Comments regarding the position paper: Mitochondrial respiratory control and the protonmotive force: a conceptual perspective on coupling states in mitochondrial preparations. MitoEAGLE recommendations Part 1

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Comments drafted by Dr Ehinger, edited and approved by Dr Elmér

General comments:
A very good initiative to harmonize terminology in a complex field, and the manuscript is an excellent starting point. Well done! We appreciate the massive wotk that clearly have been put into this very qualitative product.

To reach the goal of widespread dissipation, we think a few fundamental principles are necessary

- Uncomplicated – It is critical to make the article as accessible as possible
- Equipment independent – All definitions should be fully compatible with a HansaTech electrode, an O2k, a Seahorse or any other similar equipment
- Non-political – the paper should not be used to push a position of an organization or individual

To this end, we have made a few suggestions below. We have mainly focused on respirometry, as that is our particular area of expertise. We suggest that the paper is shortened substantially, and that more complex reasoning are included in an appendix, or as supplementary material. By doing this, the concept has a chance of reaching outside of the sphere of hardcore mito-researchers, otherwise much of this work is for nothing. Lengthy discussions on specific experimental protocols should not be included.

Specific comments

Abstract:
Possibly the abstract is focusing too much on details regarding experimental condition and related limitations, most strikingly the wording about saturating levels of ADP and P. This is very relevant, but as I see it, it's not a key message with the publication.
Does OXPHOS at present actually have "diagnostic reference values". Defined by whom and for what conditions?

Fig 1:
In Fig 1A, the focus is heavily weighted towards carbohydrate metabolism, but omits e.g. fatty acid oxidation etc. Are the "orphan arrows" pointing towards the Q-junction meant to represent fatty acid metabolism and other sources of electrons? I think it would be wise to preferable include also these fluxes, or at least acknowledging their existence in a clearer way. Fig 1B, the different shuttles and complexes in the membrane should be named

2.1:
Possibly the section about **mitochondrial preparations** should include a sentence about limitations with isolated mitochondria and permeabilized cells and fibers. After all, cytosolic factors are dramatically diluted and many cell functions are hampered.

In the last paragraph under **mitochondrial preparations**, the wording “The corresponding state is characterized by high levels of oxygen consumption without control by phosphorylation” could be somewhat misleading, as it could indicate that phosphorylation controls oxygen consumption which is somewhat inaccurate. Maybe “The corresponding state is characterized by high levels of oxygen consumption without *coupling* to phosphorylation”? Also the parenthesis (´uncontrolled state´) is a little ambiguous. Is that the suggested terminology? If not, remove.
The sentences about three coupling sites in the paragraph bridging page 7 and 8 are difficult to understand.

We understand that the concept of EAGLE may be necessary to include as this paper is drafted in the context of this specific collaboration, but it does make the external validity of the manuscript lower, and will after the project is concluded make the paper “dated”. From our perspective, the paper would benefit from focusing on terminology and definitions rather than promoting this specific concept, and not include paragraphs as the one bridging page 9 and 10.

We think the article would benefit from rather extensive editing to get shorter, conveying the main message. A web supplement could then include the more in-depth reasoning behind each concept (such as a lot of the text under 2.1 “Definitions”). We will not here list everything that could go into a supplement, but would be happt yo do so if that is something that is seriously considered.

2.2:
The definitions provided in 2.2 are very welcome. With regards to the comment in yellow: We think it would be very valuable to communicate with Dr Nicholls to hear his current position on the topic. The field of mitochondrial studies would not benefit from to alternating views being pushed. If this is not an option for some reason, we strongly suggest that the wording “caused confusion” should be removed and the wording be something more neutral, such as:
“More recently, a re-definition of State 2 was proposed, considering an alternative protocol …”

State 3: The reasoning around “high ADP” is redundant, as this seems to refer to a specific experimental set-up, which should not be the scope in a position paper like this one. If included, it is suitable as a supplement.

State 5: The reasoning about anoxia more seems like a position taken for the O2k against the Seahorse and is not within the scope of an article like this, that should be equipment independent and unpolitical.

2.3:
Table 2: A nice presentation.

OXPHOS state, p14: The second paragraph is a repetition of what previously is said, and also not within the scope of this article. What do the * in the figure means? All terminology

should be introduced and explained. We propose mentioning the term State 3_{ADP}, similar to the mention State 3_u in the ETS state section.

ETS state, p15: Why is the term noncoupled used instead of the more widely known uncoupled? Noncoupled is however equally descriptive and we are not against its use.
We think the reasoning after the mention of State 3_u is redundant. A less aggressive way to put it would be to write: "We propose the use of the terms OXPHOS state and ETS state instead of the terms State 3_{ADP} and State 3_u, as the former terms conveys more information regarding the bioenergetic conditions in the experiment, while the term State 3 requires considerable prior knowledge to be meaningful", or something similar.

LEAK state, p 15: The term LEAK is somewhat problematic, as it implies that the oxygen consumption under these circumstances serves no real purpose, and just goes to waste (as the word leak in standard English normally implies that what was leaked is lost and have gone to waste). That is not necessarily the case with the oxygen consumption registered e.g. after oligomycin administration. Even though the respiration registered is mainly due to the processes of maintaining the membrane potential, the processes "consuming" the membrane potential are all but superfluous. One could consider the proton flow through UCPS as *leak* but also UCPs serves a physiological purpose, and LEAK is not a very precise definition of these fluxes. Even less compatible with the term LEAK is the respiration associated with the proton pumping that compensates for any reduction in membrane potential caused by substrate transporters, ios channels etc. We do understand that this is a widely used term, and it would probably lead to considerable confusion it is changed.

3:
Fig 6: The figure should not include concepts that are not explained, such as the word "conservation" and "dissipation". These words do not add anything as it is now. The term ExP can easily be misunderstood as "E times P"