

Commentaries on Viewpoint: Gold standards for scientists who are conducting animal-based exercise studies

A RATIONALE FOR SDS-PAGE OF MHC ISOFORMS AS A GOLD STANDARD FOR DETERMINING CONTRACTILE PHENOTYPE

TO THE EDITOR: We believe that a better “gold standard” with regard to assessing fiber type, specifically the contractile phenotype, is with SDS-PAGE separation of MHC isoforms performed, optimally, on single isolated muscle fibers. The metabolic and contractile phenotype must be assessed separately, and the electrophoretic approach offers distinct advantages to ATPase and immunohistochemistry (IHC). While the latter methods are probably the most commonly used, there are limitations to these approaches described by Booth et al. (1). Many muscle fibers express heterogeneity of MHC isoforms. ATPase and IHC provide information only on the presence of a given isoform, not its proportional contribution. These methods are therefore only informative from a qualitative perspective.

Our suggested methodology is feasible for most labs to perform and, importantly, is quantitative. It allows for determination of precise proportions of all MHC isoforms in a given muscle fiber. Additionally, it provides a definitive means for identifying the IIX MHC isoform, something that is currently problematic with respect to ATPase and IHC techniques. Neonatal and embryonic MHCs can also be distinctly separated and quantified by electrophoresis, when performed under optimized conditions (6). Another specific advantage of this methodology is that it allows for the prediction of functional properties of the muscle, i.e., force-velocity relationship (4, 5). SDS-PAGE has been definitively shown to be a method sensitive to subtle or marked differences in MHC isoform expression in response to altered loading and hormone states, during development, and between muscles of different fiber types (2–6). To prove that interventions impact on contractile phenotype, MHC isoform composition should be determined by the electrophoretic approach.

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REPEATABILITY AS A NECESSARY BUT NOT SUFFICIENT CRITERION FOR VALIDATING MEASUREMENTS OF ENDURANCE

TO THE EDITOR: Scientists risk gathering bad data when venturing into unfamiliar territory of another discipline (2). And, to quote Darwin from his 1871 book *The Descent of Man and Selection in Relation to Sex*, “False facts are highly injurious to the progress of science, for they often long endure . . .” The most efficient way to avoid bad data is to collaborate with appropriate experts.

Working with collaborators, I have developed some expertise in measuring endurance of small vertebrates (e.g., 1, 5, 6, and references therein), including laboratory mice (4). Experience has shown this to be a difficult task. Obviously, testing equipment will differ for fish, terrestrial salamanders, and mice. Beyond this, criteria for defining exhaustion will vary, sometimes because animals must be maintained intact for subsequent study rather than killed to determine, say, tissue glycogen concentrations. Moreover, some individuals adopt defensive behaviors that preclude locomotor testing (see also 2). But in most cases, it should be possible to obtain at least two replicate measures of endurance (from cooperative individuals), which help address possible methodological issues. From these, two simple but informative analyses should always be reported (although analyses of repeatability can take various forms, e.g., 3): 1) a Pearson correlation to index relative consistency of individual differences (possibly after transforming the data to make it more nearly bivariate normal); 2) a paired *t*-test to indicate if performance changes, on average, between trials. I view consistency of individual differences as necessary to validate a measurement protocol, but not sufficient to prove that animals were performing to exhaustion.

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TO THE EDITOR: Animal model studies are useful because less unevenness is seen between individual laboratory animals, with more uniform characteristics and less sampling bias between experimental groups. These benefits are especially important when we investigate the effects of nutritional state on endurance and fatigue induced by exercise. On the basis of the criteria stated by Booth et al. (2), in this study the authors examined exercise-induced fatigue in association with energy metabolic regulation in aerobic exercise, such as running and swimming. On the other hand, forced exercise in animals entails great mental stress as well as physical stress and is strongly affected by the endocrine system, including glucocorticoids and catecholamines (1, 3). Consideration should therefore be given to not only peripheral metabolic capacity, but also emotional factors, as these can affect exercise tolerance. Taken together, researchers employing forced activity need to be aware of the effect of emotional fatigue, and strive to prepare mental stress-free conditions. It is also worthy of attention that the degree of performance elevation through training is large in animals compared with humans (4). Furthermore, it has been observed that exercise performance tends to be easily improved by nutritional intervention (5). One possible reason is that the degree of adaptation through training is large in animals if they are kept in a narrow cage from birth, and not usually given opportunities to positively perform exercise, whereas in healthy humans, the level of physical performance tends to be uniform. Considering the above points, examinations of performance and fatigue using animal models should be conducted with caution and understanding of the important differences from humans.

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FOR A PRAGMATIC APPROACH TO EXERCISE STUDIES

TO THE EDITOR: We are skeptical about the feasibility of defining a gold standard for exercise studies on animals with a huge variety in strain, age, sex, transgenesis or exercise paradigms (1). For example, the practical determination of exhaustion in rodents (and humans) is more difficult than implied by the simple theoretical definition. The criteria proposed by Booth et al. are invasive, disruptive, terminal, or ethically difficult to justify. In contrast, electric shock stimulation, in particular in combination with computer-controlled break-off criteria, is a pragmatic, non-invasive solution for comparing endurance capacity between different experimental groups, even for longitudinal studies. Problems with running style and noncompliance can be overcome by appropriate acclimatization and group size.

Second, we certainly appreciate the historic, excellent work by our predecessors. However, novel complementary and interdisciplinary methods provide additional important details about the contractile and metabolic characterization of muscle (3). Even so, individual methods such as immunohistochemical staining of myosin heavy chains, or myosin ATPase activity staining for that matter, fail to provide a comprehensive picture. Therefore, a combination of methods, optimally less delicate than myosin ATPase activity determination, should be used and optimally combined with approaches that allow true determination of peak force generation and fatigue resistance (2, 4).

Finally, we think that instead of a dogmatic approach to exercise concepts [i.e., “no increase in type I fibers” vs. increase in (5) and other references], we should remain open to novel findings and continue to improve our animal-based exercise studies accordingly.

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TO THE EDITOR: Good experimental practice can not be figured out without taking into account previous experience and answering basic questions, and those arisen by the Viewpoint of Booth et al. (1) represent the starting point toward a significant advance in the common knowledge of exercise biochemistry and physiology using animal models. The requirement of shared standards for muscle fiber-type classification becomes even more critical when the results obtained from the laboratory experimental animal are going to be translated into human studies. Skeletal muscle in humans (2) and other mammals, like pig, cat, and baboon (3), lack the type 2B myosin heavy chain. In these species, the fast fiber population is composed by 2A and 2X fiber types, the latter one corresponding to the old 2B fiber type (2, 4), and displaying a metabolic pattern, which can hardly be defined as glycolytic (5). The partial correspondence between fiber types of small and large mammals does not exclude that muscle changes induced in the laboratory animal by exercise or by inactivity would mimic the events occurring in humans, although they might imply a differential involvement of fiber type populations. In this context, only the use of standard approaches to characterize myofiber phenotype, on either contractile or metabolic or redistributive basis, leads to the formulation of sound mechanistic hypotheses.

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EXERCISE—BEYOND SKELETAL MUSCLE ENERGY METABOLISM

TO THE EDITOR: The heterogeneity of skeletal muscle fibers is a consequence of specific contractile and metabolic characteristics acquired during differentiation (muscle cell signature) (5). Nevertheless, mature skeletal muscle cells are metabolically flexible and adapt to altered conditions with short- or long-term adjustments in substrate utilization (2, 4). Skeletal muscle is a main contributor to glucose utilization and its metabolic potential is partially lost in Type 2 Diabetes Mellitus (T2DM). On the other hand, regular physical activity provokes metabolic adaptations in skeletal muscle (4) that can prevent T2DM. Hence, pharmacological interventions that mimic the metabolic “exercise responses” are in demand. Transgenic technology in mice has shown that changes in gene expression can lead to a more oxidative and insulin sensitive phenotype and consequently compounds able to reproduce such adaptations

have been claimed as a potential exercise-like alternative (1, 3). However, the key components of these pathways, controlling the metabolic signature of the different skeletal muscle fibers during developmental stages, are not necessarily of interest when attempting to prevent or treat metabolic diseases that develop during adulthood. Furthermore, regular physical activity, such as endurance exercise training, produces more functional improvements in different organs than just modifications of energy metabolism in skeletal muscle. To avoid misinterpretation, caution must be taken when attempting to extrapolate the findings obtained with genetically modified animal models and viewpoints such as Booth et al. are more than welcome (1). Given the complexity of the changes occurring within our body due to regular exercise, we are far from being able to “mimic” such adaptations with genetic or pharmacological interventions. Instead, we should put our efforts in finding a way to motivate people to exercise (3).

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VALID AND REPRODUCIBLE ENDURANCE PROTOCOLS UNDERLIE DATA INTERPRETATION, INTEGRATION, AND APPLICATION

TO THE EDITOR: In their viewpoint manuscript, Booth et al. (2) identify that biochemical or central indices of fatigue are the “gold standard” criterion when a rodent model of forced treadmill exercise to exhaustion is incorporated in an experimental design. As discussed, experienced investigators should also scrutinize the running ability/style of each rodent to assess test validity and study inclusion/exclusion. This is crucial because some animals may display inconsistencies in running gait (randomly stopping for brief periods, turning to fight the grid, etc.) and in these instances objective measures of fatigue (biochemical or otherwise) may be achieved but the test should be discarded (3). When biochemical fatigue determination is prohibited (i.e., when multiple endurance tests are required) investigators should implement protocols in which test termination has been validated previously with established fatigue markers (4). Moreover, to refine experimental design and achieve high statistical power, investigators should provide evidence of the ability to achieve high degrees of within-animal reproducibility using the specific protocol. We believe

this is assessed optimally by analysis of group means to identify possible time/familiarization effects in conjunction with within-animal coefficients of variation. The often cited correlation analysis is a poor measure of reproducibility, particularly when there is a relatively small range of data (1, 3). The inability to achieve reproducibility may lead to inappropriate physiological conclusions. The ability to assess accurately an animal's capacity to run on a treadmill and obtain and report a high degree of within-animal reproducibility is vital for accurate data interpretation, integration, and application.

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TO THE EDITOR: Dr. Booth and colleagues (2) are to be commended for identifying important criteria issues in selecting standards in the exercise sciences that necessitate deliberation and debate. However, I chastise the authors and the Editor for allowing scientific aspersions to be cast upon eight laboratories in a format that does not allow them to defend their logic or indicate whether the methodology significantly affected their conclusions. While pleased to be recognized with investigators (3) who have established “gold standards” for exercise

scientists to follow, this linkage may have more extrapolation than fact.

That said, Booth and coauthors do provide convincing documentation for the need to establish gold standards by which an exercise response is evaluated and interpreted. They are also correct in advocating the standardization of nomenclature (4) as exercise continues to be described by a myriad of terms that are difficult to quantify.

Although the root of the problem is the use of inadequate procedures and methodology, the cause of the problem is the failure of reviewers and Associate Editors to reject such manuscripts for publication in scientific journals as the *Journal of Applied Physiology*. Therefore, I advocate the Editor to seek approval from the APS Publication Committee to formulate a task force that will establish gold standards for evaluating exercise methodology and responses while standardizing the exercise nomenclature suitable for APS reviewers and Associate Editors to follow. As the precedent has been established and the procedures formulated with the publication of *Guiding Principles in the Care and Use of Animals* (1), Editor Dempsey should take action on this matter.

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