**For inclusion in: 4.3. Normalization for mitochondrial content**

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Several methods are used as markers of mitochondrial content (size, volume, and mass). As mitochondrial volume or content is an important quantitative indicator of oxidative capacity, these markers are useful in the normalization and determination of changes to intrinsic mitochondrial properties [1]. Different methods have different strengths, and the type of measure of mitochondrial content/functionality it is an important consideration in experimental design. Stereological determination of mitochondrial content via two-dimensional transmission electron microscopy (TEM) can have limitations due to the dynamics of mitochondrial size. Microscopy can also be both time consuming and statistical challenging in terms of precise volume determination. Mitochondrial specific biomarkers are often employed as a measure mitochondrial size/content. The most readily used biomarkers include measure of mtDNA normalised to nDNA via qPCR, mitochondrial membrane components (Cardiolipin), and mitochondrial marker and enzyme protein activity (complex I–IV activity, citrate synthase activity). The appropriate use of a mitochondrial biomarker depends greatly on the experimental design. For example the use of a non enzymatic marker (such as mtDNA or cardiolipin) may be applicable when investigating intrinsic functionality, as these biomarkers do not have a direct role in substrate oxidation/phosphorylation [1]. Both of these biomarkers have shown varying effectiveness, with several studies indicating a strong correlation between cardiolipin content and increase in mitochondrial functionality with exercise [1-4], while a more mixed response has been seen with mtDNA. Although several studies found that mtDNA showed a positive correlation with mitochondrial content [5-7],

others have reported that mtDNA did not have a positive correlation to increasing mitochondrial content [2, 8, 9]. The use of protein activity (e.g. citrate synthase) as a biomarker can also be advantageous due to ease of use and effectiveness in whole tissue, mitochondrial rich solution, and respiratory media [10]. Although a strong correlation between mitochondrial content and citrate synthase activity has been observed [1, 11, 12] it is important to note that its use as a biomarker may not be appropriate when measuring intrinsic mitochondrial functionality, as citrate synthase activity has been shown to be acutely modifiable by exercise [13, 14] in addition to its role in the oxidation of substrates. The activity of both complex II and IV have shown to be effective biomarkers in terms of maximal OXPHOS capacity, with emphasis on complex IV activity which has a strong correlation in a number of respiratory states [1].

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