

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

http://www.mitoeagle.org/index.php/MitoEAGLE_preprint_2018-02-08

Preprint version 22 (2018-02-08)

MitoEAGLE Network

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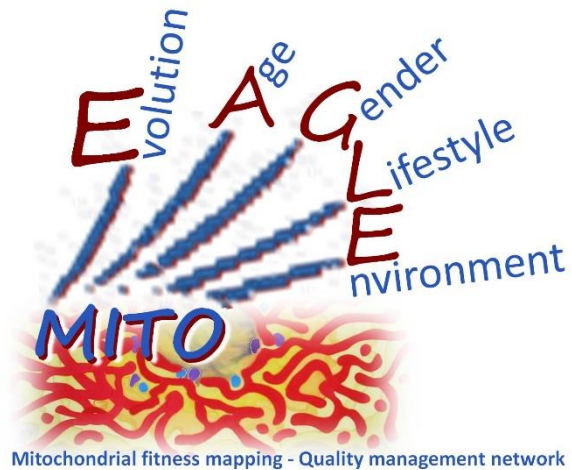
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This manuscript on 'Mitochondrial respiratory states and rates' is a position statement in the frame of COST Action CA15203 MitoEAGLE. The list of co-authors evolved beyond **phase 1** (phase 1 versions 1-44) in the **bottom-up** spirit of COST.

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.



Mitochondrial fitness mapping - Quality management network

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

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103	Contents
104	1. Introduction – Box 1: In brief: Mitochondria and Bioblasts
105	2. Oxidative phosphorylation and coupling states in mitochondrial preparations
106	Mitochondrial preparations
107	2.1. <i>Three coupling states of mitochondrial preparations and residual oxygen consumption</i>
108	Respiratory capacities in coupling control states
109	Kinetic control
110	The steady-state
111	Specification of biochemical dose
112	Phosphorylation, P»
113	Coupling
114	Uncoupling
115	LEAK, OXPHOS, ET, ROX
116	2.2. <i>Coupling states and respiratory rates</i>
117	P»/O ₂ ratio
118	Control and regulation
119	Respiratory control and response
120	Respiratory coupling control
121	ET-pathway control states
122	2.3. <i>Classical terminology for isolated mitochondria</i>
123	States 1-5
124	3. Normalization: fluxes and flows
125	3.1. <i>Normalization: system or sample</i>
126	Flow per system, I
127	Extensive quantities
128	Size-specific quantities
129	- Box 2: Metabolic fluxes and flows: vectorial and scalar
130	3.2. <i>Normalization for system-size: flux per chamber volume</i>
131	System-specific flux, J
132	3.3. <i>Normalization: per sample</i>
133	Sample concentration, C_{mX}
134	Mass-specific flux, J_{mX,O_2}
135	Number concentration, C_{NX}
136	Flow per object, I_{X,O_2}
137	3.4. <i>Normalization for mitochondrial content</i>
138	Mitochondrial concentration, C_{mtE} , and mitochondrial markers
139	Mitochondria-specific flux, J_{mtE,O_2}
140	3.5. <i>Evaluation of mitochondrial markers</i>
141	3.6. <i>Conversion: units</i>
142	4. Conclusions
143	5. References - Box 3: Mitochondrial and cell respiration
144	

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

162
163 *Keywords:* Mitochondrial respiratory control, coupling control, mitochondrial
164 preparations, protonmotive force, oxidative phosphorylation, OXPHOS, efficiency, electron
165 transfer, ET; proton leak, LEAK, residual oxygen consumption, ROX, State 2, State 3, State 4,
166 normalization, flow, flux

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169 **Executive summary**

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171 *In preparation.*

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Box 1: In brief – Mitochondria and Bioblasts

179 **Mitochondria** are the oxygen-consuming electrochemical generators which evolved from
180 endosymbiotic bacteria (Margulis 1970; Lane 2005). They were described by Richard Altmann
181 (1894) as ‘bioblasts’, which include not only the mitochondria as presently defined, but also
182 symbiotic and free-living bacteria. The word ‘mitochondria’ (Greek mitos: thread; chondros:
183 granule) was introduced by Carl Benda (1898).

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

190 We now recognize mitochondria as dynamic organelles with a double membrane that are
191 contained within eukaryotic cells. The mitochondrial inner membrane (mtIM) shows dynamic
192 tubular to disk-shaped cristae that separate the mitochondrial matrix, *i.e.*, the negatively charged
193 internal mitochondrial compartment, and the intermembrane space; the latter being positively
194 charged and enclosed by the mitochondrial outer membrane (mtOM). The mtIM contains the
195 non-bilayer phospholipid cardiolipin, which is not present in any other eukaryotic cellular
196 membrane. Cardiolipin promotes the formation of respiratory supercomplexes, which are
197 supramolecular assemblies based upon specific, though dynamic, interactions between
198 individual respiratory complexes (Greggio *et al.* 2017; Lenaz *et al.* 2017). Membrane fluidity
199 is an important parameter influencing functional properties of proteins incorporated in the
200 membranes (Waczulikova *et al.* 2007).

201 Mitochondria are the structural and functional elements of cell respiration. Cell
202 respiration is the consumption of oxygen by electron transfer coupled to electrochemical proton
203 translocation across the mtIM. In the process of oxidative phosphorylation (OXPHOS), the
204 reduction of O₂ is electrochemically coupled to the transformation of energy in the form of
205 adenosine triphosphate (ATP; Mitchell 1961, 2011). Mitochondria are the powerhouses of the
206 cell which contain the machinery of the OXPHOS-pathways, including transmembrane
207 respiratory complexes (*i.e.*, proton pumps with FMN, Fe-S and cytochrome *b*, *c*, *aa*₃ redox
208 systems); alternative dehydrogenases and oxidases; the coenzyme ubiquinone (Q); F-ATPase
209 or ATP synthase; the enzymes of the tricarboxylic acid cycle and the fatty acid oxidation
210 enzymes; transporters of ions, metabolites and co-factors; and mitochondrial kinases related to
211 energy transfer pathways. The mitochondrial proteome comprises over 1,200 proteins (Calvo
212 *et al.* 2015; 2017), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many
213 of which are relatively well known (*e.g.* apoptosis-regulating proteins), while others are still
214 under investigation, or need to be identified (*e.g.* alanine transporter).

215 There is a constant crosstalk between mitochondria and the other cellular components,
216 maintaining cellular mitostasis through regulation at both the transcriptional and post-
217 translational level, and through cell signalling including proteostatic (*e.g.* the ubiquitin-
218 proteasome and autophagy-lysosome pathways) and genome stability modules throughout the
219 cell cycle or even cell death, contributing to homeostatic regulation in response to varying
220 energy demands and stress (Quiros *et al.* 2016). In addition to mitochondrial movement along
221 the microtubules, mitochondrial morphology can change in response to energy requirements of
222 the cell via processes known as fusion and fission, through which mitochondria communicate
223 within a network, and in response to intracellular stress factors causing swelling and ultimately
224 permeability transition.

225 Mitochondria typically maintain several copies of their own genome (hundred to
226 thousands per cell; Cummins 1998), which is maternally inherited (White *et al.* 2008) and

227 known as mitochondrial DNA (mtDNA). One exception to strictly maternal inheritance in
228 animals is found in bivalves (Breton *et al.* 2007). mtDNA is 16.5 kB in length, contains 13
229 protein-coding genes for subunits of the transmembrane respiratory Complexes CI, CIII, CIV
230 and F-ATPase, and also encodes 22 tRNAs and the mitochondrial 16S and 12S rRNA.
231 Additional gene content is encoded in the mitochondrial genome, *e.g.* microRNAs, piRNA,
232 smithRNAs, repeat associated RNA, and even additional proteins (Duarte *et al.* 2014; Lee *et*
233 *al.* 2015; Cobb *et al.* 2016). The mitochondrial genome is both regulated and supplemented by
234 nuclear-encoded mitochondrial targeted proteins.

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

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

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1. Introduction

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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 Evolution, Age, Gender and sex, Lifestyle, and Environment (EAGLE) as essential background conditions intrinsic to the individual patient or subject, cohort, species, tissue and to some extent even cell line. As a large and 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.

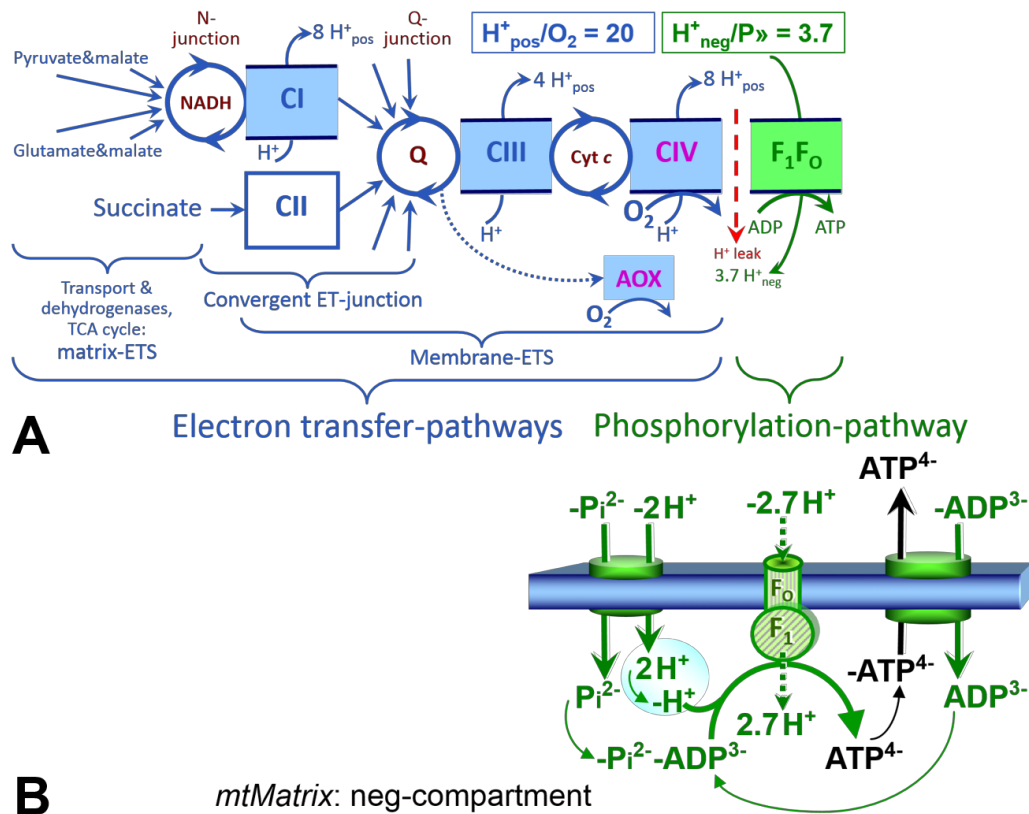
278 **2. Oxidative phosphorylation and coupling states in mitochondrial preparations**

279 *‘Every professional group develops its own technical jargon for talking about matters of*
 280 *critical concern ... People who know a word can share that idea with other members of*
 281 *their group, and a shared vocabulary is part of the glue that holds people together and*
 282 *allows them to create a shared culture’ (Miller 1991).*
 283

284 **Mitochondrial preparations** are defined as either isolated mitochondria, or tissue and
 285 cellular preparations in which the barrier function of the plasma membrane is disrupted. The
 286 plasma membrane separates the cytosol, nucleus, and organelles (the intracellular
 287 compartment) from the environment of the cell. The plasma membrane consists of a lipid
 288 bilayer, embedded proteins, and attached organic molecules that collectively control the
 289 selective permeability of ions, organic molecules, and particles across the cell boundary. The
 290 intact plasma membrane, therefore, prevents the passage of many water-soluble mitochondrial
 291 substrates, such as succinate or adenosine diphosphate (ADP), that are required for the analysis
 292 of respiratory capacity at kinetically-saturating concentrations, thus limiting the scope of
 293 investigations into mitochondrial respiratory function in intact cells. The cholesterol content of
 294 the plasma membrane is high compared to mitochondrial membranes. Therefore, mild
 295 detergents, such as digitonin and saponin, can be applied to selectively permeabilize the plasma
 296 membrane by interaction with cholesterol and allow free exchange of cytosolic components
 297 with ions and organic molecules of the immediate cell environment, while maintaining the
 298 integrity and localization of organelles, cytoskeleton, and the nucleus. Application of optimum
 299 concentrations of permeabilization agents (mild detergents or toxins) leads to the complete loss
 300 of cell viability, tested by nuclear staining and washout of cytosolic marker enzymes such as
 301 lactate dehydrogenase, while mitochondrial function remains intact. The respiration rate of
 302 isolated mitochondria remains unaltered after the addition of low concentrations of digitonin or
 303 saponin. In addition to mechanical permeabilization during homogenization of tissue,
 304 permeabilization agents may be applied to ensure permeabilization of all cells. Suspensions of
 305 cells permeabilized in the respiration chamber and crude tissue homogenates contain all
 306 components of the cell at highly diluted concentrations. All mitochondria are retained in
 307 chemically-permeabilized mitochondrial preparations and crude tissue homogenates. In the
 308 preparation of isolated mitochondria, the cells or tissues are homogenized, and the mitochondria
 309 are separated from other cell fractions and purified by differential centrifugation, entailing the
 310 loss of a fraction of mitochondria. Typical mitochondrial recovery ranges from 30% to 80%.
 311 Maximization of the purity of isolated mitochondria may compromise not only the
 312 mitochondrial yield but also the structural and functional integrity. Therefore, protocols for
 313 isolation of mitochondria need to be optimized according to the relevant questions addressed in
 314 a study. The term mitochondrial preparation does not include further fractionation of
 315 mitochondrial components, as well as submitochondrial particles.
 316

317 *2.1. Three coupling states of mitochondrial preparations and residual oxygen consumption*

318
 319 **Respiratory capacities in coupling control states:** To extend the classical nomenclature
 320 on mitochondrial coupling states (Section 2.3) by a concept-driven terminology that
 321 incorporates explicitly information on the nature of respiratory states, the terminology must be
 322 general and not restricted to any particular experimental protocol or mitochondrial preparation
 323 (Gnaiger 2009). We focus primarily on the conceptual ‘why’, along with clarification of the
 324 experimental ‘how’. In the following section, the concept-driven terminology is explained and
 325 coupling states are defined. We define respiratory capacities, comparable to channel capacity
 326 in information theory (Schneider 2006), as the upper bound of the rate of respiration measured
 327 in defined coupling control states and electron transfer-pathway (ET-pathway) states.
 328



329 **Fig. 1. The oxidative phosphorylation (OXPHOS) system.** (A) The mitochondrial electron
 330 transfer system (ETS) is fuelled by diffusion and transport of substrates across the mtOM and
 331 mtIM and consists of the matrix-ETS and membrane-ETS. Electron transfer (ET) pathways are
 332 coupled to the phosphorylation-pathway. ET-pathways converge at the N-junction and Q-
 333 junction (additional arrows indicate electron entry into the Q-junction through electron
 334 transferring flavoprotein, glycerophosphate dehydrogenase, dihydro-orotate dehydrogenase,
 335 choline dehydrogenase, and sulfide-ubiquinone oxidoreductase). The dotted arrow indicates the
 336 branched pathway of oxygen consumption by alternative quinol oxidase (AOX). The H^+_{pos}/O_2
 337 ratio is the outward proton flux from the matrix space to the positively (pos) charged
 338 compartment, divided by catabolic O_2 flux in the NADH-pathway. The H^+_{neg}/P_{\gg} ratio is the
 339 inward proton flux from the inter-membrane space to the negatively (neg) charged matrix space,
 340 divided by the flux of phosphorylation of ADP to ATP (Eq. 1). Due to ion leaks and proton slip
 341 these are not fixed stoichiometries. (B) Phosphorylation-pathway catalyzed by the proton pump
 342 F_1F_0 -ATPase, adenine nucleotide translocase, and inorganic phosphate transporter. The
 343 H^+_{neg}/P_{\gg} stoichiometry is the sum of the coupling stoichiometry in the F -ATPase reaction (-2.7
 344 H^+_{pos} from the positive intermembrane space, $2.7 H^+_{\text{neg}}$ to the matrix, *i.e.*, the negative
 345 compartment) and the proton balance in the translocation of ADP^{2-} , ATP^{3-} and P_i^{2-} . Modified
 346 from (A) Lemieux *et al.* (2017) and (B) Gnaiger (2014).

347
 348 To provide a diagnostic reference for respiratory capacities of core energy metabolism,
 349 the capacity of *oxidative phosphorylation*, OXPHOS, is measured at kinetically-saturating
 350 concentrations of ADP and inorganic phosphate, P_i . The *oxidative* ET-capacity reveals the
 351 limitation of OXPHOS-capacity mediated by the *phosphorylation*-pathway. The ET- and
 352 phosphorylation-pathways comprise coupled segments of the OXPHOS-system. ET-capacity
 353 is measured as noncoupled respiration by application of *external uncouplers*. The contribution
 354 of *intrinsically uncoupled* oxygen consumption is most easily studied in the absence of ADP,
 355 *i.e.*, by not stimulating phosphorylation, or by inhibition of the phosphorylation-pathway. The
 356 corresponding states are collectively classified as LEAK-states, when oxygen consumption

357 compensates mainly for ion leaks including the proton leak (**Table 1**). Defined coupling states
 358 are induced by: (1) adding cation chelators such as EGTA, binding free Ca^{2+} and thus limiting
 359 cation cycling; (2) adding ADP and P_i ; (3) inhibiting the phosphorylation-pathway; and (4)
 360 uncoupler titrations, while maintaining a defined ET-pathway state with constant fuel substrates
 361 and inhibitors of specific branches of the ET-pathway (**Fig. 1**).

362

363 **Table 1. Coupling states and residual oxygen consumption in mitochondrial**
 364 **preparations in relation to respiration- and phosphorylation-rate, J_{KO_2} and J_{P} ,**
 365 **and protonmotive force, pmf. Coupling states are established at kinetically-**
 366 **saturating concentrations of fuel substrates and O_2 .**

State	J_{KO_2}	J_{P}	pmf	Inducing factors	Limiting factors
LEAK	L ; low, cation leak-dependent respiration	0	max.	proton leak, slip, and cation cycling	$J_{\text{P}} = 0$: (1) without ADP, L_N ; (2) max. ATP/ADP ratio, L_T ; or (3) inhibition of the phosphorylation-pathway, L_{Omy}
OXPHOS	P ; high, ADP-stimulated respiration	max.	high	kinetically-saturating [ADP] and [P_i]	J_{P} by phosphorylation-pathway; or J_{KO_2} by ET-capacity
ET	E ; max., noncoupled respiration	0	low	optimal external uncoupler concentration for max. $J_{\text{O}_2,E}$	J_{KO_2} by ET-capacity
ROX	R_{ox} ; min., residual O_2 consumption	0	0	$J_{\text{O}_2,R_{\text{ox}}}$ in non-ET-pathway oxidation reactions	full inhibition of ET-pathway; or absence of fuel substrates

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368 **Kinetic control:** Coupling control states are established in the study of mitochondrial
 369 preparations to obtain reference values for various output variables. Physiological conditions *in*
 370 *vivo* deviate from these experimentally obtained states. Since kinetically-saturating
 371 concentrations, *e.g.* of ADP or oxygen, may not apply to physiological intracellular conditions,
 372 relevant information is obtained in studies of kinetic responses to conditions intermediate
 373 between the LEAK state at zero [ADP] and the OXPHOS-state at saturating [ADP], or of
 374 respiratory capacities in the range between kinetically-saturating [O_2] and anoxia (Gnaiger
 375 2001).

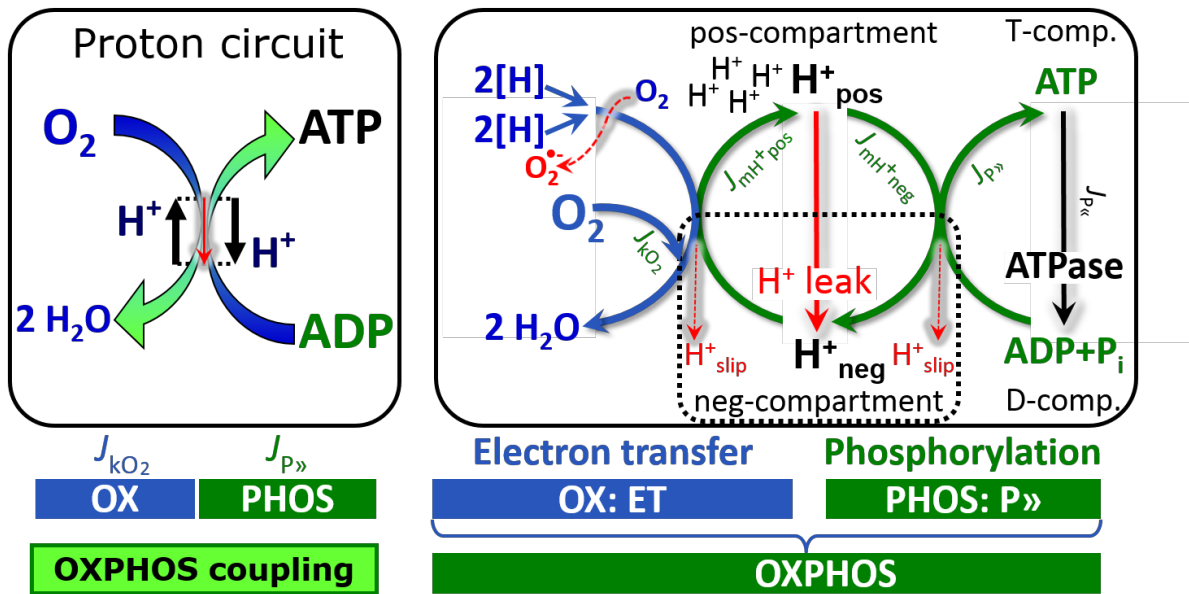
376

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

388 **Specification of biochemical dose:** Substrates, uncouplers, inhibitors, and other
 389 biochemical reagents are titrated to dissect mitochondrial function. Nominal concentrations of
 390 these substances are usually reported as initial amount of substance concentration [$\text{mol}\cdot\text{L}^{-1}$] in
 391 the incubation medium. When aiming at the measurement of kinetically saturated processes
 392 such as OXPHOS-capacities, the concentrations for substrates can be chosen in light of the
 393 apparent equilibrium constant, K_m' . In the case of hyperbolic kinetics, only 80% of maximum
 394 respiratory capacity is obtained at a substrate concentration of four times the K_m' , whereas
 395 substrate concentrations of 5, 9, 19 and 49 times the K_m' are theoretically required for reaching
 396 83%, 90%, 95% or 98% of the maximal rate (Gnaiger 2001). Other reagents are chosen to
 397 inhibit or alter some process. The amount of these chemicals in an experimental incubation is
 398 selected to maximize effect, yet not lead to unacceptable off-target consequences that would
 399 adversely affect the data being sought. Specifying the amount of substance in an incubation as
 400 nominal concentration in the aqueous incubation medium can be ambiguous (Doskey *et al.*
 401 2015), particularly when lipophilic substances (oligomycin; uncouplers, permeabilization
 402 agents) or cations (TPP^+ ; fluorescent dyes such as safranin, TMRM) are applied which
 403 accumulate in biological membranes or the mitochondrial matrix. For example, a dose of
 404 digitonin of $8 \text{ fmol}\cdot\text{cell}^{-1}$ ($10 \mu\text{g}\cdot 10^{-6} \text{ cells}$) is optimal for permeabilization of endothelial cells,
 405 and the concentration in the incubation medium has to be adjusted according to the cell density
 406 applied (Doerrier *et al.* 2018). Generally, dose/exposure can be specified per unit of biological
 407 sample, *i.e.*, (nominal moles of xenobiotic)/(number of cells) [$\text{mol}\cdot\text{cell}^{-1}$] or, as appropriate, per
 408 mass of biological sample [$\text{mol}\cdot\text{kg}^{-1}$]. This approach to specification of dose/exposure provides
 409 a scalable parameter that can be used to design experiments, help interpret a wide variety of
 410 experimental results, and provide absolute information that allows researchers worldwide to
 411 make the most use of published data (Doskey *et al.* 2015).

412 **Phosphorylation, P»:** *Phosphorylation* in the context of OXPHOS is defined as
 413 phosphorylation of ADP by P_i to ATP. On the other hand, the term phosphorylation is used
 414 generally in many different contexts, *e.g.* protein phosphorylation. This justifies consideration
 415 of a symbol more discriminating and specific than P as used in the P/O ratio (phosphate to
 416 atomic oxygen ratio; $\text{O} = 0.5 \text{ O}_2$), where P indicates phosphorylation of ADP to ATP or GDP
 417 to GTP. We propose the symbol P» for the endergonic (uphill) direction of phosphorylation
 418 $\text{ADP}\rightarrow\text{ATP}$, and likewise the symbol P« for the corresponding exergonic (downhill) hydrolysis
 419 $\text{ATP}\rightarrow\text{ADP}$ (**Fig. 2**). P» refers mainly to electrontransfer phosphorylation but may also involve
 420 substrate-level phosphorylation as part of the tricarboxylic acid cycle (succinyl-CoA ligase)
 421 and phosphorylation of ADP catalyzed by phosphoenolpyruvate carboxykinase.
 422 Transphosphorylation is performed by adenylate kinase, creatine kinase, hexokinase and
 423 nucleoside diphosphate kinase. In isolated mammalian mitochondria ATP production catalyzed
 424 by adenylate kinase, $2 \text{ ADP} \leftrightarrow \text{ATP} + \text{AMP}$, proceeds without fuel substrates in the presence
 425 of ADP (Komlódi and Tretter 2017). Kinase cycles are involved in intracellular energy transfer
 426 and signal transduction for regulation of energy flux.

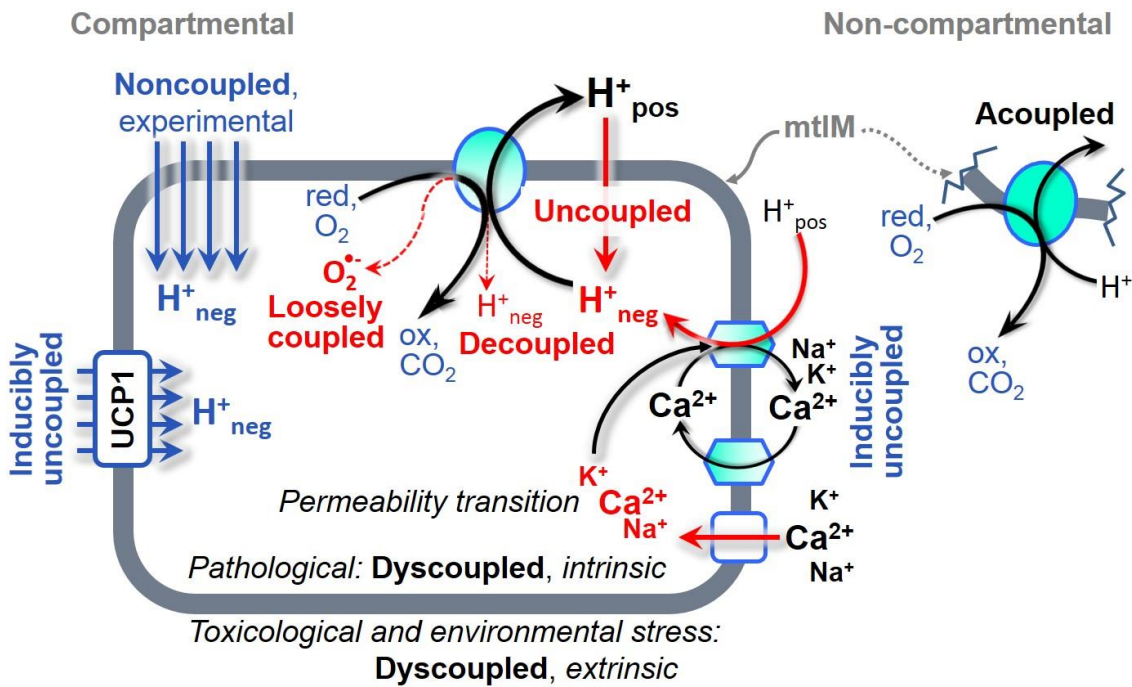
427 **Coupling:** In mitochondrial electron transfer (**Fig. 1**), vectorial transmembrane proton
 428 flux is coupled through the proton pumps CI, CIII and CIV to the catabolic flux of scalar
 429 reactions, collectively measured as oxygen flux (**Fig. 2**). Thus mitochondria are important sites
 430 of energy transformation. Energy cannot be lost or produced in any internal process (First Law
 431 of thermodynamics). Open and closed systems can gain or loose energy only by external fluxes,
 432 *i.e.*, by exchange with the environment. Energy is a fundamentally conserved quantity.
 433 Therefore, energy can neither be produced by mitochondria, nor is there any internal process
 434 without energy conservation. Exergy is defined as the ‘free energy’ with the potential to
 435 perform work. *Coupling* is the mechanistic linkage of an exergonic process (spontaneous,
 436 negative exergy change) with an endergonic process (positive exergy change) in energy
 437 transformations which conserve part of the exergy that would be irreversible lost or dissipated
 438 in an uncoupled process.



439
 440 **Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS).** Oxygen
 441 flux, J_{kO_2} , through the catabolic ET-pathway, k , is coupled to flux through the phosphorylation-
 442 pathway of ADP to ATP, $J_{P\gg}$. The proton pumps of the ET-pathway drive proton flux into the
 443 positive (pos) compartment, J_{mH^+pos} , which generates the output protonmotive force. F-ATPase
 444 is coupled to inward proton current into the negative (neg) compartment, J_{mH^+neg} , to
 445 phosphorylate ADP+P_i to ATP. 2[H] indicates the reduced hydrogen equivalents of fuel
 446 substrates of the catabolic reaction k with oxygen. Fluxes are expressed per volume, $V [m^3]$, of
 447 the system. The system defined by the boundaries (full black line) is not a black box, but is
 448 analysed as a compartmental system. The negative compartment (neg-compartment, enclosed
 449 by the dotted line) is the matrix space, separated by the mtIM from the positive compartment
 450 (pos-compartment). ADP+P_i and ATP are the substrate- and product-compartments (scalar
 451 ADP and ATP compartments, D-comp. and T-comp.), respectively. At steady-state proton
 452 turnover, $J_{\infty H^+}$, and ATP turnover, $J_{\infty P}$, maintain concentrations constant, when $J_{mH^+\infty} = J_{mH^+pos}$
 453 $= J_{mH^+neg}$, and $J_{P\infty} = J_{P\gg} = J_{P\ll}$. Modified from Gnaiger (2014).

454
 455 **Uncoupling:** Uncoupling is a general term comprising diverse mechanisms. Small
 456 differences of terms, *e.g.*, uncoupled *vs.* noncoupled, are easily overlooked, although they relate
 457 to different mechanisms of uncoupling (**Fig. 3**). An attempt at rigorous definition is required
 458 for clarification of concepts (**Table 2**).

- 459
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 uncoupled;
 5. Electron leak in the loosely coupled univalent reduction of oxygen (O₂; dioxygen) to superoxide anion radical (O₂^{•-}).
- 460
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467
 468 **Fig 3. Mechanisms of respiratory uncoupling.** An intact mitochondrial inner membrane,
 469 mtIM, is required for vectorial, compartmental coupling. ‘Acoupled’ respiration is the
 470 consequence of structural disruption with catalytic activity of non-compartmental
 471 mitochondrial fragments. Inducibly uncoupled (activation of UCP1) and experimentally
 472 noncoupled respiration (titration of protonophores) stimulate respiration to maximum oxygen
 473 flux of ET-capacity. Uncoupled, decoupled, and loosely coupled respiration are components of
 474 intrinsic LEAK respiration. Pathological dysfunction may affect all types of uncoupling,
 475 including permeability transition, causing intrinsically dyscoupled respiration. Similarly,
 476 toxicological and environmental stress factors can cause extrinsically dyscoupled respiration.

477
 478 **LEAK-state (Fig. 4):** The
 479 LEAK-state is defined as a state
 480 of mitochondrial respiration
 481 when O_2 flux mainly
 482 compensates for ion leaks in the
 483 absence of ATP synthesis, at
 484 kinetically-saturating
 485 concentrations of O_2 and
 486 respiratory fuel substrates.
 487 LEAK-respiration is measured to
 488 obtain an estimate of *intrinsic*
 489 *uncoupling* without addition of an
 490 experimental uncoupler: (1) in the
 491 absence of adenylates; (2) after
 492 depletion of ADP at a maximum
 493 ATP/ADP ratio; or (3) after
 494 inhibition of the phosphorylation-
 495 pathway by inhibitors of F-
 496 ATPase, such as oligomycin, or of adenine nucleotide translocase, such as
 497 carboxyatractyloside. It is important to consider adjustment of the nominal concentration of
 498 these inhibitors to the density of biological sample applied, to minimize or avoid inhibitory
 499 side-effects exerted on ET-capacity or even some dyscoupling.

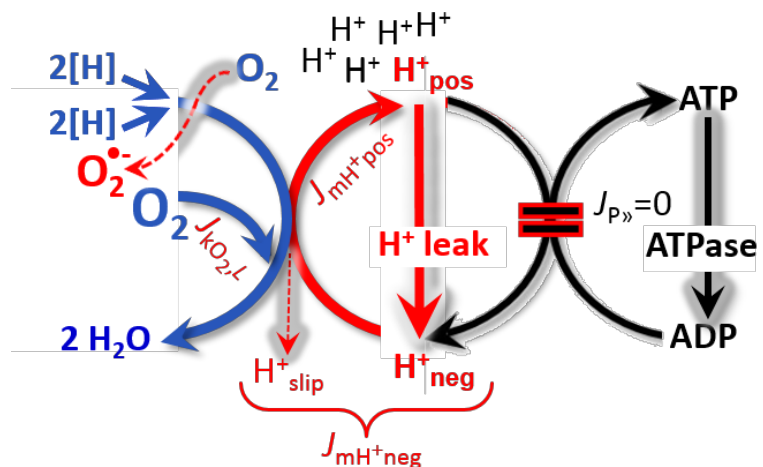



Fig. 4. LEAK-state: Phosphorylation is arrested, $J_{P\gg} = 0$, and catabolic oxygen flux, $J_{kO_2,L}$, is controlled mainly by the proton leak, $J_{mH^{+neg},L}$, at maximum protonmotive force. See also Fig. 2 and 3.

500 **Table 2. Distinction of terms related to coupling and uncoupling (Fig. 3).**

Term	Respiration	P \gg /O $_2$	Note
acoupled		0	electron transfer in mitochondrial fragments without vectorial proton translocation
uncoupled	<i>L</i>	0	non-phosphorylating intrinsic LEAK-respiration, without added protonophore
 uncoupled decoupled loosely coupled dyscoupled		0	component of LEAK-respiration, uncoupled <i>sui generis</i> , ion diffusion across the mtIM
		0	component of LEAK-respiration, proton slip
		0	component of LEAK-respiration, lower coupling due to superoxide anion radical formation and bypass of proton pumps
		0	pathologically, toxicologically, environmentally increased uncoupling, mitochondrial dysfunction
inducibly uncoupled	<i>E</i>	0	by UCP1 or cation (<i>e.g.</i> Ca $^{2+}$) cycling
noncoupled	<i>E</i>	0	non-phosphorylating respiration stimulated to maximum flux at optimum exogenous uncoupler concentration (Fig. 6)
well-coupled	<i>P</i>	high	phosphorylating respiration with an intrinsic LEAK component (Fig. 5)
fully coupled	<i>P – L</i>	max.	OXPHOS-capacity corrected for LEAK-respiration (Fig. 7)

501
 502 **Proton leak and uncoupled respiration:** Proton leak is a leak current of protons. The
 503 intrinsic proton leak is the *uncoupled* process in which protons diffuse across the mtIM in the
 504 dissipative direction of the downhill protonmotive force without coupling to phosphorylation
 505 (**Fig. 4**). The proton leak flux depends non-linearly on the protonmotive force (Garlid *et al.*
 506 1989; Divakaruni and Brand 2011), is a property of the mtIM, and may be enhanced due to
 507 possible contaminations by free fatty acids. Inducible uncoupling mediated by uncoupling
 508 protein 1 (UCP1) is physiologically controlled, *e.g.*, in brown adipose tissue. UCP1 is a member
 509 of the mitochondrial carrier family which is involved in the translocation of protons across the
 510 mtIM (Klingenberg 2017). As a consequence of this effective short-circuit, the protonmotive
 511 force diminishes, resulting in stimulation of electron transfer to O $_2$ and heat dissipation without
 512 phosphorylation of ADP.

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

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

525 **Electron leak and loosely coupled respiration:** Superoxide anion radical production by
 526 the ETS leads to a bypass of proton pumps and correspondingly lower P_{\gg}/O_2 ratio, which
 527 depends on the actual site of electron leak and the scavenging of hydrogen peroxide by
 528 cytochrome *c*, whereby electrons may re-enter the ETS with proton translocation by CIV.

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

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

540
 541 **OXPHOS-state (Fig. 5):**

542 The OXPHOS-state is defined as
 543 the respiratory state with
 544 kinetically-saturating
 545 concentrations of O_2 , respiratory
 546 and phosphorylation substrates,
 547 and absence of exogenous
 548 uncoupler, which provides an
 549 estimate of the maximal
 550 respiratory capacity in the
 551 OXPHOS-state for any given ET-
 552 pathway state. Respiratory
 553 capacities at kinetically-saturating
 554 substrate concentrations provide
 555 reference values or upper limits of
 556 performance, aiming at the
 557 generation of data sets for
 558 comparative purposes. Physiological activities and effects of substrate kinetics can be evaluated
 559 relative to the OXPHOS-capacity.

560 As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated
 561 mitochondria (Gnaiger 2001; Puchowicz *et al.* 2004); greater ADP concentration is required,
 562 particularly in permeabilized muscle fibres and cardiomyocytes, to overcome limitations by
 563 intracellular diffusion and by the reduced conductance of the mtOM (Jepihhina *et al.* 2011,
 564 Illaste *et al.* 2012, Simson *et al.* 2016), either through interaction with tubulin (Rostovtseva *et al.*
 565 2008) or other intracellular structures (Birkedal *et al.* 2014). In permeabilized muscle fibre
 566 bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks *et al.*
 567 1998), consistent with experimental evidence that >90% saturation is reached only at >5
 568 mM ADP (Pesta and Gnaiger 2012). Similar ADP concentrations are also required for accurate
 569 determination of OXPHOS-capacity in human clinical cancer samples and permeabilized cells
 570 (Klepinin *et al.* 2016; Koit *et al.* 2017). Whereas 2.5 to 5 mM ADP is sufficient to obtain the
 571 actual OXPHOS-capacity in many types of permeabilized tissue and cell preparations,
 572 experimental validation is required in each specific case.

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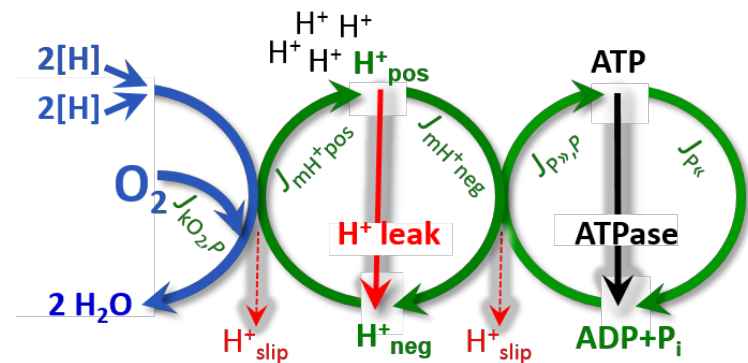
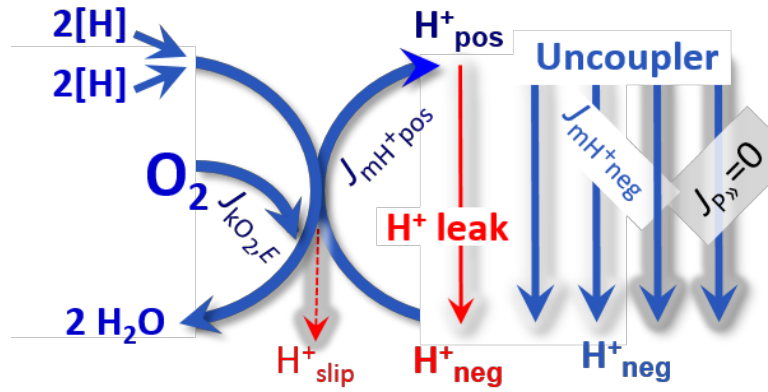


Fig. 5. OXPHOS-state: Phosphorylation, $J_{P_{\gg}}$, is stimulated by kinetically-saturating [ADP] and inorganic phosphate, [P_i], and is supported by a high protonmotive force. O_2 flux, $J_{kO_2,P}$, is well-coupled at a P_{\gg}/O_2 ratio of $J_{P_{\gg},P}/J_{kO_2,P}$. See also **Fig. 2**.

574 **Electron transfer-state**
 575 (Fig. 6): The ET-state is defined
 576 as the *noncoupled* state with
 577 kinetically-saturating
 578 concentrations of O₂, respiratory
 579 substrate and optimum
 580 *exogenous* uncoupler
 581 concentration for maximum O₂
 582 flux, as an estimate of ET-
 583 capacity. Inhibition of
 584 respiration is observed at higher
 585 than optimum uncoupler
 586 concentrations. As a consequence
 587 of the nearly collapsed
 588 protonmotive force, the driving
 589 force is insufficient for
 590 phosphorylation, and $J_{P_{\gg}} = 0$.



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Fig. 6. ET-state: Noncoupled respiration, $J_{kO_2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{P_{\gg}} = 0$. See also Fig. 2.

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

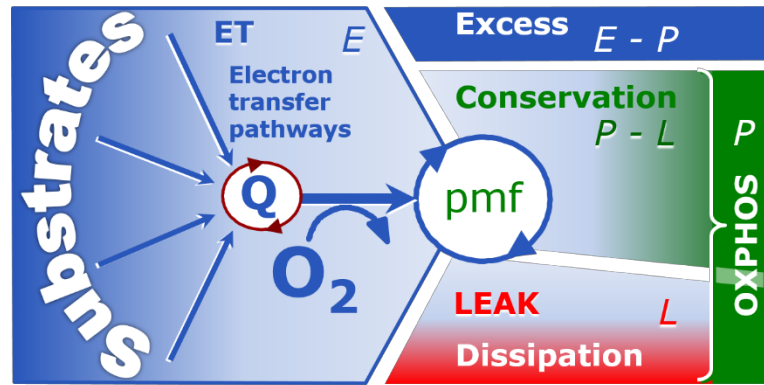
ROX state and Rox: The rate of residual oxygen consumption, *Rox*, is defined as O₂ consumption due to oxidative side reactions remaining after inhibition of ET, e.g., with rotenone, malonic acid and antimycin A. Cyanide and azide not only inhibit CIV but several peroxidases which should be involved 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), monooxygenases (cytochrome P450 monooxygenases), 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).

622 **Fig. 7. Four-compartment**
 623 **model of oxidative**
 624 **phosphorylation.** Respiratory
 625 states (ET, OXPHOS, LEAK)
 626 and corresponding rates (E , P , L)
 627 are connected by the
 628 protonmotive force, pmf.
 629 Electron transfer-capacity, E , is
 630 partitioned into (1) dissipative
 631 LEAK-respiration, L , when the
 632 Gibbs energy change of catabolic
 633 O_2 consumption is irreversibly lost, (2) net OXPHOS-capacity, $P-L$, with partial conservation
 634 of the capacity to perform work, and (3) the excess capacity, $E-P$. Modified from Gnaiger
 635 (2014).



636
 637 E may exceed or be equal to P . $E > P$ is observed in many types of mitochondria, varying
 638 between species, tissues and cell types (Gnaiger 2009). $E-P$ is the excess ET-capacity pushing
 639 the phosphorylation-flux (Fig. 1B) to the limit of its *capacity of utilizing* the protonmotive force.
 640 In addition, the magnitude of $E-P$ depends on the tightness of coupling or degree of uncoupling,
 641 since an increase of L causes P to increase towards the limit of E . The *excess* $E-P$ capacity, $E-P$,
 642 therefore, provides a sensitive diagnostic indicator of specific injuries of the
 643 phosphorylation-pathway, under conditions when E remains constant but P declines relative to
 644 controls (Fig. 7). Substrate cocktails supporting simultaneous convergent electron transfer to
 645 the Q-junction for reconstitution of tricarboxylic acid cycle (TCA cycle or Krebs cycle)
 646 function establish pathway control states with high ET-capacity, and consequently increase the
 647 sensitivity of the $E-P$ assay.

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

655 **P»/O₂ ratio:** The P»/O₂ ratio (P»/4 e⁻) is two times the 'P/O' ratio (P»/2 e⁻) of classical
 656 bioenergetics. P»/O₂ is a generalized symbol, independent of measurement of phosphorylation
 657 by determination of P_i consumption (P_i/O₂ flux ratio), ADP depletion (ADP/O₂ flux ratio), or
 658 ATP production (ATP/O₂ flux ratio).

659 The mechanistic P»/O₂ ratio, which may be referred to also as P»/O₂ stoichiometry, is
 660 calculated from the proton-to-oxygen and proton-to-phosphorylation coupling stoichiometries
 661 (Fig. 1A),
 662

$$663 \quad P\gg/O_2 = \frac{H_{\text{pos}}^+/O_2}{H_{\text{neg}}^+/P\gg} \quad (1)$$

664
 665 The H⁺_{pos}/O₂ *coupling stoichiometry* (referring to the full 4 electron reduction of O₂) depends
 666 on the ET-pathway control state which defines the relative involvement of the three coupling
 667 sites (CI, CIII and CIV) in the catabolic pathway of electrons to O₂. This varies with: (1) a
 668 bypass of CI by single or multiple electron input into the Q-junction; and (2) a bypass of CIV
 669 by involvement of AOX. H⁺_{pos}/O₂ is 12 in the ET-pathways involving CIII and CIV as proton
 670 pumps, increasing to 20 for the NADH-pathway (Fig. 1A), but a general consensus on H⁺_{pos}/O₂
 671 stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov

672 2015). The H^+_{neg}/P coupling stoichiometry (3.7; **Fig. 1A**) is the sum of 2.7 H^+_{neg} required by
 673 the F-ATPase of vertebrate and most invertebrate species (Watt *et al.* 2010) and the proton
 674 balance in the translocation of ADP, ATP and P_i (**Fig. 1B**). Taken together, the mechanistic
 675 P/O_2 ratio is calculated at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively
 676 (Eq. 1). The corresponding classical P/O ratios (referring to the 2 electron reduction of $0.5 O_2$)
 677 are 2.7 and 1.6 (Watt *et al.* 2010), in direct agreement with the measured P/O ratio for succinate
 678 of 1.58 ± 0.02 (Gnaiger *et al.* 2000).

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

688 The net OXPHOS-capacity is calculated by subtracting L from P (**Fig. 7**). Then the net
 689 P/O_2 equals $P/(P-L)$, wherein the dissipative LEAK component in the OXPHOS-state may
 690 be overestimated. This can be avoided by measuring LEAK-respiration in a state when the
 691 protonmotive force is adjusted to its slightly lower value in the OXPHOS-state, *e.g.*, by titration
 692 of an ET inhibitor (Divakaruni and Brand 2011). Any turnover-dependent components of
 693 proton leak and slip, however, are underestimated under these conditions (Garlid *et al.* 1993).
 694 In general, it is inappropriate to use the term *ATP production* or *ATP turnover* for the difference
 695 of oxygen consumption measured in states P and L . The difference $P-L$ is the upper limit of the
 696 part of OXPHOS-capacity that is freely available for ATP production (corrected for LEAK-
 697 respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry
 698 (**Fig. 7**).

699 **Control and regulation:** The terms metabolic *control* and *regulation* are frequently used
 700 synonymously, but are distinguished in metabolic control analysis: 'We could understand the
 701 regulation as the mechanism that occurs when a system maintains some variable constant over
 702 time, in spite of fluctuations in external conditions (homeostasis of the internal state). On the
 703 other hand, metabolic control is the power to change the state of the metabolism in response to
 704 an external signal' (Fell 1997). Respiratory control may be induced by experimental control
 705 signals that *exert* an influence on: (1) ATP demand and ADP phosphorylation-rate; (2) fuel
 706 substrate composition, pathway competition; (3) available amounts of substrates and oxygen,
 707 *e.g.*, starvation and hypoxia; (3) the protonmotive force, redox states, flux-force relationships,
 708 coupling and efficiency; (4) Ca^{2+} and other ions including H^+ ; (5) inhibitors, *e.g.*, nitric oxide
 709 or intermediary metabolites, such as oxaloacetate; (6) signalling pathways and regulatory
 710 proteins, *e.g.* insulin resistance, transcription factor HIF-1 or inhibitory factor 1. *Mechanisms*
 711 of respiratory control and regulation include adjustments of: (1) enzyme activities by allosteric
 712 mechanisms and phosphorylation; (2) enzyme content, concentrations of cofactors and
 713 conserved moieties (such as adenylates, nicotinamide adenine dinucleotide [$NAD^+/NADH$],
 714 coenzyme Q, cytochrome *c*); (3) metabolic channeling by supercomplexes; and (4)
 715 mitochondrial density (enzyme concentrations and membrane area) and morphology (cristae
 716 folding, fission and fusion). (5) Mitochondria are targeted directly by hormones, thereby
 717 affecting their energy metabolism (Lee *et al.* 2013; Gerö and Szabo 2016; Price and Dai 2016;
 718 Moreno *et al.* 2017). Evolutionary or acquired differences in the genetic and epigenetic basis
 719 of mitochondrial function (or dysfunction) between subjects and gene therapy; age; gender,
 720 biological sex, and hormone concentrations; life style including exercise and nutrition; and
 721 environmental issues including thermal, atmospheric, toxicological and pharmacological

722 factors, exert an influence on all control mechanisms listed above. For reviews, see Brown
723 1992; Gnaiger 1993a, 2009; 2014; Paradies *et al.* 2014; Morrow *et al.* 2017.

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

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

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

749

750 2.3. Classical terminology for isolated mitochondria

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

753

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

758

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761

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

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

762

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

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

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

793 **State 4** is a LEAK-state that is obtained only if the mitochondrial preparation is intact
 794 and well-coupled. Depletion of ADP by phosphorylation to ATP leads to a decline in the rate
 795 of oxygen consumption in the transition from State 3 to State 4. Under these conditions of State
 796 4, a maximum protonmotive force and high ATP/ADP ratio are maintained. For calculation of
 797 P_{P}/O_2 ratios the gradual decline of $Y_{P_{\text{P}}/\text{O}_2}$ towards diminishing [ADP] at State 4 must be taken
 798 into account (Gnaiger 2001). State 4 respiration, L_T (**Table 1**), reflects intrinsic proton leak and
 799 intrinsic ATP hydrolysis activity. Oxygen consumption in State 4 is an overestimation of
 800 LEAK-respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP,
 801 $J_{P_{\text{P}}}$, which stimulates respiration coupled to phosphorylation, $J_{P_{\text{P}}} > 0$. This can be tested by
 802 inhibition of the phosphorylation-pathway using oligomycin, ensuring that $J_{P_{\text{P}}} = 0$ (State 4o).
 803 Alternatively, sequential ADP titrations re-establish State 3, followed by State 3 to State 4
 804 transitions while sufficient oxygen is available. However, anoxia may be reached before
 805 exhaustion of ADP (State 5).

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

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

815
 816

817 3. Normalization: fluxes and flows

818

819 3.1. Normalization: system or sample

820

821 The term *rate* is not sufficiently defined to be useful for a database (Fig. 8). The
 822 inconsistency of the meanings of rate becomes fully apparent when considering Galileo
 823 Galilei's famous principle, that 'bodies of different weight all fall at the same rate (have a
 824 constant acceleration)' (Coopersmith 2010).

825

826 **Fig. 8. Different meanings of rate may lead to confusion, if the normalization is not**
 827 **sufficiently specified.** Results are frequently expressed as mass-
 828 specific flux, J_{mX} , per mg protein, dry or wet weight (mass). Cell
 829 volume, V_{cell} , may be used for normalization (volume-specific
 830 flux, $J_{V\text{cell}}$), which must be clearly distinguished from flow per cell,
 831 $I_{N\text{cell}}$, or flux, J_V , expressed for methodological reasons per
 832 volume of the measurement system. For details see Table 4.

841

842 **Flow per system, I :** In a generalization of electrical terms, flow as an extensive quantity
 843 (per system) is distinguished from flux as a size-specific quantity (per system size) (Fig. 8).
 844 Electric current is flow, I_{el} [$\text{A} \equiv \text{C}\cdot\text{s}^{-1}$] per system (extensive quantity). When dividing this
 845 extensive quantity by system size (cross-sectional area of a 'wire'), a size-specific quantity is
 846 obtained, which is flux (current density), J_{el} [$\text{A}\cdot\text{m}^{-2} = \text{C}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$].

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

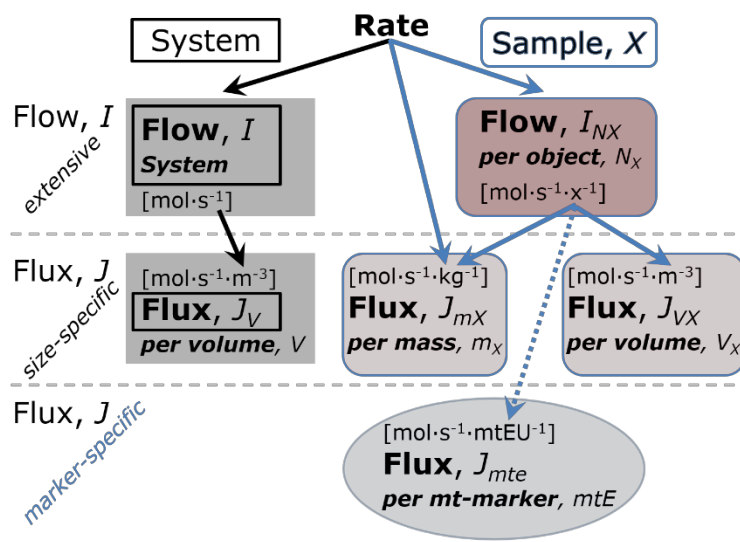
851 **Size-specific quantities:** 'The adjective *specific* before the name of an extensive quantity
 852 is often used to mean *divided by mass*' (Cohen *et al.* 2008). In this general system-paradigm,
 853 mass-specific flux is flow divided by mass of the *system* (the total mass of everything within
 854 the measuring chamber). A mass-specific quantity is independent of the extent of non-
 855 interacting homogenous subsystems. Tissue-specific quantities (related to the *sample* in
 856 contrast to the *system*) are of fundamental interest in comparative mitochondrial physiology,
 857 where *specific* refers to the *type of the sample* rather than *mass of the system*. The term *specific*,
 858 therefore, must be further clarified, such that *sample-specific*, e.g., muscle mass-specific
 859 normalization is distinguished from *system-specific* (mass or volume) quantities (Fig. 8).

860

861 **Box 2: Metabolic fluxes and flows: vectorial and scalar**

862

863 Fluxes are *vectors*, if they have *spatial* direction in addition to magnitude. A vector flux
 864 (surface-density of flow) is expressed per unit cross-sectional area, A [m^2], perpendicular to the
 865 direction of flux. *Flows* are defined as extensive quantities of the *system*, as vector or scalar
 866 flow, I or I [$\text{mol}\cdot\text{s}^{-1}$], respectively, then the corresponding vector and scalar *fluxes* are $\mathbf{J} = \mathbf{I}\cdot A^{-1}$
 867 [$\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$] and $J = I\cdot V^{-1}$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-3}$], respectively, expressing flux as an area-specific vector



868 or volume-specific scalar quantity. We suggest to define: (1) *vectorial* fluxes, which analyze
 869 translocations in continuous systems as functions of gradients; (2) *vectorial* fluxes, which
 870 describe translocations in discontinuous systems and are restricted to information on
 871 compartmental differences (**Fig. 2**, transmembrane proton flux); and (3) *scalar* fluxes, which
 872 are transformations in a homogenous system (**Fig. 2**, catabolic O₂ flux, J_{kO_2} [mol·s⁻¹·m⁻³]).

873 Vectorial transmembrane proton fluxes, J_{mH^+pos} and J_{mH^+neg} , are analyzed in a
 874 heterogenous compartmental system as a quantity with *directional* but not *spatial* information.
 875 Translocation of protons across the mtIM has a defined direction, either from the negative
 876 compartment (matrix space; negative, neg-compartment) to the positive compartment (inter-
 877 membrane space; positive, pos-compartment) or *vice versa* (**Fig. 2**). The arrows defining the
 878 direction of the translocation between the two compartments may point upwards or downwards,
 879 right or left, without any implication that these are actual directions in space. The pos-
 880 compartment is neither above nor below the neg-compartment in a spatial sense, but can be
 881 visualized arbitrarily in a figure in the upper position (**Fig. 2**). In general, the *compartmental*
 882 *direction* of vectorial translocation from the neg-compartment to the pos-compartment is
 883 defined by assigning the initial and final state as *ergodynamic compartments*, $H^+_{neg} \rightarrow H^+_{pos}$ or
 884 $0 = -1 H^+_{neg} + 1 H^+_{pos}$, related to work (erg = work) that must be performed to lift the proton from
 885 a lower to a higher electrochemical potential or from the lower to the higher ergodynamic
 886 compartment (Gnaiger 1993b).

887 In direct analogy to *vectorial* translocation, the direction of a *scalar* chemical reaction, A
 888 $\rightarrow B$ or $0 = -1 A + 1 B$, is defined by assigning substrates and products, A and B , as ergodynamic
 889 compartments. O₂ is defined as a substrate in respiratory O₂ consumption, which together with
 890 the fuel substrates comprises the substrate compartment of the catabolic reaction (**Fig. 2**).
 891 Volume-specific scalar O₂ flux is coupled to vectorial translocation, yielding the H^+_{pos}/O_2 ratio
 892 (**Fig. 1**).

893

894 3.2. Normalization for system-size: flux per chamber volume

895

896 **System-specific flux, J :** The experimental system (the experimental chamber) is part of
 897 the measurement apparatus, separated from the environment as an isolated, closed, open,
 898 isothermal or non-isothermal system (**Table 4**). It is important to distinguish between (1) the
 899 *system* with volume V and mass m defined by the system boundaries, and (2) in the experimental
 900 chamber enclosed *sample* or *objects* with volume V_X and mass m_X (**Fig. 8**). Metabolic O₂ flow
 901 per object, I_{X,O_2} , increases as the mass of the object is increased. Object mass-specific O₂ flux,
 902 J_{mX,O_2} should be independent of the mass of the object studied in the instrument chamber, but
 903 system volume-specific O₂ flux, J_{V,O_2} (per volume of the instrument chamber), should increase
 904 in direct proportion to the mass of the object in the chamber. J_{V,O_2} depends on mass-
 905 concentration of the sample in the chamber, but should be independent of the chamber (system)
 906 volume. There are practical limitations to increasing the mass-concentration of the sample in
 907 the chamber, when one is concerned about crowding effects and instrumental time resolution.

908 When the reactor volume does not change during the reaction, which is typical for liquid
 909 phase reactions, the volume-specific *flux of a chemical reaction* r is the time derivative of the
 910 advancement of the reaction per unit volume, $J_{V,rB} = d_r \zeta_B / dt \cdot V^{-1}$ [(mol·s⁻¹)·L⁻¹]. The *rate of*
 911 *concentration change* is dc_B/dt [(mol·L⁻¹)·s⁻¹], where concentration is $c_B = n_B/V$. It is important
 912 to make the fundamental distinction between (1) J_{V,rO_2} [mol·s⁻¹·L⁻¹] and (2) rate of concentration
 913 change [mol·L⁻¹·s⁻¹]. These merge to a single expression only in closed systems. In open
 914 systems, external fluxes (such as O₂ supply) are distinguished from internal transformations
 915 (metabolic flux, O₂ consumption). In a closed system, external flows of all substances are zero
 916 and O₂ consumption (internal flow of catabolic reactions k), I_{kO_2} [pmol·s⁻¹], causes a decline of
 917 the amount of O₂ in the system, n_{O_2} [nmol]. Normalization of these quantities for the volume of
 918 the system, V [L \equiv dm³], yields volume-specific O₂ flux, $J_{V,kO_2} = I_{kO_2}/V$ [nmol·s⁻¹·L⁻¹], and O₂

919 concentration, $[O_2]$ or $c_{O_2} = n_{O_2}/V$ [$\mu\text{mol}\cdot\text{L}^{-1} = \mu\text{M} = \text{nmol}\cdot\text{mL}^{-1}$]. Instrumental background O_2
 920 flux is due to external flux into a non-ideal closed respirometer, such that total volume-specific
 921 flux has to be corrected for instrumental background O_2 flux, *i.e.*, O_2 diffusion into or out of the
 922 instrumental chamber. J_{V,kO_2} is relevant mainly for methodological reasons and should be
 923 compared with the accuracy of instrumental resolution of background-corrected flux, *e.g.*, ± 1
 924 $\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}$ (Gnaiger 2001). ‘Metabolic’ or catabolic indicates O_2 flux, J_{kO_2} , corrected for: (1)
 925 instrumental background O_2 flux; (2) chemical background O_2 flux due to autoxidation of
 926 chemical components added to the incubation medium; and (3) R_{ox} for O_2 -consuming side
 927 reactions unrelated to the catabolic pathway k .

928 3.3. Normalization: per sample

931 The challenges of measuring mitochondrial respiratory flux are matched by those of
 932 normalization. Application of common and generally defined units is required for direct transfer
 933 of reported results into a database. The second [s] is the *SI* unit for the base quantity *time*. It is
 934 also the standard time-unit used in solution chemical kinetics. A rate may be considered as the
 935 numerator and normalization as the complementary denominator, which are tightly linked in
 936 reporting the measurements in a format commensurate with the requirements of a database.
 937 Normalization (**Table 4**) is guided by physicochemical principles, methodological
 938 considerations (**Fig. 9**), and conceptual strategies (**Fig. 10**).

939 **Table 4. Sample concentrations and normalization of flux.**

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

- 942 1 The SI prefix k is used for the SI base unit of mass (kg = 1,000 g). In praxis, various SI prefixes are
 943 used for convenience, to make numbers easily readable, e.g. 1 mg tissue, cell or mitochondrial mass
 944 instead of 0.000001 kg.
- 945 2 In case sample $X = \text{cells}$, the object number concentration is $C_{N\text{cell}} = N_{\text{cell}} \cdot V^{-1}$, and volume may be
 946 expressed in $[\text{dm}^3 \equiv \text{L}]$ or $[\text{cm}^3 = \text{mL}]$. See **Table 5** for different object types.
- 947 3 mt-concentration is an experimental variable, dependent on sample concentration: (1) $C_{\text{mtE}} = \text{mtE} \cdot V^{-1}$;
 948 (2) $C_{\text{mtE}} = \text{mtE}_X \cdot C_{NX}$; (3) $C_{\text{mtE}} = C_{mX} \cdot D_{\text{mtE}}$.
- 949 4 If the amount of mitochondria, mtE , is expressed as mitochondrial mass, then D_{mtE} is the mass
 950 fraction of mitochondria in the sample. If mtE is expressed as mitochondrial volume, V_{mt} , and the
 951 mass of sample, m_X , is replaced by volume of sample, V_X , then D_{mtE} is the volume fraction of
 952 mitochondria in the sample.
- 953 5 $\text{mtE}_X = \text{mtE} \cdot N_X^{-1} = C_{\text{mtE}} \cdot C_{NX}^{-1}$.
- 954 6 O_2 can be replaced by other chemicals B to study different reactions, e.g. ATP, H_2O_2 , or
 955 compartmental translocations, e.g. Ca^{2+} .
- 956 7 I_{O_2} and V are defined per instrument chamber as a system of constant volume (and constant
 957 temperature), which may be closed or open. I_{O_2r} is abbreviated for I_{O_2r} , i.e., the metabolic or internal
 958 O_2 flow of the chemical reaction r in which O_2 is consumed, hence the negative stoichiometric
 959 number, $\nu_{\text{O}_2} = -1$. $I_{\text{O}_2r} = d_r n_{\text{O}_2} / dt \cdot \nu_{\text{O}_2}^{-1}$. If r includes all chemical reactions in which O_2 participates, then
 960 $d_r n_{\text{O}_2} = dn_{\text{O}_2} - d_e n_{\text{O}_2}$, where dn_{O_2} is the change in the amount of O_2 in the instrument chamber and $d_e n_{\text{O}_2}$
 961 is the amount of O_2 added externally to the system. At steady state, by definition $dn_{\text{O}_2} = 0$, hence $d_r n_{\text{O}_2}$
 962 $= -d_e n_{\text{O}_2}$.
- 963 8 J_{V,O_2} is an experimental variable, expressed per volume of the instrument chamber.
- 964 9 I_{X,O_2} is a physiological variable, depending on the size of entity X .
- 965 10 There are many ways to normalize for a mitochondrial marker, that are used in different experimental
 966 approaches: (1) $J_{\text{mtE},\text{O}_2} = J_{V,\text{O}_2} \cdot C_{\text{mtE}}^{-1}$; (2) $J_{\text{mtE},\text{O}_2} = J_{V,\text{O}_2} \cdot C_{mX}^{-1} \cdot D_{\text{mtE}}^{-1} = J_{mX,\text{O}_2} \cdot D_{\text{mtE}}^{-1}$; (3) $J_{\text{mtE},\text{O}_2} =$
 967 $J_{V,\text{O}_2} \cdot C_{NX}^{-1} \cdot \text{mtE}_X^{-1} = I_{X,\text{O}_2} \cdot \text{mtE}_X^{-1}$; (4) $J_{\text{mtE},\text{O}_2} = I_{\text{O}_2} \cdot \text{mtE}^{-1}$. The mt-elemental unit [mtEU] varies between
 968 different mt-markers.

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

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

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

974 **Sample concentration, C_{mX} :** Normalization for sample concentration is required for
 975 reporting respiratory data. Consider a tissue or cells as the sample, X , and the sample mass, m_X
 976 [mg] from which a mitochondrial preparation is obtained. m_X is frequently measured as wet or
 977 dry weight, W_w or W_d [mg], or as amount of tissue or cell protein, m_{Protein} . In the case of
 978 permeabilized tissues, cells, and homogenates, the sample concentration, $C_{mX} = m_X / V$ [$\text{mg} \cdot \text{mL}^{-1}$
 979 $= \text{g} \cdot \text{L}^{-1}$], is simply the mass of the subsample of tissue that is transferred into the instrument
 980 chamber.

981 **Mass-specific flux, J_{mX,O_2} :** Mass-specific flux is obtained by expressing respiration per
 982 mass of sample, m_X [mg]. X is the type of sample, e.g., tissue homogenate, permeabilized fibres
 983 or cells. Volume-specific flux is divided by mass concentration of X , $J_{mX,\text{O}_2} = J_{V,\text{O}_2} / C_{mX}$, or flow
 984 per cell is divided by mass per cell, $J_{m\text{cell},\text{O}_2} = I_{\text{cell},\text{O}_2} / M_{\text{cell}}$. If mass-specific O_2 flux is constant
 985 and independent of sample size (expressed as mass), then there is no interaction between the
 986 subsystems. A 1.5 mg and a 3.0 mg muscle sample respire at identical mass-specific flux.
 987 Mass-specific O_2 flux, however, may change with the mass of a tissue sample, cells or isolated

988 mitochondria in the measuring chamber, in which case the nature of the interaction becomes an
 989 issue. Optimization of cell density and arrangement is generally important and particularly in
 990 experiments carried out in wells, considering the confluency of the cell monolayer or clumps
 991 of cells (Salabei *et al.* 2014).

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

995 **Flow per object, I_{X,O_2} :** A special case of normalization is encountered in respiratory
 996 studies with permeabilized (or intact) cells. If respiration is expressed per cell, the O_2 flow per
 997 measurement system is replaced by the O_2 flow per cell, I_{cell,O_2} (**Table 4**). O_2 flow can be
 998 calculated from volume-specific O_2 flux, $J_{V,O_2} [nmol \cdot s^{-1} \cdot L^{-1}]$ (per V of the measurement chamber
 999 [L]), divided by the number concentration of cells, $C_{N_{ce}} = N_{ce}/V [cell \cdot L^{-1}]$, where N_{ce} is the
 1000 number of cells in the chamber. Cellular O_2 flow can be compared between cells of identical
 1001 size. To take into account changes and differences in cell size, further normalization is required
 1002 to obtain cell size-specific or mitochondrial marker-specific O_2 flux (Renner *et al.* 2003).

1003 The complexity changes when the sample is a whole organism studied as an experimental
 1004 model. The well-established scaling law in respiratory physiology reveals a strong interaction
 1005 of O_2 consumption and individual body mass of an organism, since *basal* metabolic rate (flow)
 1006 does not increase linearly with body mass, whereas *maximum* mass-specific O_2 flux, $\dot{V}_{O_{2max}}$ or
 1007 $\dot{V}_{O_{2peak}}$, is approximately constant across a large range of individual body mass (Weibel and
 1008 Hoppeler 2005), with individuals, breeds, and certain species deviating substantially from this
 1009 general relationship. $\dot{V}_{O_{2peak}}$ of human endurance athletes is 60 to 80 mL $O_2 \cdot min^{-1} \cdot kg^{-1}$ body
 1010 mass, converted to $J_{M,O_{2peak}}$ of 45 to 60 $nmol \cdot s^{-1} \cdot g^{-1}$ (Gnaiger 2014; **Table 6**).

1011

1012 3.4. Normalization for mitochondrial content

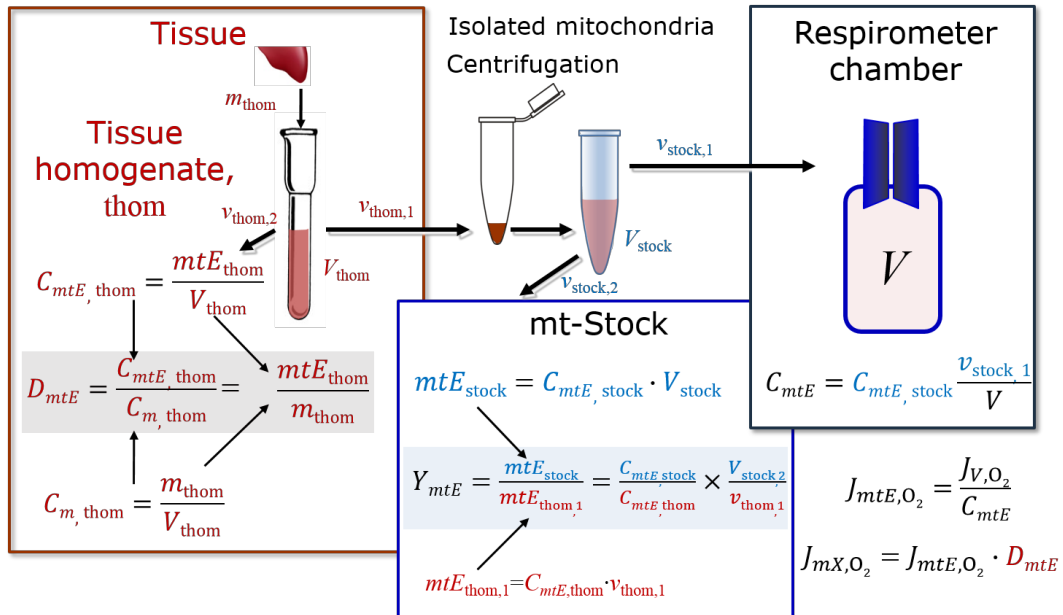
1013

1014 Tissues can contain multiple cell populations which may have distinct mitochondrial
 1015 subtypes. Mitochondria undergo dynamic fission and fusion cycles, and can exist in multiple
 1016 stages and sizes which may be altered by a range of factors. The isolation of mitochondria (often
 1017 achieved through differential centrifugation) can therefore yield a subsample of the
 1018 mitochondrial types present in a tissue, dependent on isolation protocols utilized (*e.g.*
 1019 centrifugation speed). This possible artefact should be taken into account when planning
 1020 experiments using isolated mitochondria. The tendency for mitochondria of specific sizes to be
 1021 enriched at different centrifugation speeds also has the potential to allow the isolation of specific
 1022 mitochondrial subpopulations and therefore the analysis of mitochondria from multiple cell
 1023 lineages within a single tissue.

1024 Part of the mitochondria from the tissue is lost during preparation of isolated
 1025 mitochondria. The fraction of mitochondria obtained is expressed as mitochondrial recovery
 1026 (**Fig. 9**). At a high mitochondrial recovery the sample of isolated mitochondria is more
 1027 representative of the total mitochondrial population than in preparations characterized by low
 1028 recovery. Determination of the mitochondrial recovery and yield is based on measurement of
 1029 the concentration of a mitochondrial marker in the tissue homogenate, $C_{mtE,thom}$, which
 1030 simultaneously provides information on the specific mitochondrial density in the sample (**Fig.**
 1031 **9**).

1032 Normalization is a problematic subject and it is essential to consider the question of the
 1033 study. If the study aims to compare tissue performance, such as the effects of a certain treatment
 1034 on a specific tissue, then normalization can be successful, using tissue mass or protein content,
 1035 for example. If the aim, however, is to find differences of mitochondrial function independent
 1036 of mitochondrial density (**Table 4**), then normalization to a mitochondrial marker is imperative
 1037 (**Fig. 10**). However, one cannot assume that quantitative changes in various markers such as
 1038 mitochondrial proteins necessarily occur in parallel with one another. It is important to first

1039 establish that the marker chosen is not selectively altered by the performed treatment. In
 1040 conclusion, the normalization must reflect the question under investigation to reach a satisfying
 1041 answer. On the other hand, the goal of comparing results across projects and institutions
 1042 requires some standardization on normalization for entry into a databank.
 1043



1044

Symbol	Definition [Units]
C_{mtE}	mitochondrial concentration in chamber [mtEU·L ⁻¹]
C_m	sample mass concentration in chamber [g·L ⁻¹]
D_{mtE}	specific mte-density per tissue mass [mtEU·g ⁻¹]
J_{m, O_2}	mass-specific O ₂ flux [nmol·s ⁻¹ ·g ⁻¹]
J_{mtE, O_2}	mitochondria-specific O ₂ flux [nmol·s ⁻¹ ·mtEU ⁻¹]
mtE	amount of mitochondrial elements [mtEU]
m_{thom}	mass of tissue in the homogenate [g]
Y_{mtE}	recovery of isolated mitochondria
$Y_{mtE/m}$	yield of isolated mitochondria; $Y_{mtE/m} = Y_{mtE} \cdot D_{mtE}$ [mtEU·g ⁻¹]

Respirometer chamber

Homogenate

$C_m = C_{m, thom} \frac{v_{thom,1}}{V}$

$C_{mtE} = C_m \cdot D_{mtE}$

$J_{m, O_2} = \frac{J_{V, O_2}}{C_m}$

$J_{mtE, O_2} = \frac{J_{m, O_2}}{D_{mtE}}$

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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

1060 measurement of chosen mitochondrial markers. ‘Mitochondria are the structural and functional
 1061 elemental units of cell respiration’ (Gnaiger 2014). The quantity of a mitochondrial marker can
 1062 be considered to reflect the amount of *mitochondrial elements*, mtE , expressed in various
 1063 mitochondrial elemental units [mtEU] specific for each measured mt-marker (**Table 4**).
 1064 However, since mitochondrial quality changes under certain stimuli, particularly in
 1065 mitochondrial dysfunction and after exercise training (Pesta *et al.* 2011; Campos *et al.* 2017),
 1066 some markers can vary while other markers are unchanged: (1) Mitochondrial volume and
 1067 membrane area are structural markers, whereas mitochondrial protein mass is frequently used
 1068 as a marker for isolated mitochondria. (2) Molecular and enzymatic mitochondrial markers
 1069 (amounts or activities) can be selected as matrix markers, *e.g.*, citrate synthase activity, mtDNA;
 1070 mtIM-markers, *e.g.*, cytochrome *c* oxidase activity, aa_3 content, cardiolipin, or mtOM-markers,
 1071 *e.g.*, TOM20. (3) Extending the measurement of mitochondrial marker enzyme activity to
 1072 mitochondrial pathway capacity, ET- or OXPHOS-capacity can be considered as an integrative
 1073 functional mitochondrial marker.
 1074

Flow, Performance	=	Element function	x	Element density	x	Size of entity
$\frac{\text{mol}\cdot\text{s}^{-1}}{x}$	=	$\frac{\text{mol}\cdot\text{s}^{-1}}{x_{\text{mte}}}$	·	$\frac{x_{\text{mte}}}{\text{kg}}$	·	$\frac{\text{kg}}{x}$
A						
Flow	=	mt-specific flux	x	mt-structure, functional elements		
I_{X,O_2}	=	J_{mte,O_2}	·	mte_X		
				$\frac{mte_X}{M_X}$	·	M_X
I_{X,O_2}	=	J_{mte,O_2}	·	D_{mte}	·	M_X
$\frac{I_{X,O_2}}{M_X}$	=	$\frac{I_{X,O_2}}{mte_X}$	·	$\frac{mte_X}{M_X}$		
B						
Flow	=	Entity mass- specific flux			x	Mass of entity
I_{X,O_2}	=	J_{mX,O_2}			·	M_X

1075
 1076 **Fig. 10. Structure-function analysis of performance of an organism, organ or tissue, or a**
 1077 **cell (sample entity, X). O_2 flow, I_{X,O_2} , is the product of performance per functional element**
 1078 **(element function, mitochondria-specific flux), element density (mitochondrial density,**
 1079 **D_{mte}), and size of entity X (mass, M_X). (A) Structured analysis: performance is the product of**
 1080 **mitochondrial function (mt-specific flux) and structure (functional elements; D_{mte} times mass**
 1081 **of X). (B) Unstructured analysis: performance is the product of entity mass-specific flux, J_{mX,O_2}**
 1082 **$= I_{X,O_2}/M_X = I_{O_2}/m_X$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{kg}^{-1}$] and size of entity, expressed as mass of X; $M_X = m_X N_X^{-1}$**
 1083 **[$\text{kg}\cdot\text{x}^{-1}$]. See **Table 4** for further explanation of quantities and units. Modified from Gnaiger**
 1084 **(2014).**

1085
 1086 Depending on the type of mitochondrial marker, the mitochondrial elements, mtE , are
 1087 expressed in marker-specific units. It is recommended to distinguish *experimental*
 1088 *mitochondrial concentration*, $C_{\text{mte}} = \text{mtE}/V$ and *physiological mitochondrial density*, $D_{\text{mte}} =$
 1089 mtE/m_X . Then mitochondrial density is the amount of mitochondrial elements per mass of tissue,
 1090 which is a biological variable (**Fig. 10**). The experimental variable is mitochondrial density
 1091 multiplied by sample mass concentration in the measuring chamber, $C_{\text{mte}} = D_{\text{mte}} \cdot C_{mX}$, or
 1092 mitochondrial content multiplied by sample number concentration, $C_{\text{mte}} = \text{mtE}_X \cdot C_{NX}$ (**Table 4**).

1093 **Mitochondria-specific flux, J_{mtE,O_2} :** Volume-specific metabolic O₂ flux depends on: (1)
 1094 the sample concentration in the volume of the instrument chamber, C_{mX} , or C_{NX} ; (2) the
 1095 mitochondrial density in the sample, $D_{mtE} = mtE/m_X$ or $mtE_X = mtE/N_X$; and (3) the specific
 1096 mitochondrial activity or performance per elemental mitochondrial unit, $J_{mtE,O_2} = J_{V,O_2}/C_{mtE}$
 1097 [mol·s⁻¹·mtEU⁻¹] (**Table 4**). Obviously, the numerical results for J_{mtE,O_2} vary according to the
 1098 type of mitochondrial marker chosen for measurement of mtE and $C_{mtE} = mtE/V$ [mtEU·m⁻³].
 1099

1100 3.5. Evaluation of mitochondrial markers

1101
 1102 Different methods are implicated in quantification of mitochondrial markers and have
 1103 different strengths. Some problems are common for all mitochondrial markers, mtE : (1)
 1104 Accuracy of measurement is crucial, since even a highly accurate and reproducible
 1105 measurement of O₂ flux results in an inaccurate and noisy expression normalized for a biased
 1106 and noisy measurement of a mitochondrial marker. This problem is acute in mitochondrial
 1107 respiration because the denominators used (the mitochondrial markers) are often very small
 1108 moieties whose accurate and precise determination is difficult. This problem can be avoided
 1109 when O₂ fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for
 1110 flux in a defined respiratory reference state, which is used as an *internal* marker and yields flux
 1111 control ratios, *FCRs* (**Fig. 8**). *FCRs* are independent of any *externally* measured markers and,
 1112 therefore, are statistically very robust, considering the limitations of ratios in general (Jasienski
 1113 and Bazzaz 1999). *FCRs* indicate qualitative changes of mitochondrial respiratory control, with
 1114 highest quantitative resolution, separating the effect of mitochondrial density or concentration
 1115 on J_{mX,O_2} and I_{X,O_2} from that of function per elemental mitochondrial marker, J_{mtE,O_2} (Pesta *et al.*
 1116 2011; Gnaiger 2014). (2) If mitochondrial quality does not change and only the amount of
 1117 mitochondria varies as a determinant of mass-specific flux, any marker is equally qualified in
 1118 principle; then in practice selection of the optimum marker depends only on the accuracy and
 1119 precision of measurement of the mitochondrial marker. (3) If mitochondrial flux control ratios
 1120 change, then there may not be any best mitochondrial marker. In general, measurement of
 1121 multiple mitochondrial markers enables a comparison and evaluation of normalization for a
 1122 variety of mitochondrial markers. Particularly during postnatal development, the activity of
 1123 marker enzymes, such as cytochrome *c* oxidase and citrate synthase, follows different time
 1124 courses (Drahota *et al.* 2004). Evaluation of mitochondrial markers in healthy controls is
 1125 insufficient for providing guidelines for application in the diagnosis of pathological states and
 1126 specific treatments.

1127 In line with the concept of the respiratory control ratio (Chance and Williams 1955a), the
 1128 most readily used normalization is that of flux control ratios and flux control factors (Gnaiger
 1129 2014). Selection of the state of maximum flux in a protocol as the reference state has the
 1130 advantages of: (1) internal normalization; (2) statistical linearization of the response in the range
 1131 of 0 to 1; and (3) consideration of maximum flux for integrating a very large number of
 1132 elemental steps in the OXPHOS- or ET-pathways. This reduces the risk of selecting a functional
 1133 marker that is specifically altered by the treatment or pathodology, yet increases the chance that
 1134 the highly integrative pathway is disproportionately affected, *e.g.* the OXPHOS- rather than
 1135 ET-pathway in case of an enzymatic defect in the phosphorylation-pathway. In this case,
 1136 additional information can be obtained by reporting flux control ratios based on a reference
 1137 state which indicates stable tissue-mass specific flux. Stereological determination of
 1138 mitochondrial content via two-dimensional transmission electron microscopy can have
 1139 limitations due to the dynamics of mitochondrial size (Meinild Lundby *et al.* 2017). Accurate
 1140 determination of three-dimensional volume by two-dimensional microscopy can be both time
 1141 consuming and statistically challenging (Larsen *et al.* 2012).

1142 The validity of using mitochondrial marker enzymes (citrate synthase activity, Complex
 1143 I–IV amount or activity) for normalization of flux is limited in part by the same factors that

1144 apply to flux control ratios. Strong correlations between various mitochondrial markers and
 1145 citrate synthase activity (Reichmann *et al.* 1985; Boushel *et al.* 2007; Mogensen *et al.* 2007)
 1146 are expected in a specific tissue of healthy subjects and in disease states not specifically
 1147 targeting citrate synthase. Citrate synthase activity is acutely modifiable by exercise
 1148 (Tonkonogi *et al.* 1997; Leek *et al.* 2001). Evaluation of mitochondrial markers related to a
 1149 selected age and sex cohort cannot be extrapolated to provide recommendations for
 1150 normalization in respirometric diagnosis of disease, in different states of development and
 1151 ageing, different cell types, tissues, and species. mtDNA normalised to nDNA via qPCR is
 1152 correlated to functional mitochondrial markers including OXPHOS- and ET-capacity in some
 1153 cases (Puntschart *et al.* 1995; Wang *et al.* 1999; Menshikova *et al.* 2006; Boushel *et al.* 2007),
 1154 but lack of such correlations have been reported (Menshikova *et al.* 2005; Schultz and Wiesner
 1155 2000; Pesta *et al.* 2011). Several studies indicate a strong correlation between cardiolipin
 1156 content and increase in mitochondrial function with exercise (Menshikova *et al.* 2005;
 1157 Menshikova *et al.* 2007; Larsen *et al.* 2012; Faber *et al.* 2014), but its use as a general
 1158 mitochondrial biomarker in disease remains questionable.

1159

1160 3.6. Conversion: units

1161

1162 Many different units have been used to report the rate of oxygen consumption, OCR
 1163 (**Table 6**). *SI* base units provide the common reference for introducing the theoretical principles
 1164 (**Fig. 8**), and are used with appropriately chosen *SI* prefixes to express numerical data in the
 1165 most practical format, with an effort towards unification within specific areas of application
 1166 (**Table 7**). For studies of cells, we recommend that respiration be expressed, as far as possible,
 1167 as: (1) O₂ flux normalized for a mitochondrial marker, for separation of the effects of
 1168 mitochondrial quality and content on cell respiration (this includes *FCRs* as a normalization for
 1169 a functional mitochondrial marker); (2) O₂ flux in units of cell volume or mass, for comparison
 1170 of respiration of cells with different cell size (Renner *et al.* 2003) and with studies on tissue
 1171 preparations, and (3) O₂ flow in units of attomole (10⁻¹⁸ mol) of O₂ consumed in a second by
 1172 each cell [amol·s⁻¹·cell⁻¹], numerically equivalent to [pmol·s⁻¹·10⁻⁶ cells]. This convention
 1173 allows information to be easily used when designing experiments in which oxygen consumption
 1174 must be considered. For example, to estimate the volume-specific O₂ flux in an instrument
 1175 chamber that would be expected at a particular cell number concentration, one simply needs to
 1176 multiply the flow per cell by the number of cells per volume of interest. This provides the
 1177 amount of O₂ [mol] consumed per time [s⁻¹] per unit volume [L⁻¹]. At an O₂ flow of 100
 1178 amol·s⁻¹·cell⁻¹ and a cell density of 10⁹ cells·L⁻¹ (10⁶ cells·mL⁻¹), the volume-specific O₂ flux is
 1179 100 nmol·s⁻¹·L⁻¹ (100 pmol·s⁻¹·mL⁻¹).

1180 Although volume is expressed as m³ using the *SI* base unit, the litre [dm³] is the basic unit
 1181 of volume for concentration and is used for most solution chemical kinetics. If one multiplies
 1182 $I_{\text{cell},\text{O}_2}$ by $C_{N_{\text{cell}}}$, then the result will not only be the amount of O₂ [mol] consumed per time [s⁻¹]
 1183 in one litre [L⁻¹], but also the change in the concentration of oxygen per second (for any volume
 1184 of an ideally closed system). This is ideal for kinetic modeling as it blends with chemical rate
 1185 equations where concentrations are typically expressed in mol·L⁻¹ (Wagner *et al.* 2011). In
 1186 studies of multinuclear cells, such as differentiated skeletal muscle cells, it is easy to determine
 1187 the number of nuclei but not the total number of cells. A generalized concept, therefore, is
 1188 obtained by substituting cells by nuclei as the sample entity. This does not hold, however, for
 1189 enucleated platelets.

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1196**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.

1 Unit	x	Multiplication factor	SI-unit	Note
ng.atom O \cdot s $^{-1}$	(2 e $^{-}$)	0.5	nmol O $_2$ \cdot s $^{-1}$	
ng.atom O \cdot min $^{-1}$	(2 e $^{-}$)	8.33	pmol O $_2$ \cdot s $^{-1}$	
natom O \cdot min $^{-1}$	(2 e $^{-}$)	8.33	pmol O $_2$ \cdot s $^{-1}$	
nmol O $_2$ \cdot min $^{-1}$	(4 e $^{-}$)	16.67	pmol O $_2$ \cdot s $^{-1}$	
nmol O $_2$ \cdot h $^{-1}$	(4 e $^{-}$)	0.2778	pmol O $_2$ \cdot s $^{-1}$	
mL O $_2$ \cdot min $^{-1}$ at STPD ^a		0.744	μ mol O $_2$ \cdot s $^{-1}$	1
W = J/s at -470 kJ/mol O $_2$		-2.128	μ mol O $_2$ \cdot s $^{-1}$	
mA = mC \cdot s $^{-1}$	($z_{H^+} = 1$)	10.36	nmol H $^+$ \cdot s $^{-1}$	2
mA = mC \cdot s $^{-1}$	($z_{O_2} = 4$)	2.59	nmol O $_2$ \cdot s $^{-1}$	2
nmol H $^+$ \cdot s $^{-1}$	($z_{H^+} = 1$)	0.09649	mA	3
nmol O $_2$ \cdot s $^{-1}$	($z_{O_2} = 4$)	0.38594	mA	3

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- 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 \cdot 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 \cdot mol $^{-1}$. Note that the SI standard pressure is 100 kPa.
- 2 The multiplication factor is $10^6/(z_B \cdot F)$.
- 3 The multiplication factor is $z_B \cdot F/10^6$.

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

Name	Frequently used unit	Equivalent unit	Note
volume-specific flux, J_{V,O_2}	pmol \cdot s $^{-1}$ \cdot mL $^{-1}$	nmol \cdot s $^{-1}$ \cdot L $^{-1}$	1
	mmol \cdot s $^{-1}$ \cdot L $^{-1}$	mol \cdot s $^{-1}$ \cdot m $^{-3}$	
cell-specific flow, I_{O_2}	pmol \cdot s $^{-1}$ \cdot 10 $^{-6}$ cells	amol \cdot s $^{-1}$ \cdot cell $^{-1}$	2
	pmol \cdot s $^{-1}$ \cdot 10 $^{-9}$ cells	zmol \cdot s $^{-1}$ \cdot cell $^{-1}$	3
cell number concentration, C_{Nce}	10 6 cells \cdot mL $^{-1}$	10 9 cells \cdot L $^{-1}$	
mitochondrial protein concentration, C_{mtE}	0.1 mg \cdot mL $^{-1}$	0.1 g \cdot L $^{-1}$	
mass-specific flux, J_{m,O_2}	pmol \cdot s $^{-1}$ \cdot mg $^{-1}$	nmol \cdot s $^{-1}$ \cdot g $^{-1}$	4
catabolic power, P_k	μ W \cdot 10 $^{-6}$ cells	pW \cdot cell $^{-1}$	1
volume	1,000 L	m 3 (1,000 kg)	
	L	dm 3 (kg)	
	mL	cm 3 (g)	
	μ L	mm 3 (mg)	
	fL	μ m 3 (pg)	5
amount of substance concentration	M = mol \cdot L $^{-1}$	mol \cdot dm $^{-3}$	

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- 1 pmol: picomole = 10 $^{-12}$ mol 4 nmol: nanomole = 10 $^{-9}$ mol
- 2 amol: attomole = 10 $^{-18}$ mol 5 fL: femtolitre = 10 $^{-15}$ L
- 3 zmol: zeptomole = 10 $^{-21}$ mol

1212 ET-capacity in various human cell types including HEK 293, primary HUVEC and
 1213 fibroblasts ranges from 50 to 180 $\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$, measured in intact cells in the noncoupled state
 1214 (see Gnaiger 2014). At 100 $\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$ corrected for *Rox*, the current across the mt-
 1215 membranes, $I_{e\text{H}^+}$, approximates 193 $\text{pA}\cdot\text{cell}^{-1}$ or 0.2 nA per cell. See Rich (2003) for an
 1216 extension of quantitative bioenergetics from the molecular to the human scale, with a
 1217 transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W.
 1218 Modelling approaches illustrate the link between protonmotive force and currents (Willis *et al.*
 1219 2016).

1220 We consider isolated mitochondria as powerhouses and proton pumps as molecular
 1221 machines to relate experimental results to energy metabolism of the intact cell. The cellular
 1222 $\text{P}\gg/\text{O}_2$ based on oxidation of glycogen is increased by the glycolytic (fermentative) substrate-
 1223 level phosphorylation of 3 $\text{P}\gg/\text{Glyc}$, *i.e.*, 0.5 mol $\text{P}\gg$ for each mol O_2 consumed in the complete
 1224 oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial $\text{P}\gg/\text{O}_2$ ratio of 5.4
 1225 yields a bioenergetic cell physiological $\text{P}\gg/\text{O}_2$ ratio close to 6. Two NADH equivalents are
 1226 formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either
 1227 by the malate-aspartate shuttle or by the glycerophosphate shuttle resulting in different
 1228 theoretical yields of ATP generated by mitochondria, the energetic cost of which potentially
 1229 must be taken into account. Considering also substrate-level phosphorylation in the TCA cycle,
 1230 this high $\text{P}\gg/\text{O}_2$ ratio not only reflects proton translocation and OXPHOS studied in isolation,
 1231 but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger
 1232 1993a).

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1235 4. Conclusions

1236

1237 MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects
 1238 linked to genetic variation, age-related health risks, sex-specific mitochondrial performance,
 1239 lifestyle with its effects on degenerative diseases, and thermal and chemical environment. The
 1240 present recommendations on coupling control states and rates, linked to the concept of the
 1241 protonmotive force, are focused on studies with mitochondrial preparations. These will be
 1242 extended in a series of reports on pathway control of mitochondrial respiration, respiratory
 1243 states in intact cells, and harmonization of experimental procedures.

1244 The optimal choice for expressing mitochondrial and cell respiration (**Box 3**) as O_2 flow
 1245 per biological system, and normalization for specific tissue-markers (volume, mass, protein)
 1246 and mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes,
 1247 respiratory reference state) is guided by the scientific question under study. Interpretation of
 1248 the obtained data depends critically on appropriate normalization, and therefore reporting rates
 1249 merely as $\text{nmol}\cdot\text{s}^{-1}$ is discouraged, since it restricts the analysis to intra-experimental
 1250 comparison of relative (qualitative) differences. Expressing O_2 consumption per cell may not
 1251 be possible when dealing with tissues. For studies with mitochondrial preparations, we
 1252 recommend that normalizations be provided as far as possible: (1) on a per cell basis as O_2 flow
 1253 (a biophysical normalization); (2) per g cell or tissue protein, or per cell or tissue mass as mass-
 1254 specific O_2 flux (a cellular normalization); and (3) per mitochondrial marker as mt-specific flux
 1255 (a mitochondrial normalization). With information on cell size and the use of multiple
 1256 normalizations, maximum potential information is available (Renner *et al.* 2003; Wagner *et al.*
 1257 2011; Gnaiger 2014).

1258 When using isolated mitochondria, mitochondrial protein is a frequently applied
 1259 mitochondrial marker, the use of which is basically restricted to isolated mitochondria. The
 1260 mitochondrial recovery and yield, and experimental criteria for evaluation of purity versus
 1261 integrity should be reported. Mitochondrial markers, such as citrate synthase activity as an
 1262 enzymatic matrix marker, provide a link to the tissue of origin on the basis of calculating the

1263 mitochondrial recovery, *i.e.*, the fraction of mitochondrial marker obtained from a unit mass of
 1264 tissue.
 1265

1266 **Table 8. Terms, symbols, and units.**
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 1268

1269 1270	Term	Symbol	SI unit	Links and comments
1271				
1272	alternative quinol oxidase	AOX		Fig. 1
1273	amount of substance B	n_B	[mol]	
1274	apparent equilibrium constant	K_m'		
1275	Complexes I to IV	CI to CIV		respiratory ET Complexes; Fig. 1
1276	concentration of substance B	$c_B = n_B \cdot V^{-1}$; [B]	[mol·m ⁻³]	Box 2
1277	electron transfer system	ETS		
1278	flow, for substance B	I_B	[mol·s ⁻¹]	system-related extensive quantity; Fig. 8
1279	flux, for substance B	J_B	<i>varies</i>	size-specific quantity; Fig. 8
1280	inorganic phosphate	P_i		
1281	LEAK	LEAK		Tab. 1
1282	mass of sample X	m_X	[kg]	Tab. 4
1283	mass of entity X	M_X	[kg]	Tab. 4
1284	MITOCARTA			https://www.broadinstitute.org/scientific-community/science/programs/metabolic-disease-program/publications/mitocarta/mitocarta-in-0
1285				
1286				
1287				
1288				
1289	mitochondria or mitochondrial	mt		Box 1
1290	mitochondrial DNA	mtDNA		Box 1
1291	mitochondrial concentration	$C_{mtE} = mtE \cdot V^{-1}$	[mtEU·m ⁻³]	Tab. 4
1292	mitochondrial content	$mtE_X = mtE \cdot N_X^{-1}$	[mtEU·x ⁻¹]	Tab. 4
1293	mitochondrial elemental unit	mtEU	<i>varies</i>	Tab. 4, specific units for mt-marker
1294	mitochondrial inner membrane	mtIM		MIM is widely used, and M is replaced by mt as abbreviation for mitochondria; Box 1
1295				
1296				
1297	mitochondrial outer membrane	mtOM		MOM is widely used, and M is replaced by mt as abbreviation for mitochondria; Box 1
1298				
1299				
1300	mitochondrial recovery	Y_{mtE}		Fig. 9
1301	mitochondrial yield	$Y_{mtE/m}$		Fig. 9
1302	negative	neg		Fig. 2
1303	number concentration of X	C_{NX}	[x·m ⁻³]	Tab. 4
1304	number of entities X	N_X	[x]	Tab. 4, Fig. 10
1305	number of entity B	N_B	[x]	Tab. 4
1306	oxidative phosphorylation	OXPPOS		Tab. 1
1307	oxygen concentration	$c_{O_2} = n_{O_2} \cdot V^{-1}$; [O ₂]	[mol·m ⁻³]	Section 3.2
1308	phosphorylation of ADP to ATP	$P \gg$		Section 2.2
1309	positive	pos		Fig. 2
1310	proton in the negative compartment	H^+_{neg}		Fig. 2
1311	proton in the positive compartment	H^+_{pos}		Fig. 2
1312	rate of electron transfer in ET state	E		ET-capacity; Tab. 1
1313	rate of LEAK respiration	L		Tab. 1
1314	rate of oxidative phosphorylation	P		OXPPOS capacity; Tab. 1
1315	rate of residual oxygen consumption	R_{ox}		Tab. 1
1316	residual oxygen consumption	ROX		Tab. 1
1317	specific mitochondrial density	$D_{mtE} = mtE \cdot m_X^{-1}$	[mtEU·kg ⁻¹]	Tab. 7
1318	volume	V	[m ³]	
1319	weight, dry weight	W_d	[kg]	used as mass of sample X ; Fig. 8
1320	weight, wet weight	W_w	[kg]	used as mass of sample X ; Fig. 8
1321				
1322				

1323 Terms and symbols are summarized in **Table 8**. Their use will facilitate transdisciplinary
 1324 communication and support further developments towards a consistent theory of bioenergetics
 1325 and mitochondrial physiology.
 1326

1327 **Box 3: Mitochondrial and cell respiration**

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 1329 Mitochondrial and cell respiration is the process of highly exergonic and exothermic energy
 1330 transformation in which scalar redox reactions are coupled to vectorial ion translocation across
 1331 a semipermeable membrane, which separates the small volume of a bacterial cell or
 1332 mitochondrion from the larger volume of its surroundings. The electrochemical exergy can be
 1333 partially conserved in the phosphorylation of ADP to ATP or in ion pumping, or dissipated in
 1334 an electrochemical short-circuit. Respiration is thus clearly distinguished from fermentation as
 1335 the counterpart of cellular core energy metabolism. Respiration is separated in mitochondrial
 1336 preparations from the partial contribution of fermentative pathways of the intact cell. According
 1337 to this definition, residual oxygen consumption, as measured after inhibition of mitochondrial
 1338 electron transfer, does not belong to the class of catabolic reactions and is, therefore, subtracted
 1339 from total oxygen consumption to obtain baseline-corrected respiration.

1340

1341 **Acknowledgements**

1342 We thank M. Beno for management assistance. Supported by COST Action CA15203
 1343 MitoEAGLE and K-Regio project MitoFit (E.G.).
 1344

1345 **Competing financial interests:** E.G. is founder and CEO of Oroboros Instruments, Innsbruck,
 1346 Austria.
 1347

1348

1348 **5. References**

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