<tr>
<td>apparent equilibrium constant</td>
<td>( K_m )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complexes I to IV</td>
<td>CI to CIV</td>
<td>Respiratory ET Complexes; Fig. 1</td>
<td></td>
</tr>
<tr>
<td>concentration of substance B</td>
<td>( c_B = n_B \cdot V^{-1}; \ [B] )</td>
<td>[mol·m(^{-3})]</td>
<td>Box 2</td>
</tr>
<tr>
<td>electron transfer system</td>
<td>ETS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flow, for substance B</td>
<td>( I_B )</td>
<td>[mol·s(^{-1})]</td>
<td>System-related extensive quantity; Fig. 8</td>
</tr>
<tr>
<td>flux, for substance B</td>
<td>( J_B )</td>
<td>( \text{varies} )</td>
<td>Size-specific quantity; Fig. 8</td>
</tr>
<tr>
<td>inorganic phosphate</td>
<td>( P_i )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEAK</td>
<td>LEAK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mass of sample ( X )</td>
<td>( m_X )</td>
<td>[kg]</td>
<td></td>
</tr>
<tr>
<td>mass of entity ( X )</td>
<td>( M_X )</td>
<td>[kg]</td>
<td></td>
</tr>
<tr>
<td>mitochondria or mitochondrial DNA</td>
<td>mt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mitochondrial concentration</td>
<td>( C_{mtE} = \text{mtE}\cdot V^{-1} )</td>
<td>[mtEU·m(^{-3})]</td>
<td>Tab. 4</td>
</tr>
<tr>
<td>mitochondrial content</td>
<td>( \text{mtE}_X = \text{mtE}\cdot N_X^{-1} )</td>
<td>[mtEU·x(^{-1})]</td>
<td>Tab. 4</td>
</tr>
<tr>
<td>mitochondrial elemental unit</td>
<td>( \text{mtEU} )</td>
<td>( \text{varies} )</td>
<td></td>
</tr>
<tr>
<td>mitochondrial inner membrane</td>
<td>( \text{mtIM} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mitochondrial outer membrane</td>
<td>( \text{mtOM} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mitochondrial recovery</td>
<td>( Y_{mtE} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mitochondrial yield</td>
<td>( Y_{mtEm} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>( \text{neg} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>number concentration of ( X )</td>
<td>( C_{XX} )</td>
<td>[x·m(^{-3})]</td>
<td></td>
</tr>
<tr>
<td>number of entities ( X )</td>
<td>( N_X )</td>
<td>[x]</td>
<td></td>
</tr>
<tr>
<td>number of entity ( B )</td>
<td>( N_B )</td>
<td>[x]</td>
<td></td>
</tr>
<tr>
<td>oxidative phosphorylation</td>
<td>( \text{OXPHOS} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oxygen concentration</td>
<td>( c_{O_2} = n_{O_2} \cdot V^{-1}; \ [O_2] )</td>
<td>[mol·m(^{-3})]</td>
<td>Section 3.2</td>
</tr>
<tr>
<td>phosphorylation of ADP to ATP</td>
<td>( P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>( \text{pos} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>proton in the negative compartment</td>
<td>( H^+_{\text{neg}} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>proton in the positive compartment</td>
<td>( H^+_{\text{pos}} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate of electron transfer in ET state</td>
<td>( E )</td>
<td>ET-capacity; Tab. 1</td>
<td></td>
</tr>
<tr>
<td>rate of LEAK respiration</td>
<td>( L )</td>
<td>Tab. 1</td>
<td></td>
</tr>
<tr>
<td>rate of oxidative phosphorylation</td>
<td>( P )</td>
<td>OXPHOS capacity; Tab. 1</td>
<td></td>
</tr>
<tr>
<td>rate of residual oxygen consumption</td>
<td>( \text{ROX} )</td>
<td>Tab. 1</td>
<td></td>
</tr>
<tr>
<td>residual oxygen consumption</td>
<td>( \text{ROX} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>specific mitochondrial density</td>
<td>( D_{mtE} = \text{mtE}\cdot m_X^{-1} )</td>
<td>[mtEU·kg(^{-1})]</td>
<td>Tab. 7</td>
</tr>
<tr>
<td>volume</td>
<td>( V )</td>
<td>[m(^3)]</td>
<td></td>
</tr>
<tr>
<td>weight, dry weight</td>
<td>( W_d )</td>
<td>[kg]</td>
<td>used as mass of sample ( X ); Fig. 8</td>
</tr>
<tr>
<td>weight, wet weight</td>
<td>( W_w )</td>
<td>[kg]</td>
<td>used as mass of sample ( X ); Fig. 8</td>
</tr>
</tbody>
</table>

Links and comments:
- Tab. 1: Specific units for mt-marker
- Tab. 4: Specific units for mt-marker
- Box 1: MOM is widely used, and M is replaced by mt as abbreviation for mitochondria
- Box 2: oxygen concentration

By mt as abbreviation for mitochondria;
Terms and symbols are summarized in Table 8. Their use will facilitate transdisciplinary communication and support further developments towards a consistent theory of bioenergetics and mitochondrial physiology.

Box 3: Mitochondrial and cell respiration

Mitochondrial and cell respiration is the process of highly exergonic and exothermic energy transformation in which scalar redox reactions are coupled to vectorial ion translocation across a semipermeable membrane, which separates the small volume of a bacterial cell or mitochondrion from the larger volume of its surroundings. The electrochemical exergy can be partially conserved in the phosphorylation of ADP to ATP or in ion pumping, or dissipated in an electrochemical short-circuit. Respiration is thus clearly distinguished from fermentation as the counterpart of cellular core energy metabolism. Respiration is separated in mitochondrial preparations from the partial contribution of fermentative pathways of the intact cell. According to this definition, residual oxygen consumption, as measured after inhibition of mitochondrial electron transfer, does not belong to the class of catabolic reactions and is, therefore, subtracted from total oxygen consumption to obtain baseline-corrected respiration.

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