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Mitochondrial respiratory states and rates

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Updates and discussion:

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84



85	Table of contents
86	
87	Abstract
88	Executive summary
89	1. Introduction – Box 1: In brief: Mitochondria and Bioblasts
90	2. Coupling states and rates in mitochondrial preparations
91	2.1. <i>Cellular and mitochondrial respiration</i>
92	2.1.1. Aerobic and anaerobic catabolism and ATP turnover
93	2.1.2. Specification of biochemical dose
94	2.2. <i>Mitochondrial preparations</i>
95	2.3. <i>Electron transfer pathways</i>
96	2.4. <i>Respiratory coupling control</i>
97	2.4.1. Coupling
98	2.4.2. Phosphorylation, P _o , and P _o /O ₂ ratio
99	2.4.3. Uncoupling
100	2.5. <i>Coupling states and respiratory rates</i>
101	2.5.1. LEAK-state
102	2.5.2. OXPHOS-state
103	2.5.3. Electron transfer-state
104	2.5.4. ROX state and <i>Rox</i>
105	2.5.5. Quantitative relations
106	2.5.6. The steady-state
107	2.6. <i>Classical terminology for isolated mitochondria</i>
108	2.6.1. State 1
109	2.6.2. State 2
110	2.6.3. State 3
111	2.6.4. State 4
112	2.6.5. State 5
113	2.7. <i>Control and regulation</i>
114	3. What is a rate? – Box 2: Metabolic flows and fluxes: vectorial, vectorial, and scalar
115	4. Normalization of rate per sample
116	4.1. <i>Flow: per object</i>
117	4.1.1. Number concentration
118	4.1.2. Flow per object
119	4.2. <i>Size-specific flux: per sample size</i>
120	4.2.1. Sample concentration
121	4.2.2. Size-specific flux
122	4.3. <i>Marker-specific flux: per mitochondrial content</i>
123	4.3.1. Mitochondrial concentration and mitochondrial markers
124	4.3.2. mt-Marker-specific flux
125	5. Normalization of rate per system
126	5.1. <i>Flow: per chamber</i>
127	5.2. <i>Flux: per chamber volume</i>
128	5.2.1. System-specific flux
129	5.2.2. Advancement per volume
130	6. Conversion of units
131	7. Conclusions – Box 3: Recommendations for studies with mitochondrial preparations
132	References
133	Supplement
134	S1. Manuscript phases and versions - an open-access approach
135	S2. Authors
136	S3. Joining COST Actions
137	

138 **Abstract** As the knowledge base and importance of mitochondrial physiology to human health expands,
 139 the necessity for harmonizing the terminology concerning mitochondrial respiratory states and rates has
 140 become increasingly apparent. The chemiosmotic theory establishes the mechanism of energy
 141 transformation and coupling in oxidative phosphorylation. The unifying concept of the protonmotive
 142 force provides the framework for developing a consistent theoretical foundation of mitochondrial
 143 physiology and bioenergetics. We follow IUPAC guidelines on terminology in physical chemistry,
 144 extended by considerations of open systems and thermodynamics of irreversible processes. The concept-
 145 driven constructive terminology incorporates the meaning of each quantity and aligns concepts and
 146 symbols with the nomenclature of classical bioenergetics. We endeavour to provide a balanced view of
 147 mitochondrial respiratory control and a critical discussion on reporting data of mitochondrial respiration
 148 in terms of metabolic flows and fluxes. Uniform standards for evaluation of respiratory states and rates
 149 will ultimately contribute to reproducibility between laboratories and thus support the development of
 150 databases of mitochondrial respiratory function in species, tissues, and cells. Clarity of concept and
 151 consistency of nomenclature facilitate effective transdisciplinary communication, education, and
 152 ultimately further discovery.

153
 154 *Keywords:* Mitochondrial respiratory control, coupling control, mitochondrial preparations,
 155 protonmotive force, uncoupling, oxidative phosphorylation: OXPHOS, efficiency, electron transfer: ET,
 156 electron transfer system: ETS, proton leak, ion leak and slip compensatory state: LEAK, residual oxygen
 157 consumption: ROX, State 2, State 3, State 4, normalization, flow, flux, oxygen: O₂
 158

159 **Executive summary**

160
 161 In view of the broad implications for health care, mitochondrial researchers face an increasing
 162 responsibility to disseminate their fundamental knowledge and novel discoveries to a wide range of
 163 stakeholders and scientists beyond the group of specialists. This requires implementation of a commonly
 164 accepted terminology within the discipline and standardization in the translational context. Authors,
 165 reviewers, journal editors, and lecturers are challenged to collaborate with the aim to harmonize the
 166 nomenclature in the growing field of mitochondrial physiology and bioenergetics, from evolutionary
 167 biology and comparative physiology to mitochondrial medicine. In the present communication we focus
 168 on the following concepts in mitochondrial physiology:

- 169 1. Aerobic respiration depends on the coupling of phosphorylation (ADP → ATP) to O₂ flux in
 170 catabolic reactions. Coupling in oxidative phosphorylation is mediated by the translocation of
 171 protons across the mitochondrial inner membrane (mtIM) through proton pumps generating
 172 or utilizing the protonmotive force that is maintained between the mitochondrial matrix and
 173 intermembrane compartment or outer mitochondrial space. Compartmental coupling
 174 distinguishes this vectorial component of oxidative phosphorylation from glycolytic
 175 fermentation as the counterpart of cellular core energy metabolism (**Figure 1**). Cell respiration
 176 is distinguished from fermentation: (1) Electron acceptors are supplied by external respiration
 177 for the maintenance of redox balance, whereas fermentation is characterized by an internal
 178 electron acceptor produced in intermediary metabolism. In aerobic cell respiration, redox
 179 balance is maintained by O₂ as the electron acceptor. (2) Compartmental coupling in vectorial
 180 oxidative phosphorylation contrasts to exclusively scalar substrate-level phosphorylation in
 181 fermentation.
- 182 2. When measuring mitochondrial metabolism, the contribution of fermentation and other cytosolic
 183 interactions must be excluded from analysis by disrupting the barrier function of the plasma
 184 membrane. Selective removal or permeabilization of the plasma membrane yields
 185 mitochondrial preparations—including isolated mitochondria, tissue and cellular
 186 preparations—with structural and functional integrity. Subsequently, extra-mitochondrial
 187 concentrations of fuel substrates, ADP, ATP, inorganic phosphate, and cations including H⁺
 188 can be controlled to determine mitochondrial function under a set of conditions defined as
 189 coupling control states. We strive to incorporate an easily recognized and understood concept-
 190 driven terminology of bioenergetics with explicit terms and symbols that define the nature of
 191 respiratory states.
- 192 3. Mitochondrial coupling states are defined according to the control of respiratory oxygen flux by
 193 the protonmotive force. Capacities of oxidative phosphorylation and electron transfer are

measured at kinetically saturating concentrations of fuel substrates, ADP and inorganic phosphate, and O₂, or at optimal uncoupler concentrations, respectively, in the absence of Complex IV inhibitors such as NO, CO, or H₂S. Respiratory capacity is a measure of the upper boundary of the rate of respiration; it depends on the substrate type undergoing oxidation, and provides reference values for the diagnosis of health and disease, and for evaluation of the effects of Evolutionary background, Age, Gender and sex, Lifestyle and Environment.

Figure 1. Internal and external respiration

Mitochondrial respiration is the oxidation of fuel substrates (electron donors) and reduction of O₂ catalysed by the electron transfer system, ETS: (mt) mitochondrial catabolic respiration; (ce) total cellular O₂ consumption; and (ext) external respiration. All chemical reactions, r , that consume O₂ in the cells of an organism, contribute to cell respiration, J_{rO_2} . In addition to mitochondrial catabolic respiration, O₂ is consumed by:

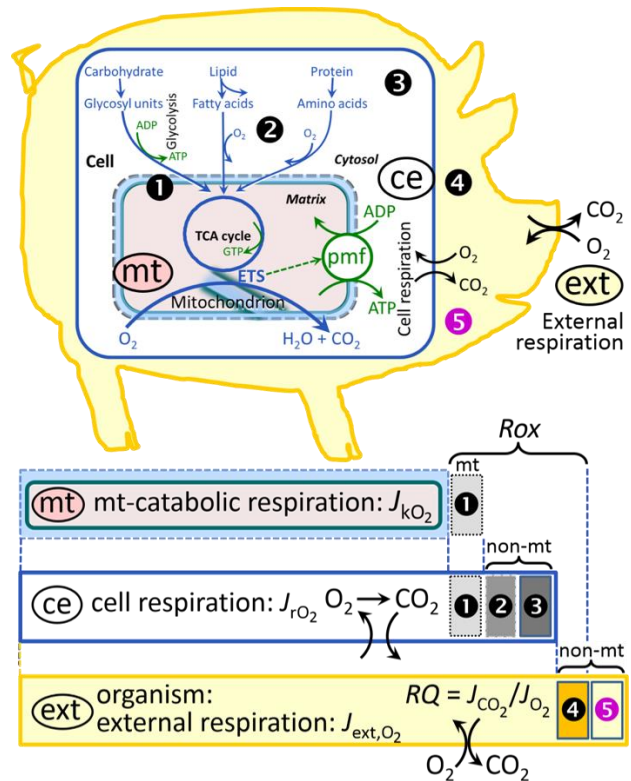
① Mitochondrial residual O₂ consumption, Rox .
 ② Non-mitochondrial O₂ consumption by catabolic reactions, particularly peroxisomal oxidases and microsomal cytochrome P450 systems. ③ Non-mitochondrial Rox by reactions unrelated to catabolism. ④ Extracellular Rox . ⑤ Aerobic microbial respiration. Bars are not at a quantitative scale.

(mt) **Mitochondrial catabolic respiration**, J_{kO_2} , is the O₂ consumption by the mitochondrial ETS excluding Rox .

(ce) **Cell respiration**, J_{rO_2} , takes into account

internal O₂-consuming reactions, r , including catabolic respiration and Rox . Catabolic cell respiration is the O₂ consumption associated with catabolic pathways in the cell, including mitochondrial catabolism in addition to peroxisomal and microsomal oxidation reactions (②).

(ext) **External respiration** balances internal respiration at steady-state, including extracellular Rox (④) and aerobic respiration by the microbiome (⑤). O₂ is transported from the environment across the respiratory cascade, *i.e.*, circulation between tissues and diffusion across cell membranes, to the intracellular compartment. The respiratory quotient, RQ , is the molar CO₂/O₂ exchange ratio; when combined with the respiratory nitrogen quotient, N/O₂ (mol N given off per mol O₂ consumed), the RQ reflects the proportion of carbohydrate, lipid and protein utilized in cell respiration during aerobically balanced steady-states. Bicarbonate and CO₂ are transported in reverse to the extracellular milieu and the organismic environment. Hemoglobin provides the molecular paradigm for the combination of O₂ and CO₂ exchange, as do lungs and gills on the morphological level.



4. Incomplete tightness of coupling, *i.e.*, some degree of uncoupling relative to the substrate-dependent coupling stoichiometry, is a characteristic of energy-transformations across membranes. Uncoupling is caused by a variety of physiological, pathological, toxicological, pharmacological and environmental conditions that exert an influence not only on the proton leak and cation cycling, but also on proton slip within the proton pumps and the structural integrity of the mitochondria. A more loosely coupled state is induced by stimulation of mitochondrial superoxide formation and the bypass of proton pumps. In addition, the use of protonophores represents an experimental uncoupling intervention to assess the transition from a well-coupled to a noncoupled state of mitochondrial respiration.

5. Respiratory oxygen consumption rates have to be carefully normalized to enable meta-analytic studies beyond the question of a particular experiment. Therefore, all raw data on rates and variables for normalization should be published in an open access data repository. Normalization of rates for: (1) the number of objects (cells, organisms); (2) the volume or

250 mass of the experimental sample; and (3) the concentration of mitochondrial markers in the
 251 experimental chamber are sample-specific normalizations, which are distinguished from
 252 system-specific normalization for the volume of the chamber (the measuring system).
 253 6. The consistent use of terms and symbols will facilitate transdisciplinary communication and
 254 support the further development of a collaborative database on bioenergetics and
 255 mitochondrial physiology. The present considerations are focused on studies with
 256 mitochondrial preparations. These will be extended in a series of reports on pathway control
 257 of mitochondrial respiration, respiratory states in intact cells, and harmonization of
 258 experimental procedures.
 259

260 **Box 1: In brief – Mitochondria and Bioblasts**

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

265 Mitochondria are oxygen-consuming electrochemical generators that evolved from the endosymbiotic
 266 alphaproteobacteria which became integrated into a host cell related to Asgard Archaea (Margulis 1970;
 267 Lane 2005; Roger *et al.* 2017). They were described by Richard Altmann (1894) as ‘bioblasts’, which
 268 include not only the mitochondria as presently defined, but also symbiotic and free-living bacteria. The
 269 word ‘mitochondria’ (Greek mitos: thread; chondros: granule) was introduced by Carl Benda (1898).

270 Contrary to current textbook dogma, mitochondria form dynamic networks within eukaryotic
 271 cells. Mitochondrial movement is supported by microtubules and morphology can change in response
 272 to energy requirements of the cell via processes known as fusion and fission; these interactions allow
 273 mitochondria to communicate within a network (Chan 2006). Mitochondria can even traverse cell
 274 boundaries in a process known as horizontal mitochondrial transfer (Torralba *et al.* 2016). Another
 275 defining characteristic of mitochondria is the double membrane. The mitochondrial inner membrane
 276 (mtIM) forms dynamic tubular to disk-shaped cristae that separate the mitochondrial matrix, *i.e.*, the
 277 negatively charged internal mitochondrial compartment, from the intermembrane space; the latter being
 278 enclosed by the mitochondrial outer membrane (mtOM) and positively charged with respect to the
 279 matrix. The mtIM contains the non-bilayer phospholipid cardiolipin, which is not present in any other
 280 eukaryotic cellular membrane. Cardiolipin has many regulatory functions (Oemer *et al.* 2018); in
 281 particular, it stabilizes and promotes the formation of respiratory supercomplexes (SC I_nIII_nIV_n), which
 282 are supramolecular assemblies based upon specific and dynamic interactions between individual
 283 respiratory complexes (Greggio *et al.* 2017; Lenaz *et al.* 2017). The mitochondrial membrane is plastic
 284 and exerts an influence on the functional properties of proteins incorporated in membranes
 285 (Waczulikova *et al.* 2007). Intracellular stress factors may cause shrinking or swelling of the
 286 mitochondrial matrix that can ultimately result in permeability transition.

287 Mitochondria are the structural and functional elementary components of cell respiration.
 288 Mitochondrial respiration is the reduction of molecular oxygen by electron transfer coupled to
 289 electrochemical proton translocation across the mtIM. In the process of oxidative phosphorylation
 290 (OXPHOS), the catabolic reaction of oxygen consumption is electrochemically coupled to the
 291 transformation of energy in the form of adenosine triphosphate (ATP; Mitchell 1961, 2011).
 292 Mitochondria are the powerhouses of the cell that contain the machinery of the OXPHOS-pathways,
 293 including transmembrane respiratory complexes (proton pumps with FMN, Fe-S and cytochrome *b*, *c*,
 294 *aa*₃ redox systems); alternative dehydrogenases and oxidases; the coenzyme ubiquinone (Q); F-ATPase
 295 or ATP synthase; the enzymes of the tricarboxylic acid cycle (TCA), fatty acid and amino acid oxidation;
 296 transporters of ions, metabolites and co-factors; iron/sulphur cluster synthesis; and mitochondrial
 297 kinases related to catabolic pathways. The mitochondrial proteome comprises over 1,200 proteins
 298 (Calvo *et al.* 2015; 2017), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many
 299 of which are relatively well known, *e.g.*, proteins regulating mitochondrial biogenesis or apoptosis,
 300 while others are still under investigation, or need to be identified, *e.g.*, permeability transition pore,
 301 alanine transporter. Only recently has it been possible to use the mammalian mitochondrial proteome to
 302 discover and characterize the genetic basis of mitochondrial diseases (Williams *et al.* 2016; Palmfeldt
 303 and Bross 2017).

304 Numerous cellular processes are orchestrated by a constant crosstalk between mitochondria and
 305 other cellular components. For example, the crosstalk between mitochondria and the endoplasmic

306 reticulum is involved in the regulation of calcium homeostasis, cell division, autophagy, differentiation,
 307 and anti-viral signaling (Murley and Nunnari 2016). Mitochondria contribute to the formation of
 308 peroxisomes, which are hybrids of mitochondrial and ER-derived precursors (Sugiura *et al.* 2017).
 309 Cellular mitochondrial homeostasis (mitostasis) is maintained through regulation at transcriptional,
 310 post-translational and epigenetic levels. Cell signalling modules contribute to homeostatic regulation
 311 throughout the cell cycle or even cell death by activating proteostatic modules, *e.g.*, the ubiquitin-
 312 proteasome and autophagy-lysosome/vacuole pathways; specific proteases like LON, and genome
 313 stability modules in response to varying energy demands and stress cues (Quiros *et al.* 2016). Several
 314 post-translational modifications, including acetylation and nitrosylation, are also capable of influencing
 315 the bioenergetic response, with clinically significant implications for health and disease (Carrico *et al.*
 316 2018).

317 Mitochondria of higher eukaryotes typically maintain several copies of their own circular genome
 318 known as mitochondrial DNA (mtDNA; hundred to thousands per cell; Cummins 1998), which is
 319 maternally inherited in humans. Biparental mitochondrial inheritance is documented in mammals, birds,
 320 fish, reptiles and invertebrate groups, and is even the norm in some bivalve taxonomic groups (Breton
 321 *et al.* 2007; White *et al.* 2008). The mitochondrial genome of the angiosperm *Amborella* contains a
 322 record of six mitochondrial genome equivalents acquired by horizontal transfer of entire genomes, two
 323 from angiosperms, three from algae and one from mosses (Rice *et al.* 2016). In unicellular organisms,
 324 *i.e.*, protists, the structural organization of mitochondrial genomes is highly variable and includes
 325 circular and linear DNA (Zikova *et al.* 2016). While some of the free-living flagellates exhibit the largest
 326 known gene coding capacity, *e.g.*, jakobid *Andalucia godoyi* mitochondrial DNA codes for 106 genes
 327 (Burger *et al.* 2013), some protist groups, *e.g.*, alveolates, possess mitochondrial genomes with only
 328 three protein-coding genes and two rRNAs (Feagin *et al.* 2012). The complete loss of mitochondrial
 329 genome is observed in highly reduced mitochondria of *Cryptosporidium* species (Liu *et al.* 2016).
 330 Reaching the final extreme, the microbial eukaryote, oxymonad *Monocercomonoides*, has no
 331 mitochondrion whatsoever and lacks all typical nuclear-encoded mitochondrial proteins, showing that
 332 while in 99% of organisms mitochondria play a vital role, this organelle is not indispensable
 333 (Karnkowska *et al.* 2016).

334 In vertebrates but not all invertebrates, mtDNA is compact (16.5 kB in humans) and encodes 13
 335 protein subunits of the transmembrane respiratory Complexes CI, CIII, CIV and ATP synthase (F-
 336 ATPase), 22 tRNAs, and two RNAs. Additional gene content has been suggested to include microRNAs,
 337 piRNA, smithRNAs, repeat associated RNA, and even additional proteins (Duarte *et al.* 2014; Lee *et al.*
 338 2015; Cobb *et al.* 2016). The mitochondrial genome requires nuclear-encoded mitochondrially
 339 targeted proteins, *e.g.*, TFAM, for its maintenance and expression (Rackham *et al.* 2012). Both genomes
 340 encode peptides of the membrane spanning redox pumps (CI, CIII and CIV) and F-ATPase, leading to
 341 strong constraints in the coevolution of both genomes (Blier *et al.* 2001).

342 Given the multiple roles of mitochondria, it is perhaps not surprising that mitochondrial
 343 dysfunction is associated with a wide variety of genetic and degenerative diseases. Robust mitochondrial
 344 function is supported by physical exercise and caloric balance, and is central for sustained metabolic
 345 health throughout life. Therefore, a more consistent set of definitions for mitochondrial physiology will
 346 increase our understanding of the etiology of disease and improve the diagnostic repertoire of
 347 mitochondrial medicine with a focus on protective medicine, lifestyle and healthy aging.

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

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353 1. Introduction

354

355 Mitochondria are the powerhouses of the cell with numerous physiological, molecular, and
 356 genetic functions (**Box 1**). Every study of mitochondrial health and disease faces **Evolution, Age,**
 357 **Gender and sex, Lifestyle, and Environment (MitoEAGLE)** as essential background conditions intrinsic
 358 to the individual person or cohort, species, tissue and to some extent even cell line. As a large and
 359 coordinated group of laboratories and researchers, the mission of the global MitoEAGLE Network is to
 360 generate the necessary scale, type, and quality of consistent data sets and conditions to address this
 361 intrinsic complexity. Harmonization of experimental protocols and implementation of a quality control

362 and data management system are required to interrelate results gathered across a spectrum of studies
363 and to generate a rigorously monitored database focused on mitochondrial respiratory function. In this
364 way, researchers from a variety of disciplines can compare their findings using clearly defined and
365 accepted international standards.

366 With an emphasis on quality of research, published data can be useful far beyond the specific
367 question of a particular experiment. For example, collaborative data sets support the development of
368 open-access databases such as those for National Institutes of Health sponsored research in genetics,
369 proteomics, and metabolomics. Indeed, enabling meta-analysis is the most economic way of providing
370 robust answers to biological questions (Cooper *et al.* 2009). However, the reproducibility of quantitative
371 results and databases depend on accurate measurements under strictly-defined conditions. Likewise,
372 meaningful interpretation and comparability of experimental outcomes requires standardisation of
373 protocols between research groups at different institutes. In addition to quality control, a conceptual
374 framework is also required to standardise and homogenise terminology and methodology. Vague or
375 ambiguous jargon can lead to confusion and may convert valuable signals to wasteful noise. For this
376 reason, measured values must be expressed in standard units for each parameter used to define
377 mitochondrial respiratory function. A consensus on fundamental nomenclature and conceptual
378 coherence, however, are missing in the expanding field of mitochondrial physiology. To fill this gap,
379 the present communication provides an in-depth review on harmonization of nomenclature and
380 definition of technical terms, which are essential to improve the awareness of the intricate meaning of
381 current and past scientific vocabulary. This is important for documentation and integration into
382 databases in general, and quantitative modelling in particular (Beard 2005).

383 In this review, we focus on coupling states and fluxes through metabolic pathways of aerobic
384 energy transformation in mitochondrial preparations as a first step in the attempt to generate a
385 conceptually-oriented nomenclature in bioenergetics and mitochondrial physiology. Respiratory control
386 by fuel substrates and specific inhibitors of respiratory enzymes, coupling states of intact cells, and
387 respiratory flux control ratios will be reviewed in subsequent communications, prepared in the frame of
388 COST Action MitoEAGLE open to global bottom-up input.

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391 **2. Coupling states and rates in mitochondrial preparations**

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

396

397 *2.1. Cellular and mitochondrial respiration*

398

399 **2.1.1. Aerobic and anaerobic catabolism and ATP turnover:** In respiration, electron transfer
400 is coupled to the phosphorylation of ADP to ATP, with energy transformation mediated by the
401 protonmotive force, pmf (**Figure 2**). Anabolic reactions are coupled to catabolism, both by ATP as the
402 intermediary energy currency and by small organic precursor molecules as building blocks for
403 biosynthesis. Glycolysis involves substrate-level phosphorylation of ADP to ATP in fermentation
404 without utilization of O₂, studied mainly in intact cells and organisms. Many cellular fuel substrates are
405 catabolized to acetyl-CoA or to glutamate, and further electron transfer reduces nicotinamide adenine
406 dinucleotide to NADH or flavin adenine dinucleotide to FADH₂. Subsequent mitochondrial electron
407 transfer to O₂ is coupled to proton translocation for the control of the protonmotive force and
408 phosphorylation of ADP (**Figure 2B and 2C**). In contrast, extra-mitochondrial oxidation of fatty acids
409 and amino acids proceeds partially in peroxisomes without coupling to ATP production: acyl-CoA
410 oxidase catalyzes the oxidation of FADH₂ with electron transfer to O₂; amino acid oxidases oxidize
411 flavin mononucleotide FMNH₂ or FADH₂ (**Figure 2A**).

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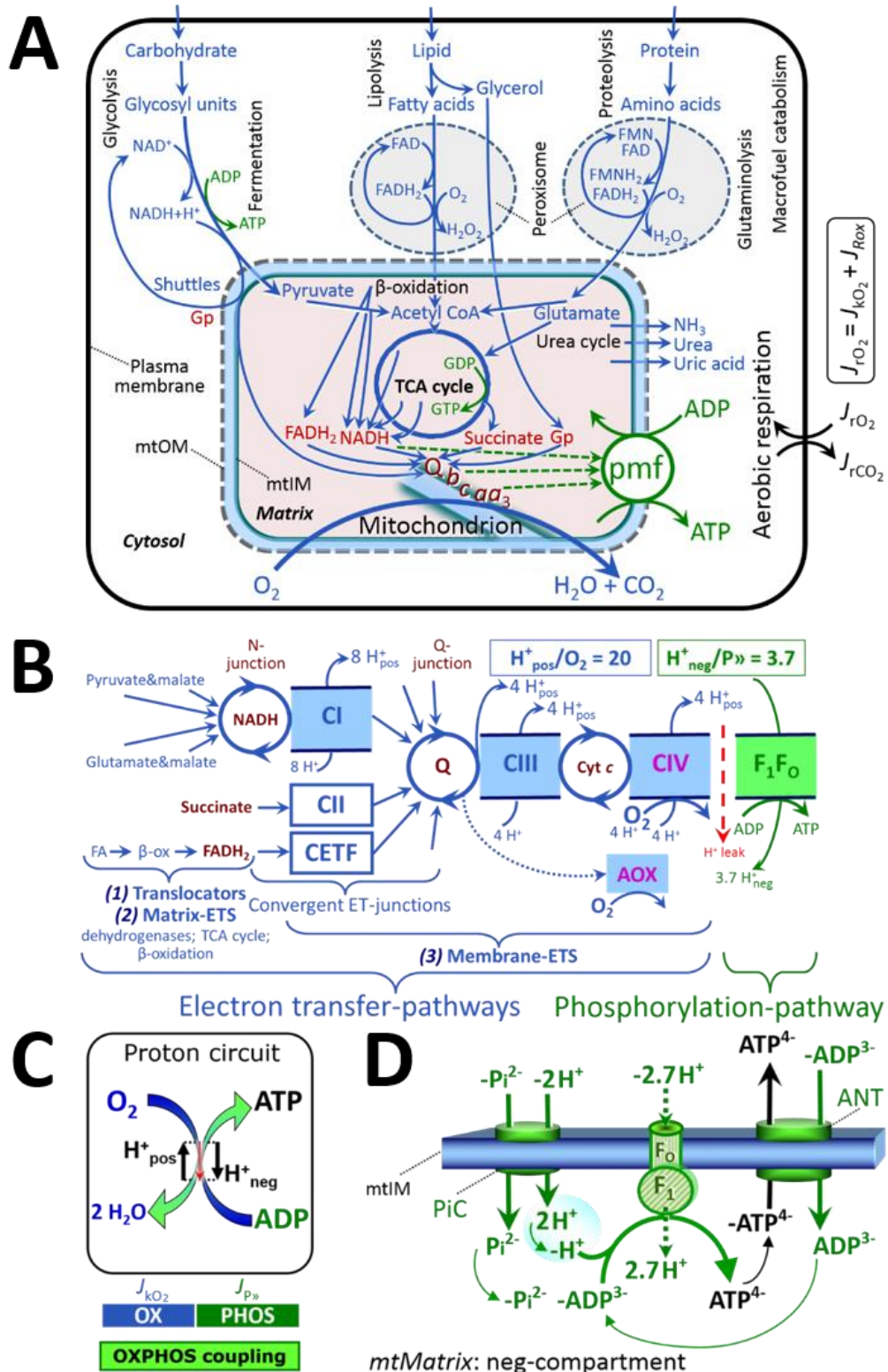


Figure 2. Cell respiration and oxidative phosphorylation (OXPHOS)

Mitochondrial respiration is the oxidation of fuel substrates (electron donors) with electron transfer to O_2 as the electron acceptor. For explanation of symbols see also **Figure 1**.

(A) Respiration of intact cells: Extra-mitochondrial catabolism of macromolecules and uptake of small molecules by the cell provide the mitochondrial fuel substrates. Dashed arrows indicate the connection between the redox proton pumps (respiratory Complexes CI, CIII and CIV) and the transmembrane protonmotive force, pmf. Coenzyme Q (Q) and the cytochromes *b*, *c*, and *aa₃* are redox systems of the mitochondrial inner membrane, mtIM. Glycerol-3-phosphate, Gp.

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423 (B) Respiration in mitochondrial preparations: The mitochondrial electron transfer system
 424 (ETS) is (1) fuelled by diffusion and transport of substrates across the mtOM and mtIM,
 425 and in addition consists of the (2) matrix-ETS, and (3) membrane-ETS. Electron transfer
 426 converges at the N-junction, and from CI, CII and electron transferring flavoprotein
 427 complex (CETF) at the Q-junction. Unlabeled arrows converging at the Q-junction indicate
 428 additional ETS-sections with electron entry into Q through glycerophosphate
 429 dehydrogenase, dihydro-orotate dehydrogenase, proline dehydrogenase, choline
 430 dehydrogenase, and sulfide-ubiquinone oxidoreductase. The dotted arrow indicates the
 431 branched pathway of oxygen consumption by alternative quinol oxidase (AOX). ET-
 432 pathways are coupled to the phosphorylation-pathway. The H^+_{pos}/O_2 ratio is the outward
 433 proton flux from the matrix space to the positively (pos) charged vesicular compartment,
 434 divided by catabolic O_2 flux in the NADH-pathway. The H^+_{neg}/P_{\gg} ratio is the inward proton
 435 flux from the inter-membrane space to the negatively (neg) charged matrix space, divided
 436 by the flux of phosphorylation of ADP to ATP. These stoichiometries are not fixed because
 437 of ion leaks and proton slip. Modified from Lemieux *et al.* (2017) and Rich (2013).
 438 (C) OXPHOS coupling: O_2 flux through the catabolic ET-pathway, J_{kO_2} , is coupled
 439 by the H^+ circuit to flux through the phosphorylation-pathway of ADP to ATP, $J_{P_{\gg}}$.
 440 (D) Chemiosmotic phosphorylation-pathway catalyzed by the proton pump F_1F_0 -ATPase
 441 (F-ATPase, ATP synthase), adenine nucleotide translocase (ANT), and inorganic
 442 phosphate carrier (PiC). The H^+_{neg}/P_{\gg} stoichiometry is the sum of the coupling
 443 stoichiometry in the F-ATPase reaction ($-2.7 H^+_{\text{pos}}$ from the positive intermembrane space,
 444 $2.7 H^+_{\text{neg}}$ to the matrix, *i.e.*, the negative compartment) and the proton balance in the
 445 translocation of ADP^{3-} , ATP^{4-} and P_i^{2-} . Modified from Gnaiger (2014).
 446

447 The plasma membrane separates the intracellular compartment including the cytosol, nucleus, and
 448 organelles from the extracellular environment. The plasma membrane consists of a lipid bilayer with
 449 embedded proteins and attached organic molecules that collectively control the selective permeability
 450 of ions, organic molecules, and particles across the cell boundary. The intact plasma membrane prevents
 451 the passage of many water-soluble mitochondrial substrates and inorganic ions—such as succinate,
 452 adenosine diphosphate (ADP) and inorganic phosphate (P_i) that must be precisely controlled at
 453 kinetically-saturating concentrations for the analysis of mitochondrial respiratory capacities.
 454 Respiratory capacities delineate, comparable to channel capacity in information theory (Schneider
 455 2006), the upper boundary of the rate of O_2 consumption measured in defined respiratory states. Despite
 456 the activity of solute carriers, *e.g.*, SLC13A3 and SLC20A2, which transport specific metabolites across
 457 the plasma membrane of various cell types, the intact plasma membrane limits the scope of
 458 investigations into mitochondrial respiratory function in intact cells.

459 **2.1.2. Specification of biochemical dose:** Substrates, uncouplers, inhibitors, and other chemical
 460 reagents are titrated to analyse cellular and mitochondrial function. Nominal concentrations of these
 461 substances are usually reported as initial amount of substance concentration [$\text{mol}\cdot\text{L}^{-1}$] in the incubation
 462 medium. When aiming at the measurement of kinetically saturated processes—such as OXPHOS-
 463 capacities—the concentrations for substrates can be chosen according to the apparent equilibrium
 464 constant, K_m' . In the case of hyperbolic kinetics, only 80% of maximum respiratory capacity is obtained
 465 at a substrate concentration of four times the K_m' , whereas substrate concentrations of 5, 9, 19 and 49
 466 times the K_m' are theoretically required for reaching 83%, 90%, 95% or 98% of the maximal rate
 467 (Gnaiger 2001). Other reagents are chosen to inhibit or alter a particular process. The amount of these
 468 chemicals in an experimental incubation is selected to maximize effect, avoiding unacceptable off-target
 469 consequences that would adversely affect the data being sought. Specifying the amount of substance in
 470 an incubation as nominal concentration in the aqueous incubation medium can be ambiguous (Doskey
 471 *et al.* 2015), particularly for cations (TPP^+ ; fluorescent dyes such as safranin, TMRM; Chowdhury *et al.*
 472 2015) and lipophilic substances (oligomycin, uncouplers, permeabilization agents; Doerrier *et al.* 2018),
 473 which accumulate in the mitochondrial matrix or in biological membranes, respectively. Generally,
 474 dose/exposure can be specified per unit of biological sample, *i.e.*, (nominal moles of
 475 xenobiotic)/(number of cells) [$\text{mol}\cdot\text{cell}^{-1}$] or, as appropriate, per mass of biological sample [$\text{mol}\cdot\text{kg}^{-1}$].
 476 This approach to specification of dose/exposure provides a scalable parameter that can be used to design
 477 experiments, help interpret a wide variety of experimental results, and provide absolute information that
 478 allows researchers worldwide to make the most use of published data (Doskey *et al.* 2015).

479 2.2. Mitochondrial preparations

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482 Mitochondrial preparations are defined as either isolated mitochondria or tissue and cellular
 483 preparations in which the barrier function of the plasma membrane is disrupted. Since this entails the
 484 loss of cell viability, mitochondrial preparations are not studied *in vivo*. In contrast to isolated
 485 mitochondria and tissue homogenate preparations, mitochondria in permeabilized tissues and cells are
 486 *in situ* relative to the plasma membrane. When studying mitochondrial preparations, substrate-
 487 uncoupler-inhibitor-titration (SUIT) protocols are used to establish respiratory coupling control states
 488 (CCS) and pathway control states (PCS) that provide reference values for various output variables
 489 (**Table 1**). Physiological conditions *in vivo* deviate from these experimentally obtained states; this is
 490 because kinetically-saturating concentrations, *e.g.*, of ADP, oxygen (O₂; dioxygen) or fuel substrates,
 491 may not apply to physiological intracellular conditions. Further information is obtained in studies of
 492 kinetic responses to variations in fuel substrate concentrations, [ADP], or [O₂] in the range between
 493 kinetically-saturating concentrations and anoxia (Gnaiger 2001).

494

495 The cholesterol content of the plasma membrane is high compared to mitochondrial membranes
 496 (Korn 1969). Therefore, mild detergents—such as digitonin and saponin—can be applied to selectively
 497 permeabilize the plasma membrane via interaction with cholesterol; this allows free exchange of organic
 498 molecules and inorganic ions between the cytosol and the immediate cell environment, while
 499 maintaining the integrity and localization of organelles, cytoskeleton, and the nucleus. Application of
 500 permeabilization agents (mild detergents or toxins) leads to washout of cytosolic marker enzymes—
 501 such as lactate dehydrogenase—and results in the complete loss of cell viability (tested by nuclear
 502 staining using plasma membrane-impermeable dyes), while mitochondrial function remains intact
 503 (tested by cytochrome *c* stimulation of respiration). Digitonin concentrations have to be optimized
 504 according to cell type, particularly since mitochondria from cancer cells contain significantly higher
 505 contents of cholesterol in both membranes (Baggetto and Testa-Perussini, 1990). For example, a dose
 506 of digitonin of 8 fmol·cell⁻¹ (10 pg·cell⁻¹; 10 μg·10⁻⁶ cells) is optimal for permeabilization of endothelial
 507 cells, and the concentration in the incubation medium has to be adjusted according to the cell density
 508 (Doerrier *et al.* 2018). Respiration of isolated mitochondria remains unaltered after the addition of low
 509 concentrations of digitonin or saponin. In addition to mechanical cell disruption during homogenization
 510 of tissue, permeabilization agents may be applied to ensure permeabilization of all cells in tissue
 511 homogenates.

512

513 Suspensions of cells permeabilized in the respiration chamber and crude tissue homogenates
 514 contain all components of the cell at highly dilute concentrations. All mitochondria are retained in
 515 chemically-permeabilized mitochondrial preparations and crude tissue homogenates. In the preparation
 516 of isolated mitochondria, however, the mitochondria are separated from other cell fractions and purified
 517 by differential centrifugation, entailing the loss of mitochondria at typical recoveries ranging from 30%
 518 to 80% of total mitochondrial content (Lai *et al.* 2018). Using Percoll or sucrose density gradients to
 519 maximize the purity of isolated mitochondria may compromise the mitochondrial yield or structural and
 520 functional integrity. Therefore, mitochondrial isolation protocols need to be optimized according to each
 521 study. The term, *mitochondrial preparation*, neither includes intact cells, nor submitochondrial particles
 522 and further fractionation of mitochondrial components.

523

524 2.3. Electron transfer pathways

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526 Mitochondrial electron transfer (ET) pathways are fuelled by diffusion and transport of substrates
 527 across the mtOM and mtIM. In addition, the mitochondrial electron transfer system (ETS) consists of
 528 the matrix-ETS and membrane-ETS (**Figure 2B**). Upstream sections of ET-pathways converge at the
 529 NADH-junction (N-junction). NADH is mainly generated in the tricarboxylic acid (TCA) cycle and is
 530 oxidized by Complex I (CI), with further electron entry into the coenzyme Q-junction (Q-junction).
 531 Similarly, succinate is formed in the TCA cycle and oxidized by CII to fumarate. CII is part of both the
 532 TCA cycle and the ETS, and reduces FAD to FADH₂ with further reduction of ubiquinone to ubiquinol
 533 downstream of the TCA cycle in the Q-junction. Thus FADH₂ is not a substrate but is the product of
 CII, in contrast to erroneous metabolic maps shown in many publications. β-oxidation of fatty acids
 (FA) supplies reducing equivalents via (1) FADH₂ as the substrate of electron transferring flavoprotein
 complex (CETF); (2) acetyl-CoA generated by chain shortening; and (3) NADH generated via 3-

534 hydroxyacyl-CoA dehydrogenases. The ATP yield depends on whether acetyl-CoA enters the TCA
535 cycle, or is for example used in ketogenesis.

536 Selected mitochondrial catabolic pathways, k , of electron transfer from the oxidation of fuel
537 substrates to the reduction of O_2 are activated by addition of fuel substrates to the mitochondrial
538 respiration medium after depletion of endogenous substrates (**Figure 2B**). Substrate combinations and
539 specific inhibitors of ET-pathway enzymes are used to obtain defined pathway control states in
540 mitochondrial preparations (Gnaiger 2014).

541

542 2.4. Respiratory coupling control

543

544 **2.4.1. Coupling:** In mitochondrial electron transfer, vectorial transmembrane proton flux is
545 coupled through the redox proton pumps CI, CIII and CIV to the catabolic flux of scalar reactions,
546 collectively measured as O_2 flux, J_{kO_2} (**Figure 2**). Thus mitochondria are elementary components of
547 energy transformation. Energy is a conserved quantity and cannot be lost or produced in any internal
548 process (First Law of Thermodynamics). Open and closed systems can gain or lose energy only by
549 external fluxes—by exchange with the environment. Therefore, energy can neither be produced by
550 mitochondria, nor is there any internal process without energy conservation. Exergy or Gibbs energy
551 (‘free energy’) is the part of energy that can potentially be transformed into work under conditions of
552 constant temperature and pressure. *Coupling* is the interaction of an exergonic process (spontaneous,
553 negative exergy change) with an endergonic process (positive exergy change) in energy transformations
554 which conserve part of the exergy that would be irreversibly lost or dissipated in an uncoupled process.

555 Pathway control states (PCS) and coupling control states (CCS) are complementary, since
556 mitochondrial preparations depend on (1) an exogenous supply of pathway-specific fuel substrates and
557 oxygen, and (2) exogenous control of phosphorylation (**Figure 2**).

558 **2.4.2. Phosphorylation, P_{\gg} , and P_{\gg}/O_2 ratio:** Phosphorylation in the context of OXPHOS is
559 defined as phosphorylation of ADP by P_i to form ATP. On the other hand, the term phosphorylation is
560 used generally in many contexts, *e.g.*, protein phosphorylation. This justifies consideration of a symbol
561 more discriminating and specific than P as used in the P/O ratio (phosphate to atomic oxygen ratio),
562 where P indicates phosphorylation of ADP to ATP or GDP to GTP (**Figure 2**). We propose the symbol
563 P_{\gg} for the endergonic (uphill) direction of phosphorylation $ADP \rightarrow ATP$, and likewise the symbol P_{\ll} for
564 the corresponding exergonic (downhill) hydrolysis $ATP \rightarrow ADP$. P_{\gg} refers mainly to electrontransfer
565 phosphorylation but may also involve substrate-level phosphorylation as part of the TCA cycle
566 (succinyl-CoA ligase, phosphoglycerate kinase) and phosphorylation of ADP catalyzed by pyruvate
567 kinase, and of GDP phosphorylated by phosphoenolpyruvate carboxykinase. Transphosphorylation is
568 performed by adenylate kinase, creatine kinase (mtCK), hexokinase and nucleoside diphosphate kinase.
569 In isolated mammalian mitochondria, ATP production catalyzed by adenylate kinase ($2 ADP \leftrightarrow ATP +$
570 AMP) proceeds without fuel substrates in the presence of ADP (Kömldi and Tretter 2017). Kinase
571 cycles are involved in intracellular energy transfer and signal transduction for regulation of energy flux.

572 The P_{\gg}/O_2 ratio ($P_{\gg}/4 e^-$) is two times the ‘P/O’ ratio ($P_{\gg}/2 e^-$). P_{\gg}/O_2 is a generalized symbol, not
573 specific for reporting P_i consumption (P_i/O_2 flux ratio), ADP depletion (ADP/O_2 flux ratio), or ATP
574 production (ATP/O_2 flux ratio). The mechanistic P_{\gg}/O_2 ratio—or P_{\gg}/O_2 stoichiometry—is calculated
575 from the proton-to- O_2 and proton-to-phosphorylation coupling stoichiometries (**Figure 2B**):

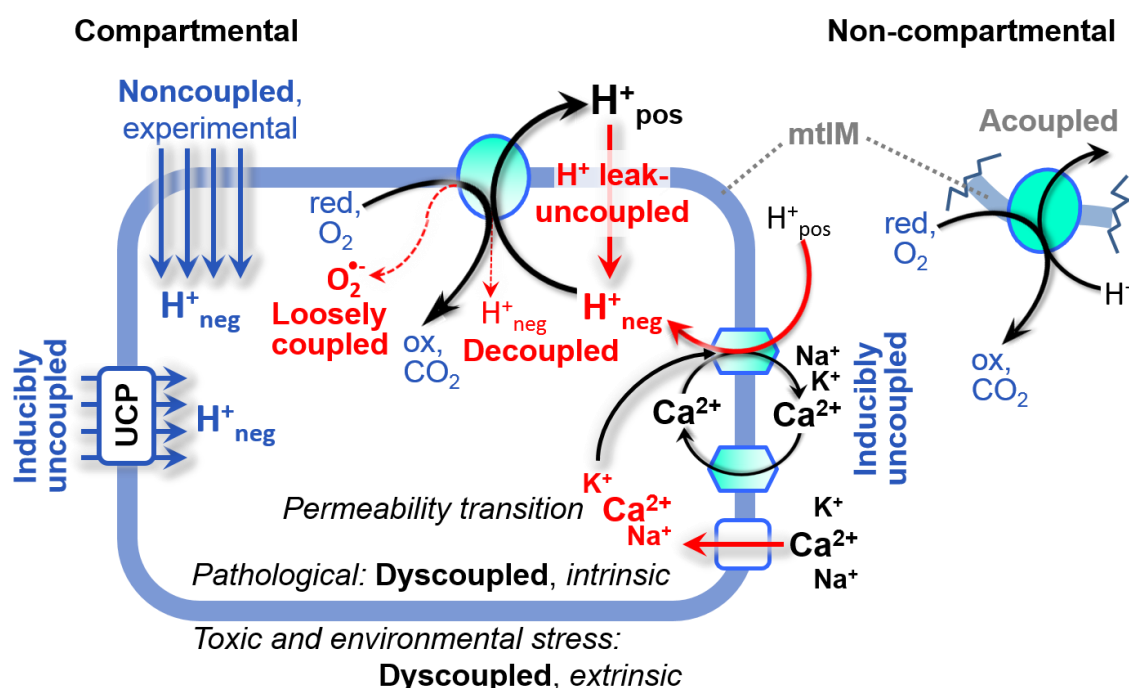
$$577 \quad P_{\gg}/O_2 = \frac{H_{pos}^+/O_2}{H_{neg}^+/P_{\gg}} \quad (1)$$

579 The H_{pos}^+/O_2 coupling stoichiometry (referring to the full four electron reduction of O_2) depends on the
580 relative involvement of the three coupling sites (respiratory Complexes CI, CIII and CIV) in the
581 catabolic ET-pathway from reduced fuel substrates (electron donors) to the reduction of O_2 (electron
582 acceptor). This varies with: (1) a bypass of CI by single or multiple electron input into the Q-junction;
583 and (2) a bypass of CIV by involvement of alternative oxidases, AOX. AOX are expressed in all plants,
584 some fungi, many protists, and several animal phyla, but are not expressed in vertebrate mitochondria
585 (McDonald *et al.* 2009).

586 The H_{pos}^+/O_2 coupling stoichiometry equals 12 in the ET-pathways involving CIII and CIV as
587 proton pumps, increasing to 20 for the NADH-pathway through CI (**Figure 2B**), but a general consensus
588 on H_{pos}^+/O_2 stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov
589 2015). The H_{neg}^+/P_{\gg} coupling stoichiometry (3.7; **Figure 2B**) is the sum of 2.7 H_{neg}^+ required by the F-
590 ATPase of vertebrate and most invertebrate species (Watt *et al.* 2010) and the proton balance in the

591 translocation of ADP, ATP and P_i (**Figure 2C**). Taken together, the mechanistic P_{\gg}/O_2 ratio is calculated
 592 at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively (Eq. 1). The corresponding
 593 classical P_{\gg}/O ratios (referring to the 2 electron reduction of $0.5 O_2$) are 2.7 and 1.6 (Watt *et al.* 2010),
 594 in agreement with the measured P_{\gg}/O ratio for succinate of 1.58 ± 0.02 (Gnaiger *et al.* 2000).

595 **2.4.3. Uncoupling:** The effective P_{\gg}/O_2 flux ratio ($Y_{P_{\gg}/O_2} = J_{P_{\gg}}/J_{kO_2}$) is diminished relative to the
 596 mechanistic P_{\gg}/O_2 ratio by intrinsic and extrinsic uncoupling or dyscoupling (**Figure 3**). Such
 597 generalized uncoupling is different from switching to mitochondrial pathways that involve fewer than
 598 three proton pumps ('coupling sites': Complexes CI, CIII and CIV), bypassing CI through multiple
 599 electron entries into the Q-junction, or CIII and CIV through AOX (**Figure 2B**). Reprogramming of
 600 mitochondrial pathways leading to different types of substrates being oxidized may be considered as a
 601 switch of gears (changing the stoichiometry by altering the substrate that is oxidized) rather than
 602 uncoupling (loosening the tightness of coupling relative to a fixed stoichiometry). In addition, Y_{P_{\gg}/O_2}
 603 depends on several experimental conditions of flux control, increasing as a hyperbolic function of [ADP]
 604 to a maximum value (Gnaiger 2001).
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607 **Figure 3. Mechanisms of respiratory uncoupling**

608 An intact mitochondrial inner membrane, mtIM, is required for vectorial, compartmental coupling.
 609 'Acoupled' respiration is the consequence of structural disruption with catalytic activity of non-
 610 compartmental mitochondrial fragments. Inducible uncoupling, *e.g.*, by activation of UCP1, increases
 611 LEAK-respiration; experimentally noncoupled respiration provides an estimate of ET-capacity obtained
 612 by titration of protonophores stimulating respiration to maximum O_2 flux. H^+ leak-uncoupled,
 613 decoupled, and loosely coupled respiration are components of intrinsic uncoupling (**Table 2**).
 614 Pathological dysfunction may affect all types of uncoupling, including permeability transition, causing
 615 intrinsically dyscoupled respiration. Similarly, toxicological and environmental stress factors can cause
 616 extrinsically dyscoupled respiration. Reduced fuel substrates, red; oxidized products, ox.

618 Uncoupling of mitochondrial respiration is a general term comprising diverse mechanisms:

- 619 1. Proton leak across the mtIM from the positive to the negative compartment (H^+ leak-uncoupled;
 620 **Figure 3**).
- 621 2. Cycling of other cations, strongly stimulated by permeability transition; comparable to the use of
 622 protonophores, cation cycling is experimentally induced by valinomycin in the presence of K^+ ;
- 623 3. Decoupling by proton slip in the redox proton pumps when protons are effectively not pumped
 624 (CI, CIII and CIV) or are not driving phosphorylation (F-ATPase);
- 625 4. Loss of vesicular (compartmental) integrity when electron transfer is acoupled;

626 5. Electron leak in the loosely coupled univalent reduction of O₂ to superoxide (O₂^{•-}; superoxide
627 anion radical).

628 Differences of terms—uncoupled *vs.* noncoupled—are easily overlooked, although they relate to
629 different meanings of uncoupling (**Figure 3** and **Table 2**).

630

631 2.5. Coupling states and respiratory rates

632

633 To extend the classical nomenclature on mitochondrial coupling states (Section 2.6) by a concept-
634 driven terminology that explicitly incorporates information on the meaning of respiratory states, the
635 terminology must be general and not restricted to any particular experimental protocol or mitochondrial
636 preparation (Gnaiger 2009). Concept-driven nomenclature aims at mapping the meaning and concept
637 behind the words and acronyms onto the forms of words and acronyms (Miller 1991). The focus of
638 concept-driven nomenclature is primarily the conceptual *why*, along with clarification of the
639 experimental *how*.

640

641 **Table 1. Coupling states and residual oxygen consumption in mitochondrial**
642 **preparations in relation to respiration- and phosphorylation-flux, J_{kO_2} and $J_{P_{\gg}}$, and**
643 **protonmotive force, pmf.** Coupling states are established at kinetically-saturating
644 concentrations of fuel substrates and O₂.

State	J_{kO_2}	$J_{P_{\gg}}$	pmf	Inducing factors	Limiting factors
LEAK	<i>L</i> ; low, cation leak-dependent respiration	0	max.	back-flux of cations including proton leak, proton slip	$J_{P_{\gg}} = 0$: (1) without ADP, L_N ; (2) max. ATP/ADP ratio, L_T ; or (3) inhibition of the phosphorylation-pathway, L_{Omy}
OXPHOS	<i>P</i> ; high, ADP-stimulated respiration, OXPHOS-capacity	max.	high	kinetically-saturating [ADP] and [P _i]	$J_{P_{\gg}}$ by phosphorylation-pathway capacity; or J_{kO_2} by ET-capacity
ET	<i>E</i> ; max., noncoupled respiration, ET-capacity	0	low	optimal external uncoupler concentration for max. $J_{O_2,E}$	J_{kO_2} by ET-capacity
ROX	<i>Rox</i> ; min., residual O ₂ consumption	0	0	$J_{O_2,Rox}$ in non-ET-pathway oxidation reactions	inhibition of all ET-pathways; or absence of fuel substrates

645

646 To provide a diagnostic reference for respiratory capacities of core energy metabolism, the
647 capacity of oxidative phosphorylation, OXPHOS, is measured at kinetically-saturating concentrations
648 of ADP and P_i. The oxidative ET-capacity reveals the limitation of OXPHOS-capacity mediated by the
649 phosphorylation-pathway. The ET- and phosphorylation-pathways comprise coupled segments of the
650 OXPHOS-system. By application of external uncouplers, ET-capacity is measured as noncoupled
651 respiration. The contribution of intrinsically uncoupled O₂ consumption is studied by preventing the
652 stimulation of phosphorylation either in the absence of ADP or by inhibition of the phosphorylation-
653 pathway. The corresponding states are collectively classified as LEAK-states when O₂ consumption
654 compensates mainly for ion leaks, including the proton leak. Defined coupling states are induced by: (1)
655 adding cation chelators such as EGTA, binding free Ca²⁺ and thus limiting cation cycling; (2) adding
656 ADP and P_i; (3) inhibiting the phosphorylation-pathway; and (4) uncoupler titrations, while maintaining
657 a defined ET-pathway state with constant fuel substrates and inhibitors of specific branches of the ET-
658 pathway.

659

660 The three coupling states, ET, LEAK and OXPHOS, are shown schematically with the
661 corresponding respiratory rates, abbreviated as *E*, *L* and *P*, respectively (**Figure 4**). We distinguish
662 metabolic *pathways* from metabolic *states* and the corresponding metabolic *rates*; for example: ET-

662 pathways, ET-states, and ET-capacities, E , respectively (Table 1). The protonmotive force is *high* in
 663 the OXPHOS-state when it drives phosphorylation, *maximum* in the LEAK-state of coupled
 664 mitochondria, driven by LEAK-respiration at a minimum back-flux of cations to the matrix side, and
 665 *very low* in the ET-state when uncouplers short-circuit the proton cycle (Table 1).

667 **Figure 4. Four-compartment**
 668 **model of oxidative**
 669 **phosphorylation**

670 Respiratory states (ET, OXPHOS,
 671 LEAK; Table 1) and corresponding
 672 rates (E , P , L) are connected by the
 673 protonmotive force, pmf. (1) ET-
 674 capacity, E , is partitioned into (2)
 675 dissipative LEAK-respiration, L ,
 676 when the Gibbs energy change of
 677 catabolic O_2 flux is irreversibly lost,
 678 (3) net OXPHOS-capacity, $P-L$, with
 679 partial conservation of the capacity to perform work, and (4) the excess capacity, $E-P$. Modified from
 680 Gnaiger (2014).

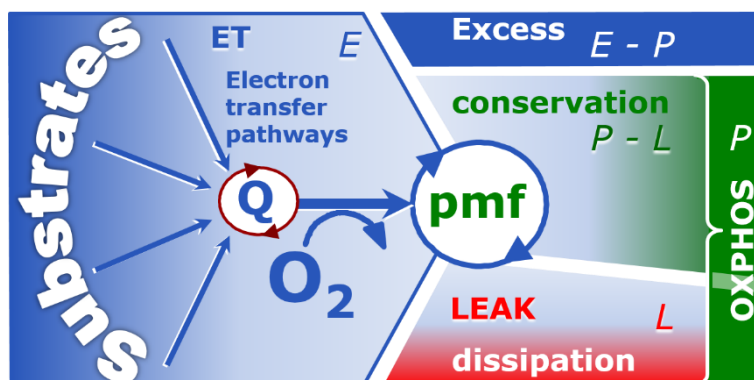


Figure 5. Respiratory coupling states

(A) LEAK-state and rate, L :

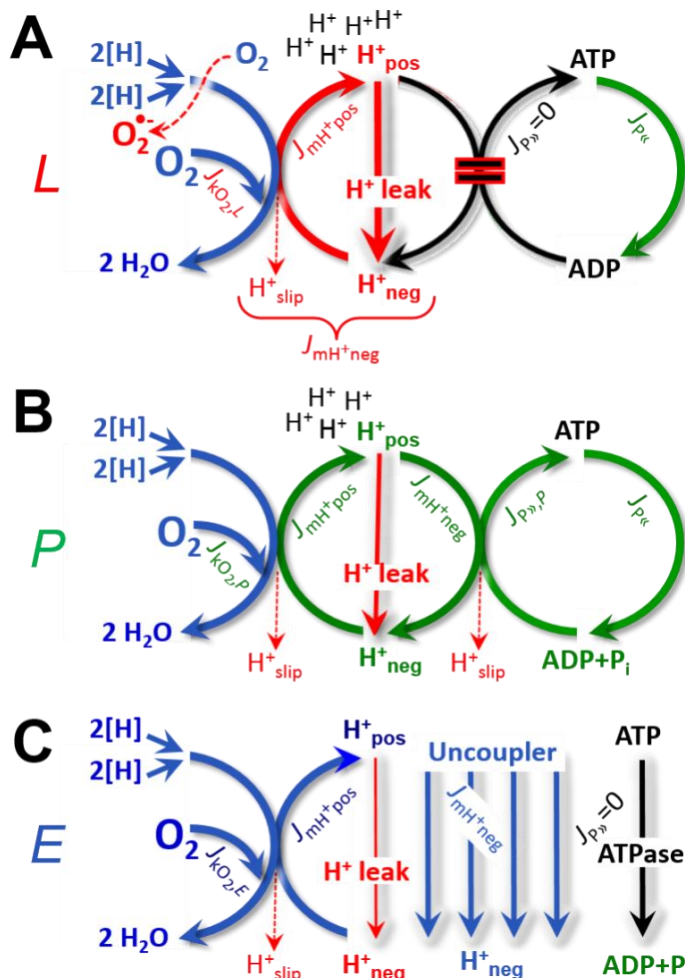
Oxidation only, since phosphorylation is arrested, $J_{P\gg} = 0$, and catabolic O_2 flux, $J_{kO_2,L}$, is controlled mainly by the proton leak and slip, J_{mH^+neg} , at maximum protonmotive force (Figure 4). Extramitochondrial ATPases, $J_{P\ll}$; then phosphorylation must be blocked.

(B) OXPHOS-state and rate, P :

Oxidation coupled to phosphorylation, $J_{P\gg}$, which is stimulated by kinetically-saturating [ADP] and $[P_i]$, supported by a high protonmotive force. O_2 flux, $J_{kO_2,P}$, is well-coupled at a $P\gg/O_2$ ratio of $J_{P\gg,P}/J_{O_2,P}$. Extramitochondrial ATPases may recycle ATP, $J_{P\ll}$.

(C) ET-state and rate, E :

Oxidation only, since phosphorylation is zero, $J_{P\gg} = 0$, at optimum exogenous uncoupler concentration when noncoupled respiration, $J_{kO_2,E}$, is maximum. The F-ATPase may hydrolyze extramitochondrial ATP.



681

682 **2.5.1. LEAK-state (Figure 5A):** The LEAK-state is defined as a state of mitochondrial
 683 respiration when O_2 flux mainly compensates for ion leaks in the absence of ATP synthesis, at
 684 kinetically-saturating concentrations of O_2 , respiratory fuel substrates and P_i . LEAK-respiration is
 685 measured to obtain an estimate of intrinsic uncoupling without addition of an experimental uncoupler:

686 (1) in the absence of adenylates, *i.e.*, AMP, ADP and ATP; (2) after depletion of ADP at a maximum
 687 ATP/ADP ratio; or (3) after inhibition of the phosphorylation-pathway by inhibitors of F-ATPase—such
 688 as oligomycin, or of adenine nucleotide translocase—such as carboxyatractyloside. Adjustment of the
 689 nominal concentration of these inhibitors to the density of biological sample applied can minimize or
 690 avoid inhibitory side-effects exerted on ET-capacity or even some dyscoupling.
 691
 692

Table 2. Terms on respiratory coupling and uncoupling.

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

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- **Proton leak and uncoupled respiration:** The intrinsic proton leak is the *uncoupled* leak current of protons in which protons diffuse across the mtIM in the dissipative direction of the downhill protonmotive force without coupling to phosphorylation (**Figure 5A**). The proton leak flux depends non-linearly on the protonmotive force (Garlid *et al.* 1989; Divakaruni and Brand 2011), which is a temperature-dependent property of the mtIM and may be enhanced due to possible contamination by free fatty acids. Inducible uncoupling mediated by uncoupling protein 1 (UCP1) is physiologically controlled, *e.g.*, in brown adipose tissue. UCP1 is a member of the mitochondrial carrier family that is involved in the translocation of protons across the mtIM (Klingenberg 2017). Consequently, this short-circuit lowers the protonmotive force and stimulates electron transfer, respiration, and heat dissipation in the absence of phosphorylation of ADP.
- **Cation cycling:** There can be other cation contributors to leak current including calcium and probably magnesium. Calcium influx is balanced by mitochondrial Na^+/Ca^{2+} or H^+/Ca^{2+} exchange, which is balanced by Na^+/H^+ or K^+/H^+ exchanges. This is another effective uncoupling mechanism different from proton leak (**Table 2**).
- **Proton slip and decoupled respiration:** Proton slip is the *decoupled* process in which protons are only partially translocated by a redox proton pump of the ET-pathways and slip back to the original vesicular compartment. The proton leak is the dominant contributor to the overall leak current in mammalian mitochondria incubated under physiological conditions at 37 °C, whereas proton slip increases at lower experimental temperature (Canton *et al.* 1995). Proton slip can also happen in association with the F-ATPase, in which the proton slips downhill across the pump to

715 the matrix without contributing to ATP synthesis. In each case, proton slip is a property of the
716 proton pump and increases with the pump turnover rate.

- 717 • **Electron leak and loosely coupled respiration:** Superoxide production by the ETS leads to a
718 bypass of redox proton pumps and correspondingly lower P_{\gg}/O_2 ratio. This depends on the actual
719 site of electron leak and the scavenging of hydrogen peroxide by cytochrome *c*, whereby electrons
720 may re-enter the ETS with proton translocation by CIV.
- 721 • **Loss of compartmental integrity and acoupled respiration:** Electron transfer and catabolic O_2
722 flux proceed without compartmental proton translocation in disrupted mitochondrial fragments.
723 Such fragments are an artefact of mitochondrial isolation, and may not fully fuse to re-establish
724 structurally intact mitochondria. Loss of mtIM integrity, therefore, is the cause of acoupled
725 respiration, which is a nonvectorial dissipative process without control by the protonmotive force.
- 726 • **Dyscoupled respiration:** Mitochondrial injuries may lead to *dyscoupling* as a pathological or
727 toxicological cause of *uncoupled* respiration. Dyscoupling may involve any type of uncoupling
728 mechanism, *e.g.*, opening the permeability transition pore. Dyscoupled respiration is
729 distinguished from the experimentally induced *noncoupled* respiration in the ET-state (**Table 2**).

730

731 **2.5.2. OXPHOS-state (Figure 5B):** The OXPHOS-state is defined as the respiratory state with
732 kinetically-saturating concentrations of O_2 , respiratory and phosphorylation substrates, and absence of
733 exogenous uncoupler, which provides an estimate of the maximal respiratory capacity in the OXPHOS-
734 state for any given ET-pathway state. Respiratory capacities at kinetically-saturating substrate
735 concentrations provide reference values or upper limits of performance, aiming at the generation of data
736 sets for comparative purposes. Physiological activities and effects of substrate kinetics can be evaluated
737 relative to the OXPHOS-capacity.

738 As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria
739 (Gnaiger 2001; Puchowicz *et al.* 2004); greater [ADP] is required, particularly in permeabilized muscle
740 fibres and cardiomyocytes, to overcome limitations by intracellular diffusion and by the reduced
741 conductance of the mtOM (Jepihhina *et al.* 2011; Illaste *et al.* 2012; Simson *et al.* 2016), either through
742 interaction with tubulin (Rostovtseva *et al.* 2008) or other intracellular structures (Birkedal *et al.* 2014).
743 In addition, saturating ADP concentrations need to be evaluated under different experimental conditions
744 such as temperature (Lemieux *et al.* 2017) and with different animal models (Blier and Guderley, 1993).
745 In permeabilized muscle fibre bundles of high respiratory capacity, the apparent K_m for ADP increases
746 up to 0.5 mM (Saks *et al.* 1998), consistent with experimental evidence that >90% saturation is reached
747 only at >5 mM ADP (Pesta and Gnaiger 2012). Similar ADP concentrations are also required for
748 accurate determination of OXPHOS-capacity in human clinical cancer samples and permeabilized cells
749 (Klepinin *et al.* 2016; Koit *et al.* 2017). 2.5 to 5 mM ADP is sufficient to obtain the actual OXPHOS-
750 capacity in many types of permeabilized tissue and cell preparations, but experimental validation is
751 required in each specific case.

752 **2.5.3. Electron transfer-state (Figure 5C):** O_2 flux determined in the ET-state yields an estimate
753 of ET-capacity. The ET-state is defined as the *noncoupled* state with kinetically-saturating
754 concentrations of O_2 , respiratory substrate and optimum exogenous uncoupler concentration for
755 maximum O_2 flux. Uncouplers are weak lipid-soluble acids which function as protonophores. These
756 disrupt the barrier function of the mtIM and thus short circuit the protonmotive system, functioning like
757 a clutch in a mechanical system. As a consequence of the nearly collapsed protonmotive force, the
758 driving force is insufficient for phosphorylation, and $J_{P_{\gg}} = 0$. The most frequently used uncouplers are
759 carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP), carbonyl cyanide *p*-
760 trifluoromethoxyphenylhydrazone (FCCP), or dinitrophenol (DNP). Stepwise titration of uncouplers
761 stimulates respiration up to or above the level of O_2 consumption rates in the OXPHOS-state; respiration
762 is inhibited, however, above optimum uncoupler concentrations (Mitchell 2011). Data obtained with a
763 single dose of uncoupler must be evaluated with caution, particularly when a fixed uncoupler
764 concentration is used in studies exploring a treatment or disease that may alter the mitochondrial content
765 or mitochondrial sensitivity to inhibition by uncouplers. The effect on ET-capacity of the reversed
766 function of F-ATPase ($J_{P_{\ll}}$; **Figure 5C**) can be evaluated in the presence and absence of
767 extramitochondrial ATP.

768 **2.5.4. ROX state and *Rox*:** Besides the three fundamental coupling states of mitochondrial
769 preparations, the state of residual O_2 consumption, ROX, which although not a coupling state, is relevant
770 to assess respiratory function (**Figure 1**). The rate of residual oxygen consumption, *Rox*, is defined as

771 O₂ consumption due to oxidative reactions measured after inhibition of ET with rotenone, malonic acid
 772 and antimycin A. Cyanide and azide inhibit not only CIV but catalase and several peroxidases involved
 773 in *Rox*. High concentrations of antimycin A, but not rotenone or cyanide, inhibit peroxisomal acyl-CoA
 774 oxidase and D-amino acid oxidase (Vamecq *et al.* 1987). *Rox* represents a baseline used to correct
 775 respiration measured in defined coupling control states. *Rox*-corrected *L*, *P* and *E* not only lower the
 776 values of total fluxes, but also change the flux control ratios *L/P* and *L/E*. *Rox* is not necessarily
 777 equivalent to non-mitochondrial reduction of O₂, considering O₂-consuming reactions in mitochondria
 778 that are not related to ET—such as O₂ consumption in reactions catalyzed by monoamine oxidases (type
 779 A and B), monooxygenases (cytochrome P450 monooxygenases), dioxygenase (sulfur dioxygenase and
 780 trimethyllysine dioxygenase), and several hydroxylases. Even isolated mitochondrial fractions,
 781 especially those obtained from liver, may be contaminated by peroxisomes, as shown by transmission
 782 electron microscopy. This fact makes the exact determination of mitochondrial O₂ consumption and
 783 mitochondria-associated generation of reactive oxygen species complicated (Schönfeld *et al.* 2009;
 784 Speijer 2016; **Figure 2**). The dependence of ROX-linked O₂ consumption needs to be studied in detail
 785 together with non-ET enzyme activities, availability of specific substrates, O₂ concentration, and
 786 electron leakage leading to the formation of reactive oxygen species.

787 **2.5.5. Quantitative relations:** *E* may exceed or be equal to *P*. $E > P$ is observed in many types
 788 of mitochondria, varying between species, tissues and cell types (Gnaiger 2009). *E-P* is the excess ET-
 789 capacity pushing the phosphorylation-flux (**Figure 2C**) to the limit of its capacity for utilizing the
 790 protonmotive force. In addition, the magnitude of *E-P* depends on the tightness of respiratory coupling
 791 or degree of uncoupling, since an increase of *L* causes *P* to increase towards the limit of *E*. The *excess*
 792 *E-P* capacity, *E-P*, therefore, provides a sensitive diagnostic indicator of specific injuries of the
 793 phosphorylation-pathway, under conditions when *E* remains constant but *P* declines relative to controls
 794 (**Figure 4**). Substrate cocktails supporting simultaneous convergent electron transfer to the Q-junction
 795 for reconstitution of TCA cycle function establish pathway control states with high ET-capacity, and
 796 consequently increase the sensitivity of the *E-P* assay.

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

804 The net OXPHOS-capacity is calculated by subtracting *L* from *P* (**Figure 4**). The net P_»/O₂ equals
 805 $P_{»}/(P-L)$, wherein the dissipative LEAK component in the OXPHOS-state may be overestimated. This
 806 can be avoided by measuring LEAK-respiration in a state when the protonmotive force is adjusted to its
 807 slightly lower value in the OXPHOS-state by titration of an ET inhibitor (Divakaruni and Brand 2011).
 808 Any turnover-dependent components of proton leak and slip, however, are underestimated under these
 809 conditions (Garlid *et al.* 1993). In general, it is inappropriate to use the term *ATP production* or *ATP*
 810 *turnover* for the difference of O₂ flux measured in the OXPHOS and LEAK states. *P-L* is the upper limit
 811 of OXPHOS-capacity that is freely available for ATP production (corrected for LEAK-respiration) and
 812 is fully coupled to phosphorylation with a maximum mechanistic stoichiometry (**Figure 4**).

813 LEAK-respiration and OXPHOS-capacity depend on (1) the tightness of coupling under the
 814 influence of the respiratory uncoupling mechanisms (**Figure 3**), and (2) the coupling stoichiometry,
 815 which varies as a function of the substrate type undergoing oxidation in ET-pathways with either two
 816 or three coupling sites (**Figure 2B**). When cocktails with NADH-linked substrates and succinate are
 817 used, the relative contribution of ET-pathways with three or two coupling sites cannot be controlled
 818 experimentally, is difficult to determine, and may shift in transitions between LEAK-, OXPHOS- and
 819 ET-states (Gnaiger 2014). Under these experimental conditions, we cannot separate the tightness of
 820 coupling *versus* coupling stoichiometry as the mechanisms of respiratory control in the shift of *L/P*
 821 ratios. The tightness of coupling and fully coupled O₂ flux, *P-L* (**Table 2**), therefore, are obtained from
 822 measurements of coupling control of LEAK-respiration, OXPHOS- and ET-capacities in well-defined
 823 pathway states, using either pyruvate and malate as substrates or the classical succinate and rotenone
 824 substrate-inhibitor combination (**Figure 2B**).

825 **2.5.6. The steady-state:** Mitochondria represent a thermodynamically open system in non-
 826 equilibrium states of biochemical energy transformation. State variables (protonmotive force; redox

827 states) and metabolic *rates* (fluxes) are measured in defined mitochondrial respiratory *states*. Steady-
 828 states can be obtained only in open systems, in which changes by internal transformations, *e.g.*, O₂
 829 consumption, are instantaneously compensated for by external fluxes, *e.g.*, O₂ supply, preventing a
 830 change of O₂ concentration in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored
 831 in closed systems satisfy the criteria of pseudo-steady states for limited periods of time, when changes
 832 in the system (concentrations of O₂, fuel substrates, ADP, P_i, H⁺) do not exert significant effects on
 833 metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media
 834 with sufficient buffering capacity and substrates maintained at kinetically-saturating concentrations, and
 835 thus depend on the kinetics of the processes under investigation.

836

837 2.6. Classical terminology for isolated mitochondria

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

840

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

845

846

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

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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

849

850 **2.6.1. State 1** is obtained after addition of isolated mitochondria to air-saturated
 851 isoosmotic/isotonic respiration medium containing P_i, but no fuel substrates and no adenylates.

852 **2.6.2. State 2** is induced by addition of a 'high' concentration of ADP (typically 100 to 300 μM),

853 which stimulates respiration transiently on the basis of endogenous fuel substrates and phosphorylates

854 only a small portion of the added ADP. State 2 is then obtained at a low respiratory activity limited by

855 exhausted endogenous fuel substrate availability (**Table 3**). If addition of specific inhibitors of

856 respiratory complexes such as rotenone does not cause a further decline of O₂ flux, State 2 is equivalent

857 to the ROX state (See below.). If inhibition is observed, undefined endogenous fuel substrates are a

858 confounding factor of pathway control, contributing to the effect of subsequently externally added

859 substrates and inhibitors. In contrast to the original protocol, an alternative sequence of titration steps is

860 frequently applied, in which the alternative 'State 2' has an entirely different meaning when this second

861 state is induced by addition of fuel substrate without ADP or ATP (LEAK-state; in contrast to State 2

862 defined in **Table 1** as a ROX state). Some researchers have called this condition as 'pseudostate 4'

863 because it has no significant concentrations of adenine nucleotides and hence it is not a near-

864 physiological condition, although it should be used for calculating the net OXPHOS-capacity, *P-L*.

865 **2.6.3. State 3** is the state stimulated by addition of fuel substrates while the ADP concentration

866 is still high (**Table 3**) and supports coupled energy transformation through oxidative phosphorylation.

867 'High ADP' is a concentration of ADP specifically selected to allow the measurement of State 3 to State

868 4 transitions of isolated mitochondria in a closed respirometric chamber. Repeated ADP titration re-

869 establishes State 3 at 'high ADP'. Starting at O₂ concentrations near air-saturation (193 or 238 μM O₂

870 at 37 °C or 25 °C and sea level at 1 atm or 101.32 kPa, and an oxygen solubility of respiration medium

871 at 0.92 times that of pure water; Forstner and Gnaiger 1983), the total ADP concentration added must

872 be low enough (typically 100 to 300 μM) to allow phosphorylation to ATP at a coupled O₂ flux that

873 does not lead to O₂ depletion during the transition to State 4. In contrast, kinetically-saturating ADP

874 concentrations usually are 10-fold higher than 'high ADP', *e.g.*, 2.5 mM in isolated mitochondria. The
 875 abbreviation State 3u is occasionally used in bioenergetics, to indicate the state of respiration after
 876 titration of an uncoupler, without sufficient emphasis on the fundamental difference between OXPHOS-
 877 capacity (*well-coupled* with an endogenous uncoupled component) and ET-capacity (*noncoupled*).

878 **2.6.4. State 4** is a LEAK-state that is obtained only if the mitochondrial preparation is intact and
 879 well-coupled. Depletion of ADP by phosphorylation to ATP causes a decline of O₂ flux in the transition
 880 from State 3 to State 4. Under the conditions of State 4, a maximum protonmotive force and high
 881 ATP/ADP ratio are maintained. The gradual decline of $Y_{P\gg O_2}$ towards diminishing [ADP] at State 4 must
 882 be taken into account for calculation of P_»/O₂ ratios (Gnaiger 2001). State 4 respiration, L_T (**Table 1**),
 883 reflects intrinsic proton leak and ATP hydrolysis activity. O₂ flux in State 4 is an overestimation of
 884 LEAK-respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, $J_{P\ll}$, which
 885 stimulates respiration coupled to phosphorylation, $J_{P\gg} > 0$. Some degree of mechanical disruption and
 886 loss of mitochondrial integrity allows the exposed mitochondrial F-ATPases to hydrolyze the ATP
 887 synthesized by the fraction of coupled mitochondria. This can be tested by inhibition of the
 888 phosphorylation-pathway using oligomycin, ensuring that $J_{P\gg} = 0$ (State 4o). On the other hand, the State
 889 4 respiration reached after exhaustion of added ADP is a more physiological condition, *i.e.*, presence of
 890 ATP, ADP and even AMP. Sequential ADP titrations re-establish State 3, followed by State 3 to State
 891 4 transitions while sufficient O₂ is available. Anoxia may be reached, however, before exhaustion of
 892 ADP (State 5).

893 **2.6.5. State 5** 'may be obtained by antimycin A treatment or by anaerobiosis' (Chance and
 894 Williams, 1955) '. These definitions give State 5 two different meanings of ROX or anoxia, respectively.
 895 Anoxia is obtained after exhaustion of O₂ in a closed respirometric chamber. Diffusion of O₂ from the
 896 surroundings into the aqueous solution may be a confounding factor preventing complete anoxia
 897 (Gnaiger 2001).

898 In **Table 3**, only States 3 and 4 are coupling control states, with the restriction that rates in State
 899 3 may be limited kinetically by non-saturating ADP concentrations.

900

901 2.7. Control and regulation

902

903 The terms metabolic *control* and *regulation* are frequently used synonymously, but are
 904 distinguished in metabolic control analysis: "We could understand the regulation as the mechanism that
 905 occurs when a system maintains some variable constant over time, in spite of fluctuations in external
 906 conditions (homeostasis of the internal state). On the other hand, metabolic control is the power to
 907 change the state of the metabolism in response to an external signal" (Fell 1997). Respiratory control
 908 may be induced by experimental control signals that exert an influence on: (1) ATP demand and ADP
 909 phosphorylation-rate; (2) fuel substrate composition, pathway competition; (3) available amounts of
 910 substrates and O₂, *e.g.*, starvation and hypoxia; (4) the protonmotive force, redox states, flux-force
 911 relationships, coupling and efficiency; (5) Ca²⁺ and other ions including H⁺; (6) inhibitors, *e.g.*, nitric
 912 oxide or intermediary metabolites such as oxaloacetate; (7) signalling pathways and regulatory proteins,
 913 *e.g.*, insulin resistance, transcription factor hypoxia inducible factor 1.

914 Mechanisms of respiratory control and regulation include adjustments of: (1) enzyme activities
 915 by allosteric mechanisms and phosphorylation; (2) enzyme content, concentrations of cofactors and
 916 conserved moieties such as adenylates, nicotinamide adenine dinucleotide [NAD⁺/NADH], coenzyme
 917 Q, cytochrome *c*; (3) metabolic channeling by supercomplexes; and (4) mitochondrial density (enzyme
 918 concentrations and membrane area) and morphology (cristae folding, fission and fusion). Mitochondria
 919 are targeted directly by hormones, *e.g.*, progesterone and glucacorticoids, which affect their energy
 920 metabolism (Lee *et al.* 2013; Gerö and Szabo 2016; Price and Dai 2016; Moreno *et al.* 2017).
 921 Evolutionary or acquired differences in the genetic and epigenetic basis of mitochondrial function (or
 922 dysfunction) between individuals; age; biological sex, and hormone concentrations; life style including
 923 exercise and nutrition; and environmental issues including thermal, atmospheric, toxic and
 924 pharmacological factors, exert an influence on all control mechanisms listed above. For reviews, see
 925 Brown 1992; Gnaiger 1993a, 2009; 2014; Paradies *et al.* 2014; Morrow *et al.* 2017.

926 Lack of control by a metabolic pathway, *e.g.*, phosphorylation-pathway, means that there will
 927 be no response to a variable activating it, *e.g.*, [ADP]. The reverse, however, is not true as the absence
 928 of a response to [ADP] does not exclude the phosphorylation-pathway from having some degree of
 929 control. The degree of control of a component of the OXPHOS-pathway on an output variable, such as

930 O₂ flux, will in general be different from the degree of control on other outputs, such as phosphorylation-
 931 flux or proton leak flux. Therefore, it is necessary to be specific as to which input and output are under
 932 consideration (Fell 1997).

933 Respiratory control refers to the ability of mitochondria to adjust O₂ flux in response to external
 934 control signals by engaging various mechanisms of control and regulation. Respiratory control is
 935 monitored in a mitochondrial preparation under conditions defined as respiratory states, preferentially
 936 under near-physiological conditions of temperature, pH, and medium ionic composition, to generate
 937 data of higher biological relevance. When phosphorylation of ADP to ATP is stimulated or depressed,
 938 an increase or decrease is observed in electron transfer measured as O₂ flux in respiratory coupling states
 939 of intact mitochondria ('controlled states' in the classical terminology of bioenergetics). Alternatively,
 940 coupling of electron transfer with phosphorylation is diminished by uncouplers. The corresponding
 941 coupling control state is characterized by a high respiratory rate without control by P» (noncoupled or
 942 'uncontrolled state').

943
 944

945 3. What is a rate?

946

947 The term *rate* is not adequately defined to be useful for reporting data. Normalization of 'rates'
 948 leads to a diversity of formats. Application of common and defined units is required for direct transfer
 949 of reported results into a database. The second [s] is the SI unit for the base quantity *time*. It is also the
 950 standard time-unit used in solution chemical kinetics.

951 The inconsistency of the meanings of rate becomes apparent when considering Galileo Galilei's
 952 famous principle, that 'bodies of different weight all fall at the same rate (have a constant acceleration)'
 953 (Coopersmith 2010). A rate may be an extensive quantity, which is a *flow*, *I*, when expressed per object
 954 (per number of cells or organisms) or per chamber (per system). 'System' is defined as the open or
 955 closed chamber of the measuring device. A rate is a *flux*, *J*, when expressed as a size-specific quantity
 956 (**Figure 6A; Box 2**).

957 • **Extensive quantities:** An extensive quantity increases proportionally with system size. For
 958 example, mass and volume are extensive quantities. Flow is an extensive quantity. The
 959 magnitude of an extensive quantity is completely additive for non-interacting subsystems.
 960 The magnitude of these quantities depends on the extent or size of the system (Cohen *et al.*
 961 2008).

962 • **Size-specific quantities:** 'The adjective *specific* before the name of an extensive quantity is
 963 often used to mean *divided by mass*' (Cohen *et al.* 2008). In this system-paradigm, mass-
 964 specific flux is flow divided by mass of the system (the total mass of everything within the
 965 measuring chamber or reactor). Rates are frequently expressed as volume-specific flux. A
 966 mass-specific or volume-specific quantity is independent of the extent of non-interacting
 967 homogenous subsystems. Tissue-specific quantities (related to the *sample* in contrast to the
 968 *system*) are of fundamental interest in the field of comparative mitochondrial physiology,
 969 where *specific* refers to the *type of the sample* rather than *mass of the system*. The term
 970 *specific*, therefore, must be clarified; *sample-specific*, *e.g.*, muscle mass-specific
 971 normalization, is distinguished from *system-specific* quantities (mass or volume; **Figure 6**).

972 • **Intensive quantities:** In contrast to size-specific properties, forces are intensive quantities
 973 defined as the change of an extensive quantity per advancement of an energy transformation
 974 (Gnaiger 1993b).

975 • N_X and m_X indicate the number format and mass format, respectively, for expressing the
 976 quantity of a sample *X*. When different formats are indicated in symbols of derived quantities,
 977 the format (\underline{N} , \underline{m}) is shown as a subscript (*underlined italic*), as in $I_{O_2/\underline{N}X}$ and $J_{O_2/\underline{m}X}$. Oxygen
 978 flow and flux are expressed in the molar format, n_{O_2} [mol], but in the volume format, V_{O_2} [m³]
 979 in ergometry. For mass-specific flux these formats can be distinguished as $J_{\underline{n}O_2/\underline{m}X}$ and $J_{V_{O_2}/\underline{m}X}$,
 980 respectively. Further examples are given in **Figure 6** and **Table 4**.

981

982 **Figure 6. Flow and flux, and**
 983 **normalization in structure-**
 984 **function analysis**

985 (A) When expressing metabolic
 986 ‘rate’ measured in a chamber, a
 987 fundamental distinction is made
 988 between relating the rate to the
 989 experimental sample (left) or
 990 chamber (right). The different
 991 meanings of rate need to be
 992 specified by the chosen
 993 normalization. Left: Results are
 994 expressed as mass-specific flux, J_{mX} ,
 995 per mg protein, dry or wet mass.
 996 Cell volume, V_{ce} , may be used for
 997 normalization (volume-specific
 998 flux, J_{Vce}). Right: Flow per chamber,
 999 I , or flux per chamber volume, J_V ,
 1000 are merely reported for
 1001 methodological reasons.

1002 (B) O_2 flow per cell, $I_{O_2/Nce}$, is the
 1003 product of mitochondria-specific
 1004 flux, mt-density and mass per cell.
 1005 Unstructured analysis: performance
 1006 is the product of mass-specific flux,
 1007 $J_{O_2/MX}$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{kg}^{-1}$], and size
 1008 (mass per cell). Structured analysis:
 1009 performance is the product of
 1010 mitochondrial function (mt-specific
 1011 flux) and structure (mt-content).
 1012 Modified from Gnaiger (2014). For
 1013 further details see **Table 4**.

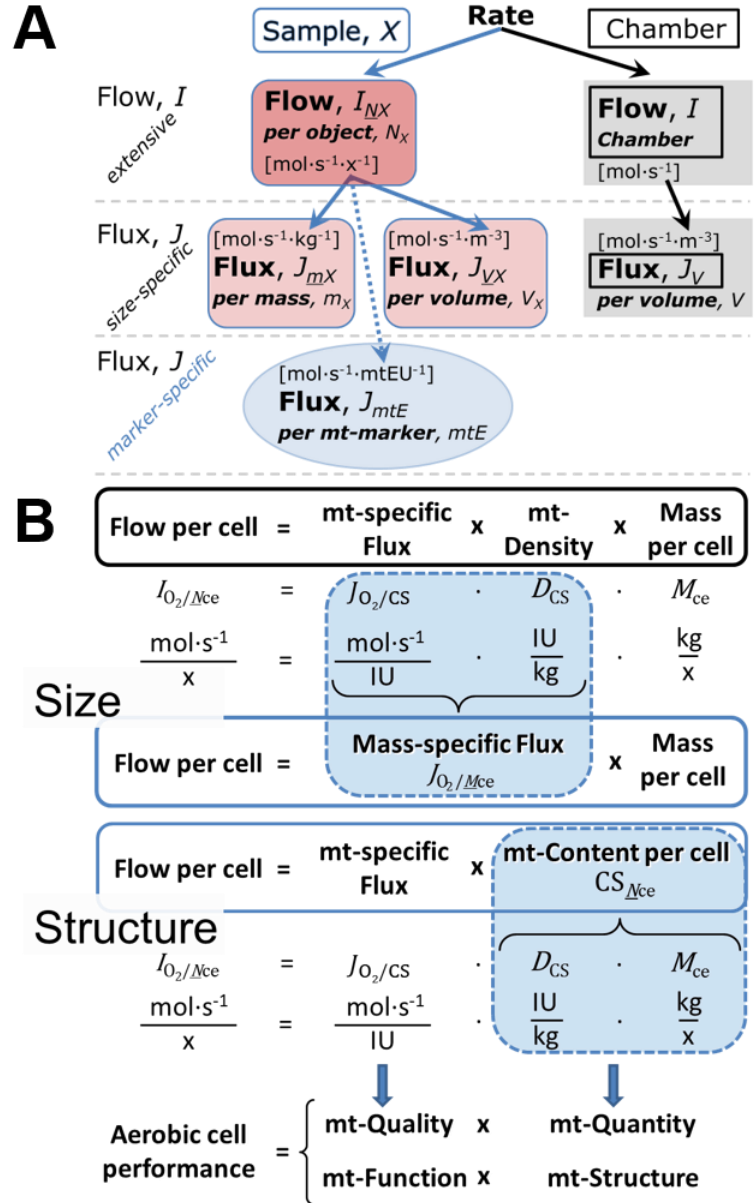
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1019 **Box 2: Metabolic flows and fluxes: vectoral, vectorial, and scalar**

1020

1021 In a generalization of electrical terms, flow as an extensive quantity (I ; per system) is
 1022 distinguished from flux as a size-specific quantity (J ; per system size). *Flows*, I_{tr} , are defined for all
 1023 transformations as extensive quantities. Electric charge per unit time is electric flow or current, $I_{el} =$
 1024 $dQ_{el}\cdot dt^{-1}$ [$A \equiv C\cdot s^{-1}$]. When dividing I_{el} by size of the system (cross-sectional area of a ‘wire’), we obtain
 1025 flux as a size-specific quantity, which is the current density (surface-density of flow) perpendicular to
 1026 the direction of flux, $J_{el} = I_{el}\cdot A^{-1}$ [$A\cdot m^{-2}$] (Cohen et al. 2008). Fluxes with *spatial* geometric direction and
 1027 magnitude are *vectors*. Vector and scalar *fluxes* are related to flows as $J_{tr} = I_{tr}\cdot A^{-1}$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$] and $J_{tr} =$
 1028 $I_{tr}\cdot V^{-1}$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-3}$], expressing flux as an area-specific vector or volume-specific vectorial or scalar
 1029 quantity, respectively (Gnaiger 1993b). We use the metre–kilogram–second–ampere (MKSA)
 1030 international system of units (SI) for general cases ([m], [kg], [s] and [A]), with decimal SI prefixes for
 1031 specific applications (**Table 4**).

1032 We suggest defining: (1) *vectoral* fluxes, which are translocations as functions of *gradients* with
 1033 direction in geometric space in continuous systems; (2) *vectorial* fluxes, which describe translocations
 1034 in discontinuous systems and are restricted to information on *compartmental differences*
 1035 (transmembrane proton flux); and (3) *scalar* fluxes, which are transformations in a *homogenous* system
 1036 (catabolic O_2 flux, J_{kO_2}).



4. Normalization of rate per sample

The challenges of measuring mitochondrial respiratory flux are matched by those of normalization. Normalization (**Table 4**) is guided by physicochemical principles, methodological considerations, and conceptual strategies (**Figure 6**).

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	1
mass of sample X	m_X		kg	2
mass of object X	M_X	$M_X = m_X \cdot N_X^{-1}$	$\text{kg} \cdot \text{x}^{-1}$	2
Mitochondria				
mitochondria	mt	$X = \text{mt}$		
amount of mt-elementary components	mtE	quantity of mt-marker	mtEU	
Concentrations				
object number concentration	C_{NX}	$C_{NX} = N_X \cdot V^{-1}$	$\text{x} \cdot \text{m}^{-3}$	3
sample mass concentration	C_{mX}	$C_{mX} = m_X \cdot V^{-1}$	$\text{kg} \cdot \text{m}^{-3}$	
mitochondrial concentration	C_{mtE}	$C_{mtE} = mtE \cdot V^{-1}$	$\text{mtEU} \cdot \text{m}^{-3}$	4
specific mitochondrial density	D_{mtE}	$D_{mtE} = mtE \cdot m_X^{-1}$	$\text{mtEU} \cdot \text{kg}^{-1}$	5
mitochondrial content, mtE per object X	mtE_{NX}	$mtE_{NX} = mtE \cdot N_X^{-1}$	$\text{mtEU} \cdot \text{x}^{-1}$	6
O₂ flow and flux				
flow, system	I_{O_2}	internal flow	$\text{mol} \cdot \text{s}^{-1}$	8
volume-specific flux	J_{V,O_2}	$J_{V,O_2} = I_{O_2} \cdot V^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-3}$	9
flow per object X	$I_{O_2/NX}$	$I_{O_2/NX} = J_{V,O_2} \cdot C_{NX}^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{x}^{-1}$	10
mass-specific flux	$J_{O_2/mX}$	$J_{O_2/mX} = J_{V,O_2} \cdot C_{mX}^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{kg}^{-1}$	
mt-marker-specific flux	$J_{O_2/mtE}$	$J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$	$\text{mol} \cdot \text{s}^{-1} \cdot \text{mtEU}^{-1}$	11

- 1045 1 The unit x for a number is not used by IUPAC. To avoid confusion, the units [$\text{kg} \cdot \text{x}^{-1}$] and [kg]
 1046 distinguish the mass per object from the mass of a sample that may contain any number of objects.
 1047 Similarly, the units for flow per system *versus* flow per object are [$\text{mol} \cdot \text{s}^{-1}$] (Note 8) and [$\text{mol} \cdot \text{s}^{-1} \cdot \text{x}^{-1}$]
 1048 (Note 10).
 1049 2 Units are given in the MKSA system (**Box 2**). The SI prefix k is used for the SI base unit of mass (kg
 1050 = 1,000 g). In praxis, various SI prefixes are used for convenience, to make numbers easily readable,
 1051 e.g., 1 mg tissue, cell or mitochondrial mass instead of 0.000001 kg.
 1052 3 In case of cells (sample $X = \text{cells}$), the object number concentration is $C_{N_{ce}} = N_{ce} \cdot V^{-1}$, and volume
 1053 may be expressed in [$\text{dm}^3 \equiv \text{L}$] or [$\text{cm}^3 = \text{mL}$]. See **Table 5** for different object types.
 1054 4 mt-concentration is an experimental variable, dependent on sample concentration: (1) $C_{mtE} = mtE \cdot V^{-1}$;
 1055 (2) $C_{mtE} = mtE_{NX} \cdot C_{NX}$; (3) $C_{mtE} = C_{mX} \cdot D_{mtE}$.
 1056 5 If the amount of mitochondria, mtE , is expressed as mitochondrial mass, then D_{mtE} is the mass
 1057 fraction of mitochondria in the sample. If mtE is expressed as mitochondrial volume, V_{mt} , and the
 1058 mass of sample, m_X , is replaced by volume of sample, V_X , then D_{mtE} is the volume fraction of
 1059 mitochondria in the sample.
 1060 6 $mtE_{NX} = mtE \cdot N_X^{-1} = C_{mtE} \cdot C_{NX}^{-1}$.
 1061 7 O₂ can be replaced by other chemicals to study different reactions, e.g., ATP, H₂O₂, or vesicular
 1062 compartmental translocations, e.g., Ca²⁺.

- 1063 8 I_{O_2} and V are defined per instrument chamber as a system of constant volume (and constant
 1064 temperature), which may be closed or open. I_{O_2} is abbreviated for I_{rO_2} , *i.e.*, the metabolic or internal
 1065 O_2 flow of the chemical reaction r in which O_2 is consumed, hence the negative stoichiometric
 1066 number, $\nu_{O_2} = -1$. $I_{rO_2} = d_r n_{O_2} / dt \cdot \nu_{O_2}^{-1}$. If r includes all chemical reactions in which O_2 participates, then
 1067 $d_r n_{O_2} = dn_{O_2} - d_e n_{O_2}$, where dn_{O_2} is the change in the amount of O_2 in the instrument chamber and $d_e n_{O_2}$
 1068 is the amount of O_2 added externally to the system. At steady state, by definition $dn_{O_2} = 0$, hence $d_r n_{O_2}$
 1069 $= -d_e n_{O_2}$. Note that in this context ‘external’, e , refers to the system, whereas in Figure 1 ‘external’,
 1070 ext , refers to the organism.
 1071 9 J_{V,O_2} is an experimental variable, expressed per volume of the instrument chamber.
 1072 10 $I_{O_2/NX}$ is a physiological variable, depending on the size of entity X .
 1073 11 There are many ways to normalize for a mitochondrial marker, that are used in different experimental
 1074 approaches: (1) $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$; (2) $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mX}^{-1} \cdot D_{mtE}^{-1} = J_{O_2/mX} \cdot D_{mtE}^{-1}$; (3) $J_{O_2/mtE} =$
 1075 $J_{V,O_2} \cdot C_{NX}^{-1} \cdot mtE_{NX}^{-1} = I_{O_2/NX} \cdot mtE_{NX}^{-1}$; (4) $J_{O_2/mtE} = I_{O_2} \cdot mtE^{-1}$. The mt-elementary unit [mtEU] varies depending
 1076 on the mt-marker.
 1077
 1078
 1079

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

Identity of sample	X	N_X	Mass ^a	Volume	mt-Marker
mitochondrial preparation		[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}
cells ^b	ce	N_{ce}	M_{ce}	V_{ce}	mtE_{ce}
intact cell, viable cell	vce	N_{vce}	M_{vce}	V_{vce}	
dead cell	dce	N_{dce}	M_{dce}	V_{dce}	
organism	org	N_{org}	M_{org}	V_{org}	

^a Instead of mass, the wet weight or dry weight is frequently stated, W_w or W_d . m_X is mass of the sample [kg], M_X is mass of the object [kg·x⁻¹] (Table 4).

^b Total cell count, $N_{ce} = N_{vce} + N_{dce}$

4.1. Flow: per object

4.1.1. Number concentration, C_{NX} : Normalization per sample concentration is routinely required to report respiratory data. C_{NX} is the experimental number concentration of sample X . In the case of animals, *e.g.*, nematodes, $C_{NX} = N_X/V$ [x·L⁻¹], where N_X is the number of organisms in the chamber. Similarly, the number of cells per chamber volume is the number concentration of permeabilized or intact cells $C_{Nce} = N_{ce}/V$ [x·L⁻¹], where N_{ce} is the number of cells in the chamber (Table 4).

4.1.2. Flow per object, $I_{O_2/NX}$: O_2 flow per cell is calculated from volume-specific O_2 flux, J_{V,O_2} [nmol·s⁻¹·L⁻¹] (per V of the measurement chamber [L]), divided by the number concentration of cells. The total cell count is the sum of viable and dead cells, $N_{ce} = N_{vce} + N_{dce}$ (Table 5). The cell viability index, $VI = N_{vce}/N_{ce}$, is the ratio of viable cells (N_{vce} ; before experimental permeabilization) per total cell count. After experimental permeabilization, all cells are permeabilized, $N_{pce} = N_{ce}$. The cell viability index can be used to normalize respiration for the number of cells that have been viable before experimental permeabilization, $I_{O_2/Nvce} = I_{O_2/Nce}/VI$, considering that mitochondrial respiratory dysfunction in dead cells should be eliminated as a confounding factor.

The complexity changes when the object is a whole organism studied as an experimental model. The scaling law in respiratory physiology reveals a strong interaction between O_2 flow and individual body mass: *basal* metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific O_2 flux, \dot{V}_{O_2max} or \dot{V}_{O_2peak} , is approximately constant across a large range of individual body mass (Weibel and Hoppeler 2005). Individuals, breeds and species, however, deviate substantially from this relationship. \dot{V}_{O_2peak} of human endurance athletes is 60 to 80 mL $O_2 \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ body mass, converted to $J_{O_2peak/Morg}$ of 45 to 60 nmol·s⁻¹·g⁻¹ (Gnaiger 2014; Table 6).

1106 4.2. Size-specific flux: per sample size

1107
1108 **4.2.1. Sample concentration, C_{mX} :** Considering permeabilized tissue, homogenate or cells as the
1109 sample, X , the sample mass is m_X [mg], which is frequently measured as wet or dry weight, W_w or W_d
1110 [mg], respectively, or as amount of protein, m_{Protein} . The sample concentration is the mass of the
1111 subsample per volume of the measurement chamber, $C_{mX} = m_X/V$ [$\text{g}\cdot\text{L}^{-1} = \text{mg}\cdot\text{mL}^{-1}$]. X is the type of
1112 sample—isolated mitochondria, tissue homogenate, permeabilized fibres or cells (**Table 5**).

1113 **4.2.2. Size-specific flux:** Cellular O_2 flow can be compared between cells of identical size. To
1114 take into account changes and differences in cell size, normalization is required to obtain cell size-
1115 specific or mitochondrial marker-specific O_2 flux (Renner *et al.* 2003).

- 1116 • **Mass-specific flux, $J_{\text{O}_2/mX}$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{kg}^{-1}$]:** Mass-specific flux is obtained by expressing
1117 respiration per mass of sample, m_X [mg]. Flow per cell is divided by mass per cell, $J_{\text{O}_2/mce} =$
1118 $I_{\text{O}_2/Nce}/M_{Nce}$. Or chamber volume-specific flux, J_{V,O_2} , is divided by mass concentration of X in
1119 the chamber, $J_{\text{O}_2/mX} = J_{V,\text{O}_2}/C_{mX}$.
- 1120 • **Cell volume-specific flux, $J_{\text{O}_2/VX}$ [$\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-3}$]:** Sample volume-specific flux is obtained by
1121 expressing respiration per volume of sample. For example, in the case of using cells as sample
1122 will be the volume of cells added to the chamber (**Figure 6**).

1123 If size-specific O_2 flux is constant and independent of sample size, then there is no interaction
1124 between the subsystems. For example, a 1.5 mg and a 3.0 mg muscle sample respire at identical mass-
1125 specific flux. Mass-specific O_2 flux, however, may change with the mass of a tissue sample, cells or
1126 isolated mitochondria in the measuring chamber, in which the nature of the interaction becomes an issue.
1127 Therefore, cell density must be optimized, particularly in experiments carried out in wells, considering
1128 the confluency of the cell monolayer or clumps of cells (Salabei *et al.* 2014).

1129 4.3. Marker-specific flux: per mitochondrial content

1130
1131 Tissues can contain multiple cell populations that may have distinct mitochondrial subtypes.
1132 Mitochondria undergo dynamic fission and fusion cycles, and can exist in multiple stages and sizes that
1133 may be altered by a range of factors. The isolation of mitochondria (often achieved through differential
1134 centrifugation) can therefore yield a subsample of the mitochondrial types present in a tissue, depending
1135 on the isolation protocols utilized, *e.g.*, centrifugation speed. This possible bias should be taken into
1136 account when planning experiments using isolated mitochondria. Different sizes of mitochondria are
1137 enriched at specific centrifugation speeds, which can be used strategically for isolation of mitochondrial
1138 subpopulations.

1139 Part of the mitochondrial content of a tissue is lost during preparation of isolated mitochondria.
1140 The fraction of isolated mitochondria obtained from a tissue sample is expressed as mitochondrial
1141 recovery. At a high mitochondrial recovery, the fraction of isolated mitochondria is more representative
1142 of the total mitochondrial population than in preparations characterized by low recovery. Determination
1143 of the mitochondrial recovery and yield is based on measurement of the concentration of a mitochondrial
1144 marker in the stock of isolated mitochondria, $C_{mtE,\text{stock}}$, and crude tissue homogenate, $C_{mtE,\text{thom}}$, which
1145 simultaneously provides information on the specific mitochondrial density in the sample, D_{mtE} (**Table**
1146 **4**).

1147
1148 When discussing concepts of normalization, it is essential to consider the question posed by the
1149 study. If the study aims at comparing tissue performance—such as the effects of a treatment on a specific
1150 tissue, then normalization for tissue mass or protein content is appropriate. However, if the aim is to
1151 find differences in mitochondrial function independent of mitochondrial density (**Table 4**), then
1152 normalization to a mitochondrial marker is imperative (**Figure 6**). One cannot assume that quantitative
1153 changes in various markers—such as mitochondrial proteins—necessarily occur in parallel with one
1154 another. It should be established that the marker chosen is not selectively altered by the performed
1155 treatment. In conclusion, the normalization must reflect the question under investigation to reach a
1156 satisfying answer. On the other hand, the goal of comparing results across projects and institutions
1157 requires standardization on normalization for entry into a databank.

1158 **4.3.1. Mitochondrial concentration, C_{mtE} , and mitochondrial markers:** Mitochondrial
1159 organelles compose a dynamic cellular reticulum in various states of fusion and fission. Hence, the
1160 definition of an ‘amount’ of mitochondria is often misconceived: mitochondria cannot be counted
1161 reliably as a number of occurring elementary components. Therefore, quantification of the amount of

1162 mitochondria depends on the measurement of chosen mitochondrial markers. “Mitochondria are the
 1163 structural and functional elementary units of cell respiration” (Gnaiger 2014). The quantity of a
 1164 mitochondrial marker can reflect the amount of *mitochondrial elementary components*, mtE , expressed
 1165 in various mitochondrial elementary units [mtEU] specific for each measured mt-marker (**Table 4**).
 1166 However, since mitochondrial quality may change in response to stimuli—particularly in mitochondrial
 1167 dysfunction (Campos *et al.* 2017) and after exercise training (Pesta *et al.* 2011) and during aging (Daum
 1168 *et al.* 2013)—some markers can vary while others are unchanged: (1) Mitochondrial volume and
 1169 membrane area are structural markers, whereas mitochondrial protein mass is commonly used as a
 1170 marker for isolated mitochondria. (2) Molecular and enzymatic mitochondrial markers (amounts or
 1171 activities) can be selected as matrix markers, *e.g.*, citrate synthase activity, mtDNA; mtIM-markers, *e.g.*,
 1172 cytochrome *c* oxidase activity, aa_3 content, cardiolipin, or mtOM-markers, *e.g.*, the voltage-dependent
 1173 anion channel (VDAC), TOM20. (3) Extending the measurement of mitochondrial marker enzyme
 1174 activity to mitochondrial pathway capacity, ET- or OXPHOS-capacity can be considered as an
 1175 integrative functional mitochondrial marker.

1176 Depending on the type of mitochondrial marker, the mitochondrial elementary component, mtE ,
 1177 is expressed in marker-specific units. Mitochondrial concentration in the measurement chamber and the
 1178 tissue of origin are quantified as (1) a quantity for normalization in functional analyses, C_{mtE} , and (2) a
 1179 physiological output that is the result of mitochondrial biogenesis and degradation, D_{mtE} , respectively
 1180 (**Table 4**). It is recommended, therefore, to distinguish *experimental mitochondrial concentration*, C_{mtE}
 1181 $= mtE/V$ and *physiological mitochondrial density*, $D_{mtE} = mtE/m_X$. Then mitochondrial density is the
 1182 amount of mitochondrial elementary components per mass of tissue, which is a biological variable
 1183 (**Figure 6**). The experimental variable is mitochondrial density multiplied by sample mass concentration
 1184 in the measuring chamber, $C_{mtE} = D_{mtE} \cdot C_{mX}$, or mitochondrial content multiplied by sample number
 1185 concentration, $C_{mtE} = mtE_X \cdot C_{NX}$ (**Table 4**).

1186 **4.3.2. mt-Marker-specific flux, $J_{O_2/mtE}$:** Volume-specific metabolic O_2 flux depends on: (1) the
 1187 sample concentration in the volume of the instrument chamber, C_{mX} , or C_{NX} ; (2) the mitochondrial
 1188 density in the sample, $D_{mtE} = mtE/m_X$ or $mtE_X = mtE/N_X$; and (3) the specific mitochondrial activity or
 1189 performance per elementary mitochondrial unit, $J_{O_2/mtE} = J_{V,O_2}/C_{mtE}$ [$\text{mol} \cdot \text{s}^{-1} \cdot \text{mtEU}^{-1}$] (**Table 4**).
 1190 Obviously, the numerical results for $J_{O_2/mtE}$ vary with the type of mitochondrial marker chosen for
 1191 measurement of mtE and $C_{mtE} = mtE/V$ [$\text{mtEU} \cdot \text{m}^{-3}$].

1192 Different methods are involved in the quantification of mitochondrial markers and have different
 1193 strengths. Some problems are common for all mitochondrial markers, mtE : (1) Accuracy of
 1194 measurement is crucial, since even a highly accurate and reproducible measurement of O_2 flux results
 1195 in an inaccurate and noisy expression if normalized by a biased and noisy measurement of a
 1196 mitochondrial marker. This problem is acute in mitochondrial respiration because the denominators used
 1197 (the mitochondrial markers) are often small moieties of which accurate and precise determination is
 1198 difficult. This problem can be avoided when O_2 fluxes measured in substrate-uncoupler-inhibitor
 1199 titration protocols are normalized for flux in a defined respiratory reference state, which is used as an
 1200 *internal* marker and yields flux control ratios, *FCRs*. *FCRs* are independent of externally measured
 1201 markers and, therefore, are statistically robust, considering the limitations of ratios in general (Jasienski
 1202 and Bazzaz 1999). *FCRs* indicate qualitative changes of mitochondrial respiratory control, with highest
 1203 quantitative resolution, separating the effect of mitochondrial density or concentration on $J_{O_2/mX}$ and
 1204 $I_{O_2/NX}$ from that of function per elementary mitochondrial marker, $J_{O_2/mtE}$ (Pesta *et al.* 2011; Gnaiger
 1205 2014). (2) If mitochondrial quality does not change and only the amount of mitochondria varies as a
 1206 determinant of mass-specific flux, any marker is equally qualified in principle; then in practice selection
 1207 of the optimum marker depends only on the accuracy and precision of measurement of the mitochondrial
 1208 marker. (3) If mitochondrial flux control ratios change, then there may not be any best mitochondrial
 1209 marker. In general, measurement of multiple mitochondrial markers enables a comparison and
 1210 evaluation of normalization for these mitochondrial markers. Particularly during postnatal development,
 1211 the activity of marker enzymes—such as cytochrome *c* oxidase and citrate synthase—follows different
 1212 time courses (Drahota *et al.* 2004). Evaluation of mitochondrial markers in healthy controls is
 1213 insufficient for providing guidelines for application in the diagnosis of pathological states and specific
 1214 treatments.

1215 In line with the concept of the respiratory control ratio (Chance and Williams 1955a), the most
 1216 readily used normalization is that of flux control ratios and flux control factors (Gnaiger 2014). Selection
 1217 of the state of maximum flux in a protocol as the reference state has the advantages of: (1) internal

1218 normalization; (2) statistically validated linearization of the response in the range of 0 to 1; and (3)
 1219 consideration of maximum flux for integrating a large number of elementary steps in the OXPHOS- or
 1220 ET-pathways. This reduces the risk of selecting a functional marker that is specifically altered by the
 1221 treatment or pathology, yet increases the chance that the highly integrative pathway is disproportionately
 1222 affected, *e.g.*, the OXPHOS- rather than ET-pathway in case of an enzymatic defect in the
 1223 phosphorylation-pathway. In this case, additional information can be obtained by reporting flux control
 1224 ratios based on a reference state that indicates stable tissue-mass specific flux.

1225 Stereological determination of mitochondrial content via two-dimensional transmission electron
 1226 microscopy can have limitations due to the dynamics of mitochondrial size (Meinild Lundby *et al.*
 1227 2017). Accurate determination of three-dimensional volume by two-dimensional microscopy can be
 1228 both time consuming and statistically challenging (Larsen *et al.* 2012).

1229 The validity of using mitochondrial marker enzymes (citrate synthase activity, CI to CIV amount
 1230 or activity) for normalization of flux is limited in part by the same factors that apply to flux control
 1231 ratios. Strong correlations between various mitochondrial markers and citrate synthase activity
 1232 (Reichmann *et al.* 1985; Boushel *et al.* 2007; Mogensen *et al.* 2007) are expected in a specific tissue of
 1233 healthy persons and in disease states not specifically targeting citrate synthase. Citrate synthase activity
 1234 is acutely modifiable by exercise (Tonkonogi *et al.* 1997; Leek *et al.* 2001). Evaluation of mitochondrial
 1235 markers related to a selected age and sex cohort cannot be extrapolated to provide recommendations for
 1236 normalization in respirometric diagnosis of disease, in different states of development and ageing,
 1237 different cell types, tissues, and species. mtDNA normalized to nDNA via qPCR is correlated to
 1238 functional mitochondrial markers including OXPHOS- and ET-capacity in some cases (Puntschart *et al.*
 1239 1995; Wang *et al.* 1999; Menshikova *et al.* 2006; Boushel *et al.* 2007; Ehinger *et al.* 2015), but lack of
 1240 such correlations have been reported (Menshikova *et al.* 2005; Schultz and Wiesner 2000; Pesta *et al.*
 1241 2011). Several studies indicate a strong correlation between cardiolipin content and increase in
 1242 mitochondrial function with exercise (Menshikova *et al.* 2005; Menshikova *et al.* 2007; Larsen *et al.*
 1243 2012; Faber *et al.* 2014), but it has not been evaluated as a general mitochondrial biomarker in disease.
 1244 With no single best mitochondrial marker, a good strategy is to quantify several different biomarkers to
 1245 minimize the decorrelating effects caused by diseases, treatments, or other factors. Determination of
 1246 multiple markers, particularly a matrix marker and a marker from the mtIM, allows tracking changes in
 1247 mitochondrial quality defined by their ratio.

1250 5. Normalization of rate per system

1251 5.1. Flow: per chamber

1252 The experimental system (experimental chamber) is part of the measurement instrument,
 1253 separated from the environment as an isolated, closed, open, isothermal or non-isothermal system
 1254 (Table 4). Reporting O₂ flows per respiratory chamber, I_{O_2} [nmol·s⁻¹], restricts the analysis to intra-
 1255 experimental comparison of relative differences.

1256 5.2. Flux: per chamber volume

1257 **5.2.1. System-specific flux, J_{V,O_2} :** We distinguish between (1) the *system* with volume V and mass
 1258 m defined by the system boundaries, and (2) the *sample* or *objects* with volume V_X and mass m_X that are
 1259 enclosed in the experimental chamber (Figure 6). Metabolic O₂ flow per object, I_{O_2/N_X} , is the total O₂
 1260 flow in the system divided by the number of objects, N_X , in the system. I_{O_2/N_X} increases as the mass of
 1261 the object is increased. Sample mass-specific O₂ flux, J_{O_2/m_X} should be independent of the mass of the
 1262 sample studied in the instrument chamber, but system volume-specific O₂ flux, J_{V,O_2} (per volume of the
 1263 instrument chamber), increases in proportion to the mass of the sample in the chamber. Although J_{V,O_2}
 1264 depends on mass-concentration of the sample in the chamber, it should be independent of the chamber
 1265 (system) volume at constant sample mass-concentration. There are practical limitations to increasing the
 1266 mass-concentration of the sample in the chamber, when one is concerned about crowding effects and
 1267 instrumental time resolution.

1268 **5.2.2. Advancement per volume:** When the reactor volume does not change during the reaction,
 1269 which is typical for liquid phase reactions, the volume-specific flux of a chemical reaction r is the time
 1270
 1271
 1272
 1273

1274 derivative of the advancement of the reaction per unit volume, $J_{V,rB} = d_r \xi_B / dt \cdot V^{-1}$ [(mol·s⁻¹)·L⁻¹]. The *rate*
 1275 *of concentration change* is dc_B/dt [(mol·L⁻¹)·s⁻¹], where concentration is $c_B = n_B/V$. There is a difference
 1276 between (1) J_{V,rO_2} [mol·s⁻¹·L⁻¹] and (2) rate of concentration change [mol·L⁻¹·s⁻¹]. These merge into a
 1277 single expression only in closed systems. In open systems, internal transformations (catabolic flux, O₂
 1278 consumption) are distinguished from external flux (such as O₂ supply). External fluxes of all substances
 1279 are zero in closed systems. In a closed chamber O₂ consumption (internal flux of catabolic reactions k ;
 1280 I_{kO_2} [pmol·s⁻¹]) causes a decline in the amount of O₂ in the system, n_{O_2} [nmol]. Normalization of these
 1281 quantities for the volume of the system, V [L \equiv dm³], yields volume-specific O₂ flux, $J_{V,kO_2} = I_{kO_2}/V$
 1282 [nmol·s⁻¹·L⁻¹], and O₂ concentration, [O₂] or $c_{O_2} = n_{O_2}/V$ [μ mol·L⁻¹ = μ M = nmol·mL⁻¹]. Instrumental
 1283 background O₂ flux is due to external flux into a non-ideal closed respirometer, so total volume-specific
 1284 flux has to be corrected for instrumental background O₂ flux—O₂ diffusion into or out of the
 1285 instrumental chamber. J_{V,kO_2} is relevant mainly for methodological reasons and should be compared with
 1286 the accuracy of instrumental resolution of background-corrected flux, *e.g.*, ± 1 nmol·s⁻¹·L⁻¹ (Gnaiger
 1287 2001). ‘Catabolic’ indicates O₂ flux, J_{kO_2} , corrected for: (1) instrumental background O₂ flux; (2)
 1288 chemical background O₂ flux due to autoxidation of chemical components added to the incubation
 1289 medium; and (3) *Rox* for O₂-consuming side reactions unrelated to the catabolic pathway k .

1290
1291

1292 6. Conversion of units

1293

1294 Many different units have been used to report the O₂ consumption rate, OCR (Table 6). SI base
 1295 units provide the common reference to introduce the theoretical principles (Figure 6), and are used with
 1296 appropriately chosen SI prefixes to express numerical data in the most practical format, with an effort
 1297 towards unification within specific areas of application (Table 7). Reporting data in SI units—including
 1298 the mole [mol], coulomb [C], joule [J], and second [s]—should be encouraged, particularly by journals
 1299 that propose the use of SI units.

1300

1301 **Table 6. Conversion of various formats and units used in respirometry and**
 1302 **ergometry.** e^- is the number of electrons or reducing equivalents. z_B is the charge number
 1303 of entity B.
 1304

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

1305 1 At standard temperature and pressure dry (STPD: 0 °C = 273.15 K and 1 atm = 101.325 kPa =
 1306 760 mmHg), the molar volume of an ideal gas, V_m , and V_{m,O_2} is 22.414 and 22.392 L·mol⁻¹,
 1307 respectively. Rounded to three decimal places, both values yield the conversion factor of 0.744.
 1308 For comparison at normal temperature and pressure dry (NTPD: 20 °C), V_{m,O_2} is 24.038 L·mol⁻¹.
 1309 Note that the SI standard pressure is 100 kPa.

1310 2 The multiplication factor is $10^6/(z_B \cdot F)$.

1311 3 The multiplication factor is $z_B \cdot F/10^6$.

1312

1313 **Table 7. Conversion of units with preservation of numerical values.**

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

1314 1 pmol: picomole = 10^{-12} mol1315 2 amol: attomole = 10^{-18} mol1316 3 zmol: zeptomole = 10^{-21} mol

1317

1318 Although volume is expressed as m^3 using the SI base unit, the litre [dm^3] is a conventional unit
 1319 of volume for concentration and is used for most solution chemical kinetics. If one multiplies $I_{O_2/Nce}$ by
 1320 C_{Nce} , then the result will not only be the amount of O_2 [mol] consumed per time [s^{-1}] in one litre [L^{-1}],
 1321 but also the change in O_2 concentration per second (for any volume of an ideally closed system). This
 1322 is ideal for kinetic modeling as it blends with chemical rate equations where concentrations are typically
 1323 expressed in $\text{mol}\cdot\text{L}^{-1}$ (Wagner *et al.* 2011). In studies of multinuclear cells—such as differentiated
 1324 skeletal muscle cells—it is easy to determine the number of nuclei but not the total number of cells. A
 1325 generalized concept, therefore, is obtained by substituting cells by nuclei as the sample entity. This does
 1326 not hold, however, for non-nucleated platelets.

1327 For studies of cells, we recommend that respiration be expressed, as far as possible, as: (1) O_2
 1328 flux normalized for a mitochondrial marker, for separation of the effects of mitochondrial quality and
 1329 content on cell respiration (this includes $FCRs$ as a normalization for a functional mitochondrial
 1330 marker); (2) O_2 flux in units of cell volume or mass, for comparison of respiration of cells with different
 1331 cell size (Renner *et al.* 2003) and with studies on tissue preparations, and (3) O_2 flow in units of attomole
 1332 (10^{-18} mol) of O_2 consumed per second by each cell [$\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$], numerically equivalent to
 1333 [$\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6}$ cells]. This convention allows information to be easily used when designing experiments
 1334 in which O_2 flow must be considered. For example, to estimate the volume-specific O_2 flux in an
 1335 instrument chamber that would be expected at a particular cell number concentration, one simply needs
 1336 to multiply the flow per cell by the number of cells per volume of interest. This provides the amount of
 1337 O_2 [mol] consumed per time [s^{-1}] per unit volume [L^{-1}]. At an O_2 flow of 100 $\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$ and a cell
 1338 density of 10^9 cells $\cdot\text{L}^{-1}$ (10^6 cells $\cdot\text{mL}^{-1}$), the volume-specific O_2 flux is 100 $\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}$ (100
 1339 $\text{pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$).

1340 ET-capacity in human cell types including HEK 293, primary HUVEC, and fibroblasts ranges
 1341 from 50 to 180 $\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$, measured in intact cells in the noncoupled state (see Gnaiger 2014). At
 1342 100 $\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$ corrected for Rox , the current across the mt-membranes, I_{H^+e} , approximates 193
 1343 $\text{pA}\cdot\text{cell}^{-1}$ or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the
 1344 molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a
 1345 catabolic power of -110 W. Modelling approaches illustrate the link between protonmotive force and
 1346 currents (Willis *et al.* 2016).

1347 We consider isolated mitochondria as powerhouses and proton pumps as molecular machines to
 1348 relate experimental results to energy metabolism of the intact cell. The cellular P_{\gg}/O_2 based on oxidation

of glycogen is increased by the glycolytic (fermentative) substrate-level phosphorylation of 3 P_»/Glyc or 0.5 mol P_» for each mol O₂ consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial P_»/O₂ ratio of 5.4 yields a bioenergetic cell physiological P_»/O₂ ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either by the malate-aspartate shuttle or by the glycerophosphate shuttle (**Figure 2A**) resulting in different theoretical yields of ATP generated by mitochondria, the energetic cost of which potentially must be taken into account. Considering also substrate-level phosphorylation in the TCA cycle, this high P_»/O₂ ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993a).

7. Conclusions

Catabolic cell respiration is the process of exergonic and exothermic energy transformation in which scalar redox reactions are coupled to vectorial ion translocation across a semipermeable membrane, which separates the small volume of a bacterial cell or mitochondrion from the larger volume of its surroundings. The electrochemical exergy can be partially conserved in the phosphorylation of ADP to ATP or in ion pumping, or dissipated in an electrochemical short-circuit. Respiration is thus clearly distinguished from fermentation as the counterpart of cellular core energy metabolism. An O₂ flux balance scheme illustrates the relationships and general definitions (**Figures 1 and 2**).

Box 3: Recommendations for studies with mitochondrial preparations

- Normalization of respiratory rates should be provided as far as possible:
 1. *Biophysical normalization*: on a per cell basis as O₂ flow; this may not be possible when dealing with coenocytic organisms, *e.g.*, filamentous fungi, or tissues without cross-walls separating individual cells, *e.g.*, muscle fibers.
 2. *Cellular normalization*: per g protein; per cell- or tissue-mass as mass-specific O₂ flux; per cell volume as cell volume-specific flux.
 3. *Mitochondrial normalization*: per mitochondrial marker as mt-specific flux.
- With information on cell size and the use of multiple normalizations, maximum potential information is available (Renner *et al.* 2003; Wagner *et al.* 2011; Gnaiger 2014). Reporting flow in a respiratory chamber [nmol·s⁻¹] is discouraged, since it restricts the analysis to intra-experimental comparison of relative (qualitative) differences.
- Catabolic mitochondrial respiration is distinguished from residual O₂ consumption. Fluxes in mitochondrial coupling states should be, as far as possible, corrected for residual O₂ consumption.
- Different mechanisms of uncoupling should be distinguished by defined terms. The tightness of coupling relates to these uncoupling mechanisms, whereas the coupling stoichiometry varies as a function the substrate type involved in ET-pathways with either three or two redox proton pumps operating in series. Separation of tightness of coupling from the pathway-dependent coupling stoichiometry is possible only when the substrate type undergoing oxidation remains the same for respiration in LEAK-, OXPHOS-, and ET-states. In studies of the tightness of coupling, therefore, simple substrate-inhibitor combinations should be applied to exclude a shift in substrate competition that may occur when providing physiological substrate cocktails.
- In studies of isolated mitochondria, the mitochondrial recovery and yield should be reported. Experimental criteria such as transmission electron microscopy for evaluation of purity versus integrity should be considered. Mitochondrial markers—such as citrate synthase activity as an enzymatic matrix marker—provide a link to the tissue of origin on the basis of calculating the mitochondrial recovery, *i.e.*, the fraction of mitochondrial marker obtained from a unit mass of tissue. Total mitochondrial protein is frequently applied as a mitochondrial marker, which is restricted to isolated mitochondria.
- In studies of permeabilized cells, the viability of the cell culture or cell suspension of origin should be reported. Normalization should be evaluated for total cell count or viable cell count.
- Terms and symbols are summarized in **Table 8**. Their use will facilitate transdisciplinary communication and support further development of a consistent theory of bioenergetics and mitochondrial physiology. Technical terms related to and defined with normal words can be used as

1405 index terms in databases, support the creation of ontologies towards semantic information processing
 1406 (MitoPedia), and help in communicating analytical findings as impactful data-driven stories.
 1407 ‘Making data available without making it understandable may be worse than not making it available
 1408 at all’ (National Academies of Sciences, Engineering, and Medicine 2018). Success will depend on
 1409 taking further steps: (1) exhaustive text-mining considering Omics data and functional data; (2)
 1410 network analysis of Omics data with bioinformatics tools; (3) cross-validation with distinct
 1411 bioinformatics approaches; (4) correlation with functional data; (5) guidelines for biological
 1412 validation of network data. This is a call to carefully contribute to FAIR principles (Findable,
 1413 Accessible, Interoperable, Reusable) for the sharing of scientific data.

1415
 1416 **Table 8. Terms, symbols, and units.**

1417 1418 1419 1420	Term	Symbol	Unit	Links and comments
1421	1422 alternative quinol oxidase	AOX		Figure 2B
1423	adenosine monophosphate	AMP		2 ADP ↔ ATP+AMP
1424	adenosine diphosphate	ADP		Table 1, Figures 2 and 5
1425	adenosine triphosphate	ATP		Figures 2 and 5
1426	adenylates	AMP, ADP, ATP		Section 2.5.1
1427	amount of substance B	n_B	[mol]	
1428	ATP yield per O ₂	$Y_{P\gg/O_2}$		P _» /O ₂ ratio measured in any respiratory state
1429				
1430	catabolic reaction	k		Figures 1 and 3
1431	catabolic respiration	J_{kO_2}	<i>varies</i>	Figures 1 and 3
1432	cell number	N_{ce}	[x]	$N_{ce} = N_{vce} + N_{dce}$; Table 5
1433	cell respiration	J_{rO_2}	<i>varies</i>	Figure 1
1434	cell viability index	VI		$VI = N_{vce}/N_{ce} = 1 - N_{dce}/N_{ce}$
1435	charge number of entity B	z_B		Table 6; $z_{O_2} = 4$
1436	Complexes I to IV	CI to CIV		respiratory ET Complexes; Figure 2B
1437				
1438	concentration of substance B	$c_B = n_B \cdot V^{-1}$; [B]	[mol·m ⁻³]	Box 2
1439	coupling control state	CCS		Section 2.4.1
1440	dead cell number	N_{dce}	[x]	non-viable cells, loss of plasma membrane barrier function; Table 5
1441				
1442	electric format	e	[C]	Table 6
1443	electron transfer system	ETS		state; Figures 2B and 4
1444	ET state	ET		Table 1, Figures 2B and 4; State 3u
1445	ET-capacity	E	<i>varies</i>	Table 1, Figure 4
1446	flow, for substance B	I_B	[mol·s ⁻¹]	system-related extensive quantity; Figure 6
1447				
1448	flux, for substance B	J_B	<i>varies</i>	size-specific quantity; Figure 6
1449	inorganic phosphate	P _i		Figure 2C
1450	inorganic phosphate carrier	PiC		Figure 2C
1451	intact cell number,			
1452	viable cell number	N_{vce}	[x]	viable cells, intact plasma membrane barrier function; Table 5
1453				
1454	LEAK state	LEAK		state; Table 1, Figure 4; compare State 4
1455				
1456	LEAK-respiration	L	<i>varies</i>	Table 1; Figure 4
1457	mass format	m	[kg]	Table 4, Figure 6
1458	mass of sample X	m_X	[kg]	Table 4
1459	mass, dry mass	m_d	[kg]	mass of sample X; Figure 6 (frequently called dry weight)
1460				
1461	mass, wet mass	m_w	[kg]	mass of sample X; Figure 6 (frequently called wet weight)
1462				

1463	mass of object X	$M_X = m_X \cdot N_X^{-1}$	$[\text{kg} \cdot \text{x}^{-1}]$	mass of entity X ; Table 4
1464	MITOCARTA			https://www.broadinstitute.org/scientific-community/science/programs/metabolic-disease-program/publications/mitocarta/mitocarta-in-0
1465				
1466				
1467				
1468				
1469	MitoPedia			http://www.bioblast.at/index.php/MitoPedia
1470	mitochondria or mitochondrial	mt		Box 1
1471	mitochondrial DNA	mtDNA		Box 1
1472	mitochondrial concentration	$C_{mtE} = mtE \cdot V^{-1}$	$[\text{mtEU} \cdot \text{m}^{-3}]$	Table 4
1473	mitochondrial content	mtE_X	$[\text{mtEU} \cdot \text{x}^{-1}]$	$mtE_X = mtE \cdot N_X^{-1}$; Table 4
1474	mitochondrial			
1475	elementary component	mtE	$[\text{mtEU}]$	quantity of mt-marker; Table 4
1476	mitochondrial elementary unit	mtEU	<i>varies</i>	specific units for mt-marker; Table 4
1477	mitochondrial inner membrane	mtIM		MIM is widely used; the first M is replaced by mt; Figure 2; Box 1
1478				MOM is widely used; the first M is replaced by mt; Figure 2; Box 1
1479	mitochondrial outer membrane	mtOM		
1480				
1481	mitochondrial recovery	Y_{mtE}		fraction of mtE recovered in sample from the tissue of origin
1482				
1483	mitochondrial yield	$Y_{mtE/m}$		mt-yield per tissues mass; $Y_{mtE/m} = Y_{mtE} \cdot D_{mtE}$
1484				
1485	molar format	\underline{n}	$[\text{mol}]$	Table 6
1486	negative	neg		Figure 4
1487	number concentration of X	C_{NX}	$[\text{x} \cdot \text{m}^{-3}]$	Table 4
1488	number format	\underline{N}	$[\text{x}]$	Table 4, Figure 6
1489	number of entities X	N_X	$[\text{x}]$	Table 4, Figure 6
1490	number of entity B	N_B	$[\text{x}]$	Table 4
1491	oxidative phosphorylation	OXPHOS		state; Table 1, Figure 4
1492	OXPHOS state	OXPHOS		Table 1; State 3 if $[\text{ADP}]$ and $[\text{P}_i]$ are saturating
1493				
1494	OXPHOS-capacity	P	<i>varies</i>	Table 1, Figure 4
1495	oxygen concentration	$c_{\text{O}_2} = n_{\text{O}_2} \cdot V^{-1}$	$[\text{mol} \cdot \text{m}^{-3}]$	$[\text{O}_2]$; Section 3.2
1496	oxygen flux, in reaction r	$J_{r\text{O}_2}$	<i>varies</i>	Figure 1
1497	pathway control state	PCS		Section 2.2
1498	permeabilized cell number	N_{pce}	$[\text{x}]$	experimental permeabilization of plasma membrane; Table 5
1499				
1500	phosphorylation of ADP to ATP	P_{\gg}		Section 2.2
1501	P_{\gg}/O_2 ratio	P_{\gg}/O_2		mechanistic Y_{P_{\gg}/O_2} , calculated from pump stoichiometries; Figure 2B
1502				
1503	positive	pos		Figure 4
1504	proton in the negative compartment	H^+_{neg}		Figure 4
1505	proton in the positive compartment	H^+_{pos}		Figure 4
1506	protonmotive force	pmf	$[\text{V}]$	Figures 1, 2A and 4; Table 1
1507	rate of electron transfer in ET state	E	<i>varies</i>	ET-capacity; Table 1
1508	rate of LEAK-respiration	L	<i>varies</i>	Table 1
1509	rate of oxidative phosphorylation	P	<i>varies</i>	OXPHOS-capacity; Table 1
1510	rate of residual oxygen consumption	R_{ox}		Table 1, Figure 1
1511	residual oxygen consumption	ROX		state; Table 1
1512	respiratory supercomplex	$\text{SC I}_n\text{III}_n\text{IV}_n$		supramolecular assemblies composed of variable copy numbers (n) of CI, CIII and CIV; Box 1
1513				
1514				
1515	specific mitochondrial density	$D_{mtE} = mtE \cdot m_X^{-1}$	$[\text{mtEU} \cdot \text{kg}^{-1}]$	Table 4
1516	substrate-uncoupler-inhibitor-titration protocol	SUIT		Section 2.2
1517				
1518	volume	V	$[\text{m}^{-3}]$	Table 7

1520
1521
1522 Experimentally, respiration is separated in mitochondrial preparations from the interactions with
1523 the fermentative pathways of the intact cell. OXPHOS analysis is based on the study of mitochondrial
1524 preparations complementary to bioenergetic investigations of intact cells and organisms—from model
1525 organisms to the human species including healthy and diseased persons (patients). Different mechanisms
1526 of respiratory uncoupling have to be distinguished (**Figure 3**). Metabolic fluxes measured in defined
1527 coupling and pathway control states (**Figures 5 and 6**) provide insights into the meaning of cellular and
1528 organismic respiration.

1529 The optimal choice for expressing mitochondrial and cell respiration as O₂ flow per biological
1530 sample, and normalization for specific tissue-markers (volume, mass, protein) and mitochondrial
1531 markers (volume, protein, content, mtDNA, activity of marker enzymes, respiratory reference state) is
1532 guided by the scientific question under study. Interpretation of the data depends critically on appropriate
1533 normalization (**Figure 6**).

1534 MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory adaptations and
1535 defects linked to genetic variation, age-related health risks, sex-specific mitochondrial performance,
1536 lifestyle with its effects on degenerative diseases, and thermal and chemical environment. The present
1537 recommendations on coupling control states and rates, linked to the concept of the protonmotive force,
1538 are focused on studies using mitochondrial preparations (**Box 3**). These will be extended in a series of
1539 reports on pathway control of mitochondrial respiration, respiratory states in intact cells, and
1540 harmonization of experimental procedures.

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1546
1547
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1549

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1833 Supplement

1834 S1. Manuscript phases and versions - an open-access approach

1835 This manuscript on ‘Mitochondrial respiratory states and rates’ is a position statement in the frame of COST Action
 1836 CA15203 MitoEAGLE. The global MitoEAGLE network made it possible to collaborate with a large number of
 1837 coauthors to reach consensus on the present manuscript. Nevertheless, we do not consider scientific progress to be
 1838 supported by ‘declaration’ statements (other than on ethical or political issues). Our manuscript aims at providing
 1839 arguments for further debate rather than pushing opinions. We hope to initiate a much broader process of
 1840 discussion and want to raise the awareness of the importance of a consistent terminology for reporting of scientific
 1841 data in the field of bioenergetics, mitochondrial physiology and pathology. Quality of research requires quality of
 1842 communication. Some established researchers in the field may not want to re-consider the use of jargon which has
 1843 become established despite deficiencies of accuracy and meaning. In the long run, superior standards will become
 1844 accepted. We hope to contribute to this evolutionary process, with an emphasis on harmonization rather than
 1845 standardization.

1846 *Phase 1* The protonmotive force and respiratory control

1847 http://www.mitoeagle.org/index.php/The_protonmotive_force_and_respiratory_control

- 1848 • 2017-04-09 to 2017-09-18 (44 versions)
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1852 *Phase 2* Mitochondrial respiratory states and rates: Building blocks of mitochondrial physiology Part 1

1853 http://www.mitoeagle.org/index.php/MitoEAGLE_preprint_2018-02-08

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1855 *Phase 3* Mitochondrial respiratory states and rates. Submission to a preprint server: [BioRxiv](https://www.biorxiv.org/)

1856 *Phase 4* Journal submission: CELL METABOLISM, aiming at indexing by *The Web of Science* and *PubMed*.

1860 S2. Authors

1861 This manuscript developed as an open invitation to scientists and students to join as coauthors in the bottom-up
 1862 spirit of COST, to provide a balanced view of mitochondrial respiratory control and a consensus statement on
 1863 reporting data of mitochondrial respiration in terms of metabolic flows and fluxes.

1864 Coauthors are added in alphabetical order based upon a first draft written by the corresponding author, who
 1865 edited all versions. *Coauthors confirm that they have read the final manuscript, possibly have made additions or*
 1866 *suggestions for improvement, and agree to implement the recommendations into future manuscripts, presentations*
 1867 *and teaching materials.*

1868 We continue to invite comments and suggestions, particularly if you are an early career investigator adding
 1869 an open future-oriented perspective, or an established scientist providing a balanced historical basis. Your critical
 1870 input into the quality of the manuscript will be most welcome, improving our aims to be educational, general,
 1871 consensus-oriented, and in practice be helpful to students working in mitochondrial respiratory physiology.

1872 To join as a coauthor, please feel free to focus on a particular section, providing direct input and references,
 1873 and contributing to the scope of the manuscript from the perspective of your expertise. Your comments will be
 1874 considered as appropriate in the manuscript and will be largely posted on the discussion page of the MitoEAGLE
 1875 preprint website.
 1876
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1878 S3. Joining COST Actions

- 1879 • CA15203 MitoEAGLE - http://www.cost.eu/COST_Actions/ca/CA15203
- 1880 • CA16225 EU-CARDIOPROTECTION - http://www.cost.eu/COST_Actions/ca/CA16225
- 1881 • CA17129 CardioRNA - http://www.cost.eu/COST_Actions/ca/CA17129



Mitochondrial respiratory states and rates:



Building blocks of mitochondrial physiology

Part 1 - www.mitoeagle.org/index.php/MitoEAGLE_preprint_2018-02-08

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Aims Clarity of concept and consistency of nomenclature facilitate effective transdisciplinary communication, education, and ultimately further discovery.

Adhering to uniform standards and harmonizing the terminology concerning mitochondrial respiratory states and rates will support the development of databases of mitochondrial respiratory function in cells, tissues, and species.

Summary Recommendations on coupling control states and rates are focused on studies with mitochondrial preparations.

Fig. 1: Respiration is defined by O₂ flux balance.

Fig. 2: OXPHOS analysis is based on the study of mt- preparations. Metabolic fluxes measured in defined coupling and pathway control states provide insights into the meaning of cellular respiration.

Fig. 3: Interpretation of respiratory rates depends critically on appropriate normalization.

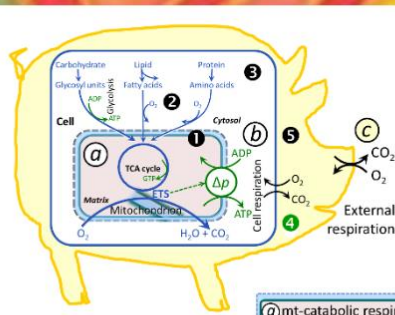
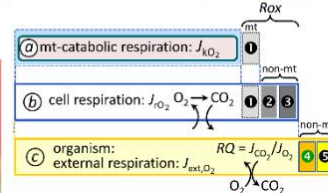


Figure 1. From mitochondrial to external respiration

Mitochondrial (mt) respiration is the oxidation of fuel substrates (electron donors) and reduction of O₂ catalysed by the electron transfer system, ETS:

- (a) mt-catabolic respiration, excluding
- (b) mt-residual oxygen consumption, *Rox*.
- (c) Total cellular O₂ consumption, including mt-*Rox*,
- (d) non-mt catabolic *Rox*, particularly by peroxisomal oxidases, and
- (e) non-mt *Rox* unrelated to catabolism.



(c) External respiration, including aerobic microbial respiration, and extracellular O₂ consumption.

MIPart by Odra Noel

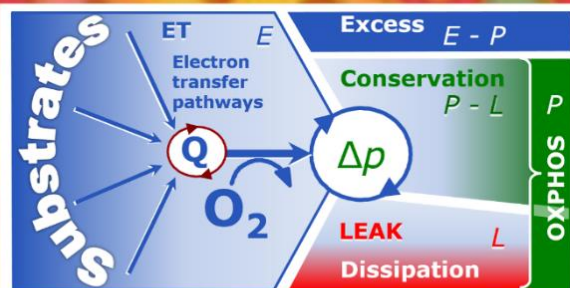


Figure 2. Respiratory states (ET, OXPHOS, LEAK) and corresponding rates (E, P, L)

Net OXPHOS-capacity, *P-L*, and excess capacity, *E-P*.

Table 1. Coupling states and residual oxygen consumption in mitochondrial preparations in relation to respiration- and phosphorylation-flux, *J_{ko}*, and *J_p*, and protonmotive force, Δp . Coupling states are established at kinetically-saturating concentrations of fuel substrates and O₂.

State	<i>J_{ko}</i>	<i>J_p</i>	Δp	Inducing factors	Limiting factors
LEAK	<i>L</i> ; low, cation leak-dependent respiration	0	max.	proton leak, slip, and cation cycling	<i>J_p</i> = 0: (1) without ADP, <i>L_{sc}</i> ; (2) max. ATP/ADP ratio, <i>L_r</i> ; or (3) inhibition of the phosphorylation-pathway, <i>L_{only}</i>
OXPHOS	<i>P</i> ; high, ADP-stimulated respiration	max.	high	kinetically-saturating [ADP] and [P _i]	<i>J_p</i> , by phosphorylation-pathway; or <i>J_{ko}</i> , by ET-capacity
ET	<i>E</i> ; max., noncoupled respiration	0	low	optimal external uncoupler concentration for max. <i>J_{o,e}</i>	<i>J_{ko}</i> , by ET-capacity
ROX	<i>Rox</i> ; min., residual O ₂ consumption	0	0	<i>J_{o,leak}</i> in non-ET-pathway oxidation reactions	inhibition of all ET-pathways; or absence of fuel substrates

Figure 3. Normalization of rate

A: Cell respiration is normalized for (1) the experimental **Sample** (flow per object, mass-specific flux, or cell-volume-specific flux); or (2) for methodological reasons for the **Chamber** volume.

B: Flow per cell [amol O₂ s⁻¹ cell⁻¹] is flux per chamber volume, *J_v* [nmol O₂ s⁻¹ L⁻¹], divided by cell concentration in the chamber, *N_{ce}*/V [cells L⁻¹], which is **Number** analysis. In **Structure** analysis, aerobic cell performance is mt-quality (mt-specific flux, e.g., per citrate synthase, CS) times mt-quantity, or mt-function times mt-structure.



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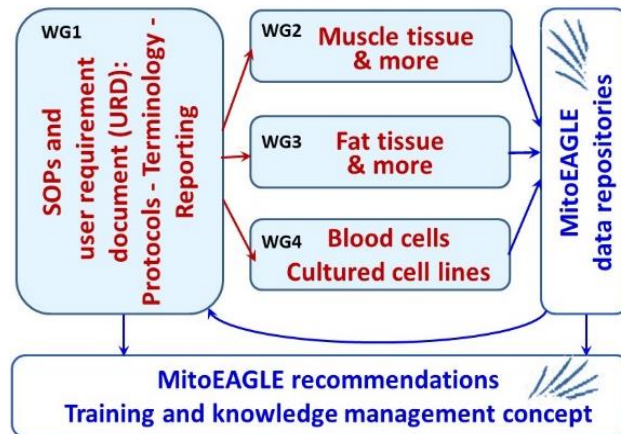
Evolution Age Gender
Lifestyle Environment



Mission of the global MitoEAGLE network

in collaboration with the Mitochondrial Physiology Society, MiPs

- Improve our knowledge on mitochondrial function in health and disease with regard to Evolution, Age, Gender, Lifestyle and Environment
- Interrelate studies across laboratories with the help of a MitoEAGLE data management system
- Provide standardized measures to link mitochondrial and physiological performance to understand the myriad of factors that play a role in mitochondrial physiology



Join the COST Action MitoEAGLE - contribute to the quality management network.



More information:
www.mitoeagle.org



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