A METABOLOMIC EXPLORATION OF THE EXERCISE RESPONSE: ACUTE AEROBIC AND ANAEROBIC EXERCISE RESPONSES IN ENDURANCE AND RESISTANCE TRAINED MALES AND FEMALES

by

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ABSTRACT OF THE DISSERTATION

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Exercise acts as a potent modifier of metabolic status both acutely and through adaptations from chronic participation in physical activity. Changes in metabolism elicited by exercise manifest across systems throughout the body and impact health and fitness status of the individual. Metabolomics has proven a sensitive measure in investigating these perturbations in the context of acute and chronic changes. Here, our aim was to apply a metabolomics approach in order to characterize differences in the responses to both aerobic and anaerobic exercise, as well as to investigate the roles of training background and sex on these responses. Participants (N=40) were equally distributed into groups based on sex (M/F) and training background [endurance (END) or resistance- trained (RES)]. Participants performed 45-minute cycling (AE) or weight-training (AN) bouts on separate days. Serum was collected before (T0), immediately after (T1), and 60-min post-exercise (T2) and an analyzed via UHPLC/MS by Metabolon for identification of 754 known individual metabolites. A variety of statistical techniques, including clustering, pathway mapping and RM-ANOVAs were performed for data analysis. Both exercise conditions served to significantly alter the metabolome of all participants, with bioenergetic pathways predominating the response (TCA Cycle, purine

salvage, substrate metabolism). Between conditions individual metabolic pathways differed by magnitude and/or timing of response, but not direction. At T1, changes within fatty acid metabolism were larger and more extensive in response to AE, while AN elicited greater perturbations in carbohydrate and anaerobic metabolites. However, by T2, both exercises presented similar patterns of substrate use, relying heavily on fatty acid oxidation. Inflammatory signaling, oxidative stress and energy balance all indicated greater stress at T2 following AN than AE. Principle components analysis was unable to discern between either END and RES or M and F groups at T0. Further, resting metabolomes were only significantly different at 3% and 1% of metabolites by training background or sex respectively ($p < 0.05$, $q < 0.1$). In response to exercise, training background differences were characterized by larger responses from the group more familiar with each exercise bout (END-AD, RES-AN) and a generally faster recovery for END as compared to RES following both exercises, and particularly AE. Thus, training background comparisons recapitulated differences observed between acute sessions. Sex differences were highlighted in substrate selection. Fatty acid use during AE (change T0-T1) was greater in F than M while amino acid catabolism, glycolytic flux and purine salvage were greater during AN in M than F. These results present the first direct comparisons of the metabolomic response to exercise across sex and/or training background for AE and AN. We report both similarities and differences in the exercise response between mode (AE/AN), training status (END/RES) and sex. Despite observed phenotypic differences between groups, groups exhibited a uniform resting metabotype and comparable responses across training background and sex. These observations illustrate the potency of regular participation in physical activity of any kind in normalizing the metabolome at rest and in response to exercise. They further suggest the possibility of a healthy resting metabotype and, a characteristic metabolomic signature in response to AE and AN in active individuals.

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INTRODUCTION

EXERCISE BACKGROUND

It is no secret that exercise is good for you. In fact, recent publications have intimated that exercise is as good as or better than modern pharmacology at treating a multitude of ailments.[1-4] Though there have been centuries of circumstantial evidence and decades of research to support the claim since made, the benefits of exercise were recognized as far back as the ancient Greeks:

> *All parts of the body which have a function, if used in moderation, and exercised in labors to which each is accustomed, become healthy and well developed and age slowly; but if unused and left idle, they become liable to disease, defective in growth and age quickly.*
>
> –Hippocrates, c. 450BC.

Today, we recognize the ability to foster good health through lifestyle choices (diet and exercise). In fact, it has been posited that our bodies were built with the purpose of movement, and a sedentary condition, adopted all-to-often in modern man, actually acts to bring about accrual of fat, escalation of the stress response, an upsurge in inflammation and inception of a diseased state.[*5] Consequently, the resultant dysfunction of pro/anti-inflammatory signaling has been purported as a possible mechanism behind the epidemic of obesity[6] and metabolic syndrome.[7] This hypothesis is bolstered by observations that regular exercise can arrest, and even reverse these conditions.[8,9]

Most exercise studies compare the trained and sedentary conditions. While certainly useful in establishing the benefit of physical activity, we have evolved beyond this in our current understanding of exercise. What is largely unexplored are the potential benefits of long-term exercise. Implementation of multi-year training interventions are impractical, if not impossible, but the inclusion of previously trained individuals in study designs offers data on the continued effects of exercise after waning of the initial gains.

Exercise Response. Exercise is a global effector that influences myriad biochemical pathways and physiological phenomena. This fact is exploited in medicine and sport to engender positive health and fitness changes. A complexity within the study of exercise is the mode of exercise, aerobic versus anaerobic. These stimulate vastly different, sometimes oppositional, responses on both the intracellular and systemic levels.[10-13] While data on the nature of these disparities are not completely lacking, a full understanding is far from clear at present.[10,11]

Acutely exercise is a potent stressor. Challenges to homeostatic balances abound during exercise and continue beyond completion of the work-bout. The body attempts to maintain homeostasis by recruiting resources to meet the increased demands imposed by exercise. Endocrine and other humoral factors respond to changes and restore the *status quo* as well as cause adaptations in the body for an augmented resistance to future exposure to this same stressor. This acute response than dictates adaptations to be had from long-term training. However, with training comes the ability to perform greater workloads, and so exercising at the same absolute intensity equates to a reduced relative load, and the same relative intensity an increased absolute load. It is therefore impossible to simultaneously match both relative and absolute intensity, and even within-subjects designs result in training adaptations that can muddle comparisons made pre-post.

Due to the convoluted nature of the exercise response, its characterization is often parceled out into relevant discrete subsets for study, i.e. endocrinology, muscle physiology, cellular biology. This proposal sought to employ metabolomic profiling, a global assessment of the metabolic fingerprint which includes hundreds of intermediates and products of various biochemical pathways, to provide specific insight into the acute

effects of 2 different modalities of acute exercise. Moreover, this project was purposefully designed such that comparisons could be made between sexes as well as training history.

Exercise Mode. Although there exist many types of exercise, from hiking and marathon running to cross-fit and power-lifting, we can categorize all of them into one of two groups: aerobic or anaerobic. This, of course, is a bit of an oversimplification. There is a grey area where the exercise has varying degrees of both aerobic and anaerobic components, i.e. soccer or middle-distance running, but for simplicity, these mixed-activities are not discussed here. Aerobic exercise involves oxidative metabolism of the macronutrients for energy, i.e. aerobic metabolism. Typically, sessions are continuous bouts of activities like cycling or running. Anaerobic exercise, which utilizes anaerobic metabolism for replenishment of adenosine triphosphate (ATP), is by default of a significantly higher intensity, and therefore is sustainable only for short bursts before interspersing rest intervals is required. This often takes the form of resistance training. Though the upshot of both exercise types is positive health and fitness gains, inherent differences in the response to each also brings about unique adaptations.

It is evident that the type of exercise performed affects the biochemistry, physiology, and anatomy of an individual. Most research supports the use of resistance training to compliment endurance training,[12-14] but cautions against over-prescription of aerobic exercise for strength/power athletes.[12-16] There is a plethora of papers examining concurrent training –use of both aerobic and anaerobic training together, and the compatibility of training for both endurance and strength[12-18] and countless others focusing on one or the other. Surprisingly few direct comparisons of aerobic and anaerobic exercise bouts have been carried out examining the acute responses responsible for yielding training adaptations.

Aerobic Exercise. In brief, aerobic exercise stimulates principally type I oxidative fibers with a prolonged challenge to ATP turnover in the muscle. A mix of intramuscular energy

stores such as glycogen and triglycerides, along with some amino acid (AA) use and mobilization of fatty acids (FA) and glucose all act as energy substrates during exercise. As intensity increases, glycogen, mobilized glucose, and lactate produced during glycolysis play a larger role.[19] Following glycolytic breakdown of glucose, lactate is largely converted to pyruvate and aerobically metabolized in the tricarboxylic acid cycle (TCAC) and electron transport chain within the mitochondria. Intramuscularly, continued ATP use yields diminished energy stores and a need for a high rate of mitochondrial enzyme activity. Systemically, the cardiopulmonary system manages blood flow for provision of sufficient nutrients and oxygen, heat dissipation and waste removal.[19,20] Acutely, this leads to elevated heart rate, stroke volume, cardiac output, and blood pressure. Chronically, cellular and systemic adaptations help lessen the magnitude of these distresses during recurrent bouts of aerobic activity.[19,20] Within the active muscle this means mitochondrial biogenesis and optimized substrate utilization. Signaling to and from the muscle initiates and carries out intracellular as well as cardiovascular changes to allow for enhanced delivery of nutrients and oxygen: greater capacity of cardiac output through left ventricular growth, higher blood-O_2 carrying capacity and proliferation of capillaries for increased uptake at the muscle.[19] It is the strain induced from exercise that drives these adaptations, and many of the molecules driving these processes can be seen shortly following an acute bout of aerobic exercise.[10,19] Therefore much is to be learned from study of the acute response.

Anaerobic Exercise. Conversely, anaerobic exercise stimulates type IIa mixed and IIx glycolytic fibers along with type I during brief, but very high rates of ATP turnover via largely anaerobic processes. As the nature of this exercise involves intermittent work and rest periods, metabolic activity will shift from predominantly anaerobic to aerobic during active and resting

periods, respectively.[21] More direct ATP-re-synthesis via adenylate kinase, creatine kinase and anaerobic glycolysis allow for faster replenishment of ATP and a higher work output than aerobic metabolism, but also fatigue faster, underlying why anaerobic processes cannot continue for prolonged durations without rest intervals. Also, in contrast to aerobic metabolism, glycolysis here ends with lactate production and yields H^+ ions, lowering muscle and blood pH, challenging the body's buffering capacity. Intramuscularly, there is greater Ca^{2+} cycling and higher force production by the myofilaments.[22] Extracellularly, acute increases in cardiac output, heart rate, and stroke volume are less than that of aerobic exercise, but blood pressure can reach much higher levels.[23] There is also greater neural activity than with aerobic activity.[24]

Chronically anaerobic training leads to increased contractility, greater high-energy phosphate stores like phosphocreatine in anaerobically-trained individuals.[10,25] As with chronic aerobic exercise, left ventricular mass and contractility both increase, but chamber volume remains unchanged.[26,27] A unique result of resistance training is increased anabolism, largely accomplished through hypertrophy of the type II fibers in humans via satellite cell activation.[28] This gives the more muscular physique of anaerobic as compared with aerobic athletes. The acute responses during and after an exercise bout reflect the unique stressors induced by said bout. The intramuscular and systemic disparities between aerobic and anaerobic exercise underly the differences in metabolic status of the body and drive the subsequent training adaptations.

Muscle Fiber Type Recruitment. During exercise, there is variation in muscle fiber recruitment based on exercise type. It is important to note that muscle fibers do not simply take over for one another when fatigued. It is the relative force of contraction, and not the degree of fatigue, that leads to recruitment of glycolytic fibers.[24] This means contraction of

these fibers *en masse* depends on the percentage of max force output rather than perceived difficulty of the movement. The high intensity associated with anaerobic training demands glycolytic fiber recruitment, whereas even exhaustive prolonged aerobic exercise relies near exclusively on oxidative fibers.

Furthermore, disparities are exaggerated with repeated exercise training. Aerobic training shifts intermediate IIa fibers to a more oxidative status and allows for some fiber type conversions IIx → IIa and IIa/x→I, resulting in highly oxidative muscle types for endurance trained individuals.[29,30] Anaerobic training also converts IIx → IIa, but it produces a glycolytic phenotype of the fiber differentiating from endurance training effects.[29,30] Moreover, anaerobic training produces disproportionately greater hypertrophy in type II as opposed to type I fibers giving a muscle cross-section that predominantly consists of fast twitch fibers in resistance trained individuals. Aerobic training on the other hand, yields almost no hypertrophy.

As an upshot of their training adaptations, endurance-trained individuals simply lack the abundance of type II fibers and so lean more heavily on type I fibers throughout the continuum of submaximal intensities. Paradoxically, resistance-trained individuals with comparatively higher max force outputs should also be recruiting more type I fibers at matched force production during low-intensity aerobic exercise[31] and turn to type II fibers only as force generation rises.[31-33] However, this does not seem to translate into improved aerobic performance without concurrent aerobic training.[34] Fiber type selection is therefore pertinent to the type of exercise being performed acutely, as well as training adaptations that shift the fiber-type ratios. This is a crucial difference in separating exercise modalities, as inherent to these fiber type differences are variants of several structural and functional proteins within the muscle, influencing rates of metabolite

synthesis, degradation, release and uptake. Sensitivity to and signaling from the muscle brought on through exercise are largely related to the muscle fiber type being utilized.[35-37] This can have wide-ranging effects on downstream substrate mobilization, energy dynamics, cytokine signaling, etc. Fiber type differences manifest in the metabolism of the cell and so directly impact the metabolome of the individual, particularly during exercise. Notably, no difference in fiber type has been identified between males and females.[38]

Male-Female Cardiovascular Response. The cardiovascular response may offer the greatest area of differences between exes in the exercise response. In addition to the greater cardiovascular response to aerobic exercise and in endurance-trained individuals, discrepancies between sexes in general cardiac health,[39-44] as well as in response to exercise, most notably with aerobic exercise, are widely reported.[45,46] Females have shown less strain on the heart during low-intensity aerobic exercise.[45-47] Sex discrepancies are attributed largely to the difference in estrogen levels and its cardioprotective properties.[47] These effects of estrogen in response to exercise seem to be at least partially mediated through modulation of the inflammatory response, as estrogen has been shown to alter expression of various inflammatory cytokines and their receptors *in vitro,*[48,49] as well as *in vivo* for both the murine model[50] and in humans.[51,52] There appears to be a compensatory mechanism in males, whereby heat shock protein-70 offers cardioprotective benefits similar to estrogen in the response to ischemic-reperfusion events.[53] While that investigation did not specifically look at exercise, it is feasible that heat shock protein activation during exercise could possibly serve as a cardioprotectin, particularly in males. This is a demonstration of the metabolic flexibility present in physiologic response systems that methodologies like comprehensive metabolomics mapping can potentially uncover, whereas targeted assay might otherwise fail.

Exercise Comparisons. In spite of the noted differences, recent evidence shows that high intensity interval training may precipitate several of the same effects as more moderate intensity endurance training.[54] Common responses to aerobic and anaerobic training are numerous. They each lead to increased muscle glycogen, improved overall longevity, reduced body fat, increased insulin sensitivity, and positive affective responses.[55] These similarities in the acute response lead to parallel adaptations.

Acute bouts of either exercise type stimulate similar global changes: increased energy demand met by substrate partitioning, mobilization and delivery; increased flux through energy-providing pathways; elevated ATP re-synthesis to meet these energy demands; inflammation followed by an anti-inflammatory response; oxidative stress followed by an antioxidant response; and altered circulation of various hormones regulating or related to many of these processes.

Still, outcome differences are evident. Pathways triggered from the specific stresses of acute exercise bouts are not identical, and acute stress caused by aerobic or anaerobic exercise is managed accordingly. Specific exercise-associated adaptations are commonly used in the sporting world, but also in medical treatment plans. Use of aerobic training in managing hypertension is common practice, whereas anaerobic training engenders an anabolic response, making it more effective at increasing lean mass in healthy individuals[28] or in mitigation of sarcopenia.[56]

Often, differences between aerobic and anaerobic training manifest on the phenotypic level. However, divergence between aerobic and anaerobic exercise is apparent from gene transcription through protein modification to metabolic outcomes and intra/extra-cellular signaling. Inside the muscle, long-term aerobic training is characterized by mitochondrial biogenesis, while chronic anaerobic exercise increases

myofilaments and sarcoplasmic reticulum.[25] Acute energy pathways appear relatively incompatible.[10,19] However, the systemic effects and organismal-level health and wellness changes are similar.[14,57] It is these phenotypic outcomes that are of most importance. Such longitudinal responses are just the protective adaptations elicited by the acute stressor.

Therefore, examination of the acute response elucidates drivers of phenotypic changes that take place with exercise. Studying individuals in a trained state provides insight into the impact of exercise in terms of continued benefits beyond going from sedentary to active. The biochemical measurement most directly tied to phenotype is metabolomics. Use of an exploratory metabolomics panel combines this proximity to phenotype with a comprehensive mechanism of assessment befit for investigating the far-reaching responses to exercise. A better understanding of how and why varying exercises bring about sometimes-concordant, and sometimes-discordant responses is needed. Examining the global metabolic response pattern following 2 different acute exercise exposures, and doing so within differentially trained individuals, provided a unique perspective in attempting to answer such questions.

METABOLOMICS BACKGROUND

Exercise is a profound stressor that spawns an intricate holistic response. Isolating a single pathway or a few select markers, as is often done in research designs, falls short in considering cross-systemic responses that involve such a multitude of reactions. Furthermore, the underlying redundancy inherent to human physiology inevitably leads to multiple explanations from scientists probing for answers within functionally adjacent systems. This gives rise to a fragmented picture and a piecemeal understanding of the issue at hand. Metabolomics allows for high-throughput analysis of an ‘omics-wide response across systems.

Metabolomics involves the assessment of small molecules (<1.5KD) in a biological medium. Of the ‘omics technologies, metabolomics is downstream of genomics,

transcriptomics, and even proteomics, providing the nearest approximation to actual phenotype currently available. Moreover, a far-reaching approach such as metabolomics is well-suited to characterize the whole-body response to exercise.

Documentation of the acute response to aerobic and anaerobic exercise serves to further our understanding of how to utilize physical activity to improve health and fitness. Herein we employed an exploratory metabolomics approach to identify biochemical signatures at rest and following two different exercise sessions (aerobic as steady-state cycling and anaerobic as resistance training).

Using a systems biology approach like metabolomics to compare modes of exercise was unique. Doing so in healthy humans, with disparate training backgrounds, across sexes, and within a repeated measures design were all completely novel approaches. The body of literature on the exercise metabolome is paltry, and addition of these findings serves to close our knowledge gap.

PURPOSE

The purpose of the proposed study was to characterize the resting metabolome and metabolomic shifts caused by acute bouts of aerobic and anaerobic exercise for differentially trained, healthy males and females. Taking the initial steps towards understanding the physiological underpinnings of the exercise response, we enrolled healthy individuals. To gain some indirect insight into longitudinal effects of training, we recruited endurance- or resistance-trained individuals. Males and females were included to identify sex differences. A repeated-measures, crossover design of both exercise bouts was implemented to increase power for our sample size and enable comparisons between conditions and within groups.

This study was the first direct comparison of the metabolomic responses to aerobic and anaerobic exercise in a healthy population. It also served as one of the only attempts at employing metabolomics in examination of a translational resistance-training protocol. The inclusion of endurance- and resistance-trained individuals made this the first metabolomics investigation to compare training background effects on the response to exercise of any kind. Further, this project is the first to utilize a comprehensive metabolomics panel to examine sex differences in the exercise response, and one of very few to consider the female metabolome on any level in the context of exercise. We hoped to gain insight into the acute changes in the metabolome in response to differing bouts of exercise, as well as how sex and training background differences may affect or relate to these responses.

DESIGN

Recruitment, Design and Analysis

Careful selection of participants for any study includes consideration of prior exposure to treatment variables. Within exercise conditions one might expect an incongruity in responses based upon training history. In fact, there is evidence that training background may have a stronger effect even than mode of exercise on certain intracellular pathways.[20] Rather than avoid familiarity with exercise, a targeted recruitment was purposefully implemented. Using individuals who were already in a trained state, rather than a sedentary sample, allowed a glimpse into the long-term effects of exercise training without the complications of a longitudinal training design. Using experienced individuals also had the advantages of improving accuracy of exercise testing and decreasing the incidence of dropouts or failure to complete an exercise session.

Previous adaptations attained through a history of similar training were thought to provide endurance-trained individuals protection from some of the stress of aerobic but not

anaerobic exercise. The inverse was expected for resistance-trained individuals. Though observed differences in resting biomarkers between these groups were largely attributed to their contrasting training histories, other considerations, like genetic predisposition, and associated lifestyle differences, may have also played a role. Observation of acute changes during and after an exercise bout is important to our understanding of chronic adaptations. Though a longitudinal intervention could reveal the consequent improvements in health and fitness, it also is susceptible to bias towards initial improvements in an untrained population[58] and requires a greater investment on all levels, and therefore typically follows an acute study such as this one.

Just as there are possible differences between individuals with different training histories, there are likely sex differences in the exercise response. It has been demonstrated in the murine model that the metabolome is determined by genetics, sex, age, diet, temperature, gut microflora, hormone levels and circadian rhythm.[59-62] Specific to humans, studies have found variation in the urinary metabolome based on sex, diet, time of day, cultural differences, and geographic location[63-67] For these reasons, targeted recruitment was used to enroll an even split between endurance and resistance-trained individuals, as well as males and females. Further, location was standardized, time of day was controlled for between-subjects and matched between-conditions, and diet was matched between-conditions.

Both males and females with experience in either primarily endurance or strength/power training were evaluated. We compared the serum metabolome of these individuals before and after acute bouts of aerobic and anaerobic exercise. Following preliminary examination (principal components analysis, hierarchical clustering, pathway visualization), targeted statistical analysis of 3-way ANOVA and 2-way follow-ups, of the

fold-of changes for the log-transformed median scaled data plus t-tests on change scores between the time points for samples collected was completed.

LIMITATIONS

Regarding limitations of this study, a targeted recruitment strategy was used to enroll males and females with either endurance or resistance training backgrounds. Inclusion of males and females in a single training group and endurance- and resistance-trained individuals in a single sex group increased heterogeneity of the sample. Collapsing across sex for analysis of training background differences and training background for analysis of sex differences also made results subject to “cross-contamination”. By this, male/female differences impacted (by exacerbating or reducing) endurance/resistance differences and vice versa. Though using groups of 10, rather than 20 would have eliminated this issue and resulted in highly homogeneous groupings, an n of 40 is on the cusp of minimal sample size in human metabolomic research. Therefore, the loss of power from cutting sample size in half for each comparison precluded such an evaluation. All participants were young and healthy individuals. Moreover, the majority of participants were Caucasian, with inclusion of 10 Asian, 1 African-American and 4 Hispanic individuals. Though this limits genetic heterogeneity, findings cannot be extended beyond the scope of young, healthy individuals of largely European descent. Modes of exercise chosen may have also impacted results. It is possible that running as opposed to cycling, or sprint intervals as opposed to resistance training could engender somewhat different responses. Still, cycling and weight training are two of the most common forms of aerobic and anaerobic exercise respectively, and represent the desired exercise modes well. Specifically, the use of cycling *in lieu* of running eliminated variations in biomechanics and eccentric contraction. From an ease of use standpoint, both cycling and lifting allow for accurate quantification of workload and enable simple adjustments to be

made to account for fatigue etc. The exercise bouts themselves were of a specific intensity and duration, and as would be in-line with our hypothesis, other exercise intensities and durations might elicit different responses than those observed in this study. Further, exercise duration was matched between the two bouts to provide translational validity, but along with matching relative intensity between subjects resulted in both higher total workloads during the cycling bout and bigger discrepancies in total caloric expenditure between modes for endurance- as compared to resistance-trained individuals. Finally, the time-line of post-exercise data collection also confined conclusions to that window. However, limiting sampling to this acute response reduces exposure to extraneous variables experienced throughout the day. The time-course was chosen due to the exploratory nature of the metabolomics response, the practicality of the design, and the vast body of literature showing changes within this period following exercise.

DELIMITATIONS

As with any research study, there are several aspects of the design that could have bene expanded upon. Funding, time, and feasibility acted to limit the scope of the design. Metabolite measures were assessed from blood collection. Having tissue samples to analyze would have better characterized intracellular mechanisms providing a tissue-specific metabolome. With muscle samples specifically, identification of differential responses by fiber type would have been possible. The metabolomic analysis was performed on relative values, and therefore does not provide actual concentrations for any of the metabolites. It is possible that non-significant differences were actually physiologically relevant and that statistically significant ones were not. Especially susceptible to type I errors are metabolites with inherently low concentrations and to type II errors are metabolites with very high concentrations. Additionally, though diet logs were

used for the 24+ hours leading up to each session, no standardized dietary intervention was implemented. This was done to allow for collection from actual free-living humans, and so participants were permitted to eat whatever they chose. They were instructed to duplicate their dietary intake prior to the first exercise condition for the subsequent visit –including a 2-hour fast prior to the pre-exercise blood draw and resting metabolome results indicated high compliance. If adhered to, use of a standardized diet would have eliminated this variable entirely. All testing was completed for a given participant within a 3-6 week period. This eliminated seasonal affects, but made female participants susceptible to menstrual cycle variation. Participants did, however, get to select session dates, and so likely would have avoided testing during the late-luteal or menstrual phase. Though time-of day was controlled to within 1-hour between conditions and within 4-hours between subjects (range: 8:00 AM – 12:00 PM), it is still possible that individual circadian rhythms could have impacted results.

REVIEW OF LITERATURE

ACUTE EXERCISE EFFECTS: GENERAL

Within the intricacies of human movement lies a complex network of molecular and humoral communication. Alterations of blood flow, mechano-sensing, bi-directional neural communication, shifts in energy balance, redox status, endocrinology, and cytokine/myokine release all serve to stimulate intracellular reactions in the form of 2nd messengers, gene expression, phosphorylation, cleavage, and other molecular processing events leading to some final outcome. In turn, whole-body changes result from activation of these many systems.

Specific responses to exercise will vary with intensity and duration, which by default, tend to inversely relate to one another. Aerobic exercise of longer duration and lower intensity often differs from anaerobic exercise of shorter duration and higher intensity. It is an oversimplification to simply think of aerobic and anaerobic exercise as differing on intensity and duration alone. Though these differences play a large role in driving many of the divergent phenomena, the differences go deeper than that. Specific muscles and nerves stimulated differ both in type and number recruited. Rate and strain of the mechanical pull on the muscles and other connective tissues are typically greater with anaerobic than aerobic. Cardiac output and oxygen consumption are typically higher with aerobic exercise, while blood flow to and from the working muscle is more impeded, leading to higher blood pressures during anaerobic exercise. Fuel mobilization, substrate use, and metabolic balance are all markedly distinct, while cell signaling and mechanistic underpinnings also vary between the two. These differences can be found throughout the literature summarized in various reviews.[10,11,19] Our current understanding of the exercise response presents an elaborate convergence of multiple systems, signaling cascades, and cell types in the body.

An essential factor guiding the design and execution of this investigation is the aggregate exercise response and the crosstalk between pathways.

All exercise results in observable changes in the protein turnover of muscle fibers. Rates of both transcription and translation are up-regulated to bring about longitudinal changes associated with aerobic training, i.e. mitochondrial biogenesis and anaerobic training, i.e. muscle hypertrophy.[68,69,70] These processes are tightly regulated during the acute exercise response, and studies looking at the transcriptional level have found changes in muscle mRNA generally peak within 4 hours.[71-73] Many regulatory signals are activated well in advance of this, and serve to directly and indirectly activate or repress the various transcription factors controlling gene expression. In this way, the metabolic status during and shortly after exercise dictate the eventual changes in gene expression.

ACUTE EXERCISE EFFECTS: AEROBIC VS ANAEROBIC

Energy Status. Regardless of fiber type, the energy for muscular contractions comes from cycling of ATP. This places a strain on the homeostasis of the total adenylate charge and the phosphorylation potential of the cell.

Total Adenylate Charge = ([ATP]+0.5x[ADP])/([ATP]+[ADP]+[AMP])

Phosphorylation Potential = [ATP]/([ADP]+[Pi])

Muscle cells maintain adequate rates of ATP hydrolysis during the stress of exercise without disrupting the balance of these kinetic parameters. As levels of ATP fall and Adenosine Diphosphate (ADP) simultaneously climbs, the concentration of Adenosine Monophosphate (AMP) (and the AMP:ATP ratio) becomes increasingly significant in balancing these equations. Particularly responsive to this ratio is AMP-activated protein kinase (AMPK).[74] Because of this sensitivity, AMPK is able to serve as an energy sensor in muscle cells. And while comprehensive reviews exist for its different functions in muscle (i.e. directional substrate processing,

hypertrophy and gene expression),[10,75,76] AMPK basically acts to conserve and generate ATP.[74] When active, AMPK increases ATP production through increased glucose uptake[77,78] and oxidative capacity for fatty acids.[79,80] There is also a net effect of AMPK to conserve ATP through its attenuation of anabolic processes. Aerobic and anaerobic bring about divergent AMPK functions, largely through fiber type selection, with aerobic engaging type I fibers showing the greatest AMPK activation.[81-91] In a rodent study comparing low frequency (aerobic) and high frequency (anaerobic) electrical stimulation, only the low frequency stimulation up-regulated AMPK phosphorylation.[87] However, Dreyer, et al. (2000)[92] revealed increased AMPK activity following a 30-second maximal sprint on the bike in humans. It has been speculated that AMPK phosphorylation from a bout of anaerobic exercise results from transient energy deficit, and serves to replace glucose used in glycolysis, though research supporting this is lacking. Regardless, the primary challenge of exercise is energy homeostasis, and aerobic and anaerobic exercise present entirely different energy dynamics to manage.

In an attempt to settle this discrepancy, a study exposed trained athletes to bouts of acute aerobic and anaerobic exercise.[20] Perhaps counter-intuitively, they found a dissociation between exercise mode and training. Whereas aerobic exercise elicited an AMPK response in power-lifters, anaerobic exercise stimulated AMPK in endurance-cyclists. The most likely explanation to this observation is that each group of individuals was equipped to meet the energy demand of their corresponding familiar exercise session. Meanwhile, the novel exercise bout provided a sufficient challenge to disrupt cellular energy stores and activate AMPK. In other words, regardless of exercise, power lifters more quickly turned to type II fibers and cyclists type I and became energy depleted either by the long duration of the aerobic bout for power lifters or the high intensity of the anaerobic bout for

the cyclists. While it must be noted that the individuals used for both groups in this study were highly trained athletes, this data clearly demonstrates the robust impact training background can have on energy metabolism during aerobic and anaerobic exercise.

Substrate Metabolism and Mobilization. Aerobic exercise is generally thought to derive energy from a mixture of fuels, while anaerobic, by nature of its higher intensity, requires a greater reliance on more quickly metabolized substrates, e.g. carbohydrate (glucose) for glycolysis and other non-aerobic pathways (creatine phosphate and adenylate kinase). Temporal constraints and sustained availability of carbohydrate during anaerobic exercise are the underlying factors.[93] This is reflected in the storage capacity of the active fiber type: type I fibers recruited during aerobic exercise generally contain relatively higher intra-muscular triglycerides and slightly lower glycogen stores than the type II fibers predominantly recruited during anaerobic exercise. The complete oxidation of free fatty acids (FFA) simply takes too long to contribute to the immediate and fleeting high-energy demand during anaerobic exercise. Similarly, mobilized FFA and glucose during short anaerobic activity are thought to play a relatively smaller role, particularly when contrasted with aerobic exercise of a longer duration.

Use of intra-muscular triglycerides has been seen to inversely relate to use of muscle glycogen,[94] and so is functionally most relevant during aerobic exercise of moderate to low intensity. However, Shepherd, et al., (2014) demonstrated increased intramuscular triglyceride stores in type I fibers following just 6-weeks of resistance training in previously sedentary individuals.[95] And multiple studies have shown increased fat oxidation in the hours following an acute bout of anaerobic exercise.[96-98]

All exercise induces muscular contraction, which results in an insulin-independent contraction/CaMK-dependent migration of the GLUT IV glucose transporter to the membrane, as well as increased insulin sensitivity in response to acute and chronic exercise.[35,36] So, glucose

influx via GLUT IV is mediated through two independent pathways during muscular activity, promoting differential use of mobilized blood-glucose dependent on exercise-type.

Within the muscle, release of glucose-1-P from glycogen provides the major starting substrate for anaerobic glycolysis during anaerobic exercise, with relatively minor contributions from hepatic glucose release.[99] Alternatively, aerobic exercise promotes a mixture of intra-muscular and mobilized extra-muscular substrate use. The exact ratios are dependent on intensity, duration, and feeding status, with higher intensities utilizing more carbohydrate and lower intensities more FAs. Intake of a given macronutrient before or during exercise leading to heightened use of that substrate. While some evidence exists for glycogen levels to affect cellular functioning,[100,101] this is still a contested notion. It seems likely that intramuscular energy stores do impact substrate use[94] and possibly other cellular functions. If true, exercises resulting in greater use of muscle-glycogen would have a bigger impact on such pathways.

Increased glycolytic enzymes and glycogen stores seen in resistance-trained individuals and increased aerobic enzymes, including those of β-oxidation, and intramuscular triglyceride availability seen in endurance-trained individuals, it would make intuitive sense carbohydrate metabolism would be favored in the former, and FA metabolism in the latter.[10] However, training impact on substrate oxidation is largely limited to trained versus untrained individuals. Direct comparisons are lacking. What is known is that as fitness improves reliance shifts from anaerobic to aerobic and from carbohydrate to FA oxidation at the same absolute intensity.[102]

In addition to intensity-driven fuel selection, research has exhibited a clear difference between male and female substrate use at rest and during exercise.[103,104] At the same relative intensity, females tend to utilize significantly more fat and less carbohydrate than

their male counterparts as an energy source. This is evidenced by a lower respiratory exchange ratio across submaximal intensities (equivalent percentage of VO_{2MAX}) for females. This observation has been made regardless of whether groups were matched for fitness[105-107] or not,[102] and may even extend to amino acid metabolism during exercise (similar to carbohydrate use, lower in females).[105,108,109] Work done using stable isotope infusion ([6,6 $2H_2$] glucose) showed a disproportionately lower use of liver and muscle glycogen and greater use of fats during exercise at 67% of VO_{2MAX} by women compared to men, even after exogenous carbohydrate feeding.[110] However, though overall and endogenous carbohydrate oxidation was lower in women across trials, relative and absolute changes in substrate utilization between water and glucose treatments were not different between sexes.

Extending this to anaerobic exercise, Hill and Smith (1993)[111] exposed a sex difference in the aerobic-anaerobic energy production ratio during maximal sprinting on the bike using gas exchange during a 30-second Wingate anaerobic test. In this study, women were estimated to rely on aerobic metabolism for 25% of their energy versus just 20% for men. Interestingly, as fiber type differences between men and women have not been observed, this difference may stem from endocrine status or some extracellular factor. It appears sex differences in energy derivation are important during both types of exercise.

Fuel selection is a determinant of the lipidomic profile,[112] as well as oxidative stress [113] and consequent antioxidant activity. The difference in use of substrates between aerobic and anaerobic exercise and the effects of training background and sex are important guiding factors for downstream events beyond merely the scope of ATP re-synthesis. Such differences include metabolites that might not differ at rest, but would be susceptible to differential regulation in response to exercise. Therefore, differences in substrate selection play a major role in impacting the metabolomic fingerprint of exercise.

Oxidative Stress. Over the past few decades much attention has been given to oxidative stress created during exercise and the antioxidant response.[114] Reactive oxygen species (ROS) were originally thought to be generated from leakage of electrons across the mitochondrial membrane during aerobic respiration.[115] Observations of ROS resulting from presumably anaerobic isometric exercise[116] debunked this, and now several sources of ROS production are recognized.[117] Production of ROS has been shown in response to auto-oxidation of the catecholamines[118] and oxyhemoglobin,[119] both of which increase with exercise intensity. Additionally, reperfusion-ischemia events, as are common with resistance training, are known to activate the ROS generating xanthine oxidase system. Lastly, eccentric contractions have yielded a greater elevation of ROS when compared to equivalent intensity concentric exercise.[120] This has led to the belief that ROS are thought to be released during the inflammatory response [121] with an exacerbated response due to muscle damage incurred through eccentric contractions. Presence of ROS has an impact on the metabolome in both activity levels of antioxidant systems (glutathione and γ-glutamyl amino acids) as well as redox status of specific metabolites, i.e. biliverdin/bilirubin.

Studies looking at the oxidative response to exercise have displayed elevated ROS levels immediately following exercise to be directly related to exercise load,[122] and subsequent attenuation of this response with training.[123] It is important to note that ROS generation seems to be associated with the overall challenge, rather than intensity or volume specifically. A prolonged bout of aerobic exercise may generate a more pronounced oxidative response than a shorter, though more intense weight-training session, or vice versa depending on the overall difficulty of each session. Research comparing exercise at different absolute intensities and using disparate exercises has found that, when the exercise is evenly matched by total workload, HR and/or rating of perceived exertion, the

ROS responses are similar,[123-126] with the caveat that the stress placed on the individual must exceed a minimal threshold for a marked response.[122] Exercise under such a threshold produces no change in ROS or antioxidant status, while even very short bouts of anaerobic exercise have proven sufficient to produce an oxidative response if the intensity is suitably high. [125,127]

It seems likely that certain markers of oxidative stress may simply have a higher threshold than others in terms of stimulus required to elicit a response, but no specific pattern of acute ROS generation or antioxidant response has emerged separating aerobic from anaerobic exercise. Though most report no difference between aerobic and anaerobic exercise,[128] multiple papers have found conflicting results in attempting to answer this question.[114,129] All that can be decisively stated is that acute bouts of both aerobic and anaerobic exercise are associated with oxidative stress within the body.

Long-term training of either kind offers protection from future bouts in the form of heightened endogenous antioxidant capacity. There is some evidence that endurance trained individuals have higher resting antioxidant levels, but this difference does not persist following a bout of exercise.[130] Similarly, sex differences for ROS generation and antioxidant activity have emerged at rest, with females having lower ROS levels and higher antioxidant activity.[131-134] Once again however most studies have failed to find any effect of sex in response to exercise.

Hormonal Signaling. The body's stress-response to metabolic disruptions from exercise is met via the coordination of numerous physiological adjustments, including the endocrine system. Exercise has such far-reaching effects in large part through its activation of a multitude of hormonal signals.[135] The major function of the endocrine system during and after exercise is to maintain homeostasis in the face of systemic perturbations in metabolic status. By presenting numerous challenges simultaneously (e.g. increased metabolism, substrate mobilization, disruptions in blood solutes, extremes in blood pressure and flow, thermal regulation, and

removal of “waste” products), exercise serves as a potent activator of the endocrine response. Circulating levels of the individual hormones are generally increased during and transiently following acute exercise of any type, however differences at rest are less often observed in trained versus untrained individuals.

Aerobic and anaerobic exercise do not greatly differ in their approximate endocrine response, yet there are subtle differences in the magnitude and time course involved. For example, some studies have reported anaerobic exercise to yield higher levels of growth hormone, [136] catecholamines,[137,138] and different cortisol time-courses between aerobic and anaerobic exercise.[139] Exercise endocrinology studies generally report intensity-dependent hormonal responses, and therefore higher levels with an anaerobic bout, but caution must be taken when interpreting these results, as work bouts not matched for duration make the anaerobic bout both higher in intensity and volume of work.[140-143] Exercise intensity may be the principal driving force differentiating specific exercise bouts, making anaerobic exercise a more potent stimulator of the endocrine system, but the influence of volume also seems to be of importance too.[137,138,144-147,148-153]

Because the endocrine system is a system of signaling molecules, but is also activated by a signal itself, it both impacts and responds to metabolite levels in the body. Cross-talk between the metabolome and hormones is likely impacted by training status in the context of exercise. With chronic training, relative submaximal workloads elicit comparable responses in trained and untrained individuals, however; matched absolute levels show a curtailed hormonal response post-training,[154] Moreover, individuals who regularly partake in high-intensity exercise exhibit an enhanced capacity for some responses like growth hormone[155] and epinephrine secretion during maximal intensity exercise, known as a “sport adrenal”.[156] Anaerobically trained individuals have been observed to have elevated

anaerobic signaling in response to exercise and at rest.[157] Still, other than hormones linked to anabolism, there seems to be little difference between endurance- and resistance-trained individuals, particularly in the context of the variable exercise response. Outside of the sex steroids and influences of the menstrual cycle, [149,158,159] males and females appear to have minimal difference in their endocrine response to exercise as well.[146,147,154,160,161,162]

Inflammation. Traditionally, inflammation was thought of as a purely negative occurrence. However, it serves to promote repair of damaged tissue and fight pathogens. As with other forms of stress, exercise induces release of key regulators of the inflammatory response, to help ameliorate strain placed on the system.[163] The inflammation following an acute exercise bout functions to hasten regenerative processes through release of pro-inflammatory cytokines, eicosanoids and other signaling molecule.[164] Further, this is followed by release of anti-inflammatory mediators, and accounts of a balanced pro- and anti-inflammatory response to acute exercise abound within the literature.[165-168] Such findings have led to the current notion that exercise's subsequent inflammatory cascade is not only inevitable, but also even necessary to bring about adaptations to exercise training.[169,170] The ideal training program would consist of acute bouts that minimized duration in pro-inflammatory status and balanced this with a concomitant anti-inflammatory response.

Direct comparisons of different types of exercise have identified incongruent inflammatory responses.[171] An early review revealed a pattern of lesser cytokine responsiveness to (anaerobic) eccentric exercise than prolonged aerobic bouts, although, at the time of this review (2002), very little research examining anaerobic exercise had been carried out.[172] More recently, cytokine response data compiled from acute and longitudinal anaerobic interventions proved inconclusive, citing variation in response sizes.[168] Authors did note post-exercise measurements should immediately or soon after exercise to capture effects.[168] Notably, discrepancies in

protocols, training status of the individuals and the context in which measurements were made (resting versus following an exercise bout) further contributed to a lack of findings.

Cytokine release is undeniably dependent on duration of exercise;[163] perhaps more so than intensity.[163] As the breadth of literature using controlled anaerobic sessions is still expands, the importance of exercise type and intensity may continue to evolve Not enough is known to fully differentiate the aerobic from anaerobic exercise response. Moreover, much research has focused on cytokines, and other mediators of inflammation may response differently. Indeed, the dose-response pattern for exercise intensity is well-documented for cortisol, a major anti-inflammatory hormone.[144,145]

One commonality in response to long-term training (weeks to months) of either modality, is that resting levels of cytokines hormones and other humoral signaling molecules seem to remain unchanged.[167] Anti-inflammatory signaling post-exercise, however, seems to be augmented in more fit individuals.[167] In fact, it has been proposed that the training effect of repeated exercise bouts on cytokines is better observed in the post-exercise condition rather than at rest.[173] Finally, studies specifically targeting sex differences have not identified any predictable differences between men and women.[146,147,174,175]

Inflammatory status is a major component of the metabolic state of the body. Inflammatory mediators consistently sow responsiveness to acute bouts of exercise, and reductions in chronic low-grade inflammation are seen with habitual exercise due to upregulation of anti-inflammatory mediators.[164] Presently, only an incomplete picture exists in regards to regulation of this multi-tiered response system through acute and chronic exercise.

THE METABOLOME & EXERCISE

Introduction. The effects of exercise reach across the miscellaneous systems and cell types in the body. Physiologic and epigenetic responses to exposures accumulate to give distinct phenotypes. Being so pervasive, the study of exercise demands a systems biology approach. Metabolomics offers a simultaneous look at a diversity of biomarkers and insight into a variety of pathways providing a means to further our understanding of exercise physiology. An advantage of examining the metabolome as compared to other 'omics science lies in its comparative proximity to the downstream observable phenotype. Age, Genetics (including sex), time of day, nutrition, health habits, prior toxin exposures, the microbiome and physical activity all influence individual health and the metabolome.[93,94,176-180]

Characterization of the acute metabolomic response to exercise provides a snapshot of the stress-response elicited by exercise, and it is this response that drives the adaptations leading to improved health and fitness of the individual. Employing such a comprehensive view of the exercise response provides a unique opportunity to holistically examine differences between the effects of aerobic and anaerobic exercise. A better understanding of the metabolic response to exercise will help guide targeted workout prescriptions for performance enhancement of athletes and improve the fitness and well-being of healthy and diseased populations. By accounting for the influence of sex and training background on these responses, findings can be more meaningfully applied to clinical settings, performance science, or to direct further mechanistic research.

Several papers defining the human metabolome have been published, but a variety of laboratory techniques (GC-MS, NMR), and statistical methods (PCA, OPLS, ASCA, etc.) make direct comparisons of results somewhat convoluted.[93,181] In early research, examining relatively few metabolites and/or including many "unknown" markers was common, rendering

comparisons of multiple reports impossible. In conjunction with the typical variance in investigative design factors, drawing conclusions across the literature is difficult.

Over the past decade the technology of metabolomics has advanced considerably, broadening its application across disciplines. Recently, this expansion has included exercise science. While application of metabolomics to exercise research is burgeoning, it is still in a nascent stage. The majority of training interventions have been limited to study of the resting metabolome, and have often been carried out in special populations, both of which reduce impact and translation of findings. Many investigations have employed *post* hoc analyses, non-experimental designs, included confounding factors, or other limiting factors. In turn, this has acted to limit the scope of findings. Given the difficulties in cross-examination of results and paucity of exercise metabolomics studies in the literature, much still remains to be discovered.

Animal Studies. Reports in animal studies have some useful information to be applied to the human metabolome. In a series of papers, Duggan and colleagues found energy metabolism and substrate mobilization to be major pathways responding to aerobic exercise in mice.[182-184] Another examination of the *murine* model similarly cited energy, oxidative stress, and branched-chain AA (BCAA) metabolism as the most effected pathways, and differ by exercise condition, a run to exhaustion at either peak velocity or critical velocity.[185] Because these are akin to VO_{2MAX} and lactate threshold pace, respectively, mice were able to run much longer at the latter speed. The conclusion regarding conditions, that duration rather than intensity was the determining factor, however, is a dubious one considering the 20% faster speed of peak velocity over critical velocity. Endurance horses (130-160Km distances) were found to have elevated creatine, but not creatinine post-race in plasma metabolites intimating protein catabolism and muscular breakdown.[186] This

metabolomics panel revealed perturbations of protein, energy and lipid metabolism as well as glycoprotein content of the plasma following races.[186] Meanwhile, both resting and post-race metabolomes proved predictive of race performance, showing efficacy for the use of metabolomics as a tool in a sports setting.[186]

Metabolomics & Health Status. Knowledge of the efficacy of exercise in the treatment of various disease states has prompted the application of metabolomics in this arena. A comparison of exercise training modalities was carried out with type 2 diabetics identifying higher TCAC and antioxidant activity in aerobically-trained and increased hypertrophy plus urea markers in resistance-trained participants.[187] Reports on obese and diabetic populations abound, but these findings may not tell us much about the healthy response to exercise. Illustrating this, plasma samples were examined in myocardial ischemia patients and healthy age- and sex-matched controls immediately and 60-minutes following a graded exercise test. Activation of multiple pathways in the healthy cohort were observed to be more evident. These included glycolysis/glycogenolysis, lipolysis, AA catabolism, ketolysis, adenine metabolism, antioxidation and TCAC at both post-exercise time points.[188] Markers of fat oxidation, in particular, varied directly with markers of fitness and health, and were nearly absent in the myocardial ischemia group. Moreover, Brugnara et al. (2012) identified disrupted mitochondrial function (blunted fatty acid and protein oxidation) before and after stationary cycling in Type 1 Diabetic patients when compared with age- and fitness-matched controls.[189] It seems responses in a diseased population are more blunted when compared to a healthy group.

This concept can be carried over to comparison of trained versus untrained individuals. Urinary metabolites of 9 competitive male master's cyclists and age-matched controls measured at rest and in response to an exhaustive endurance ride on a stationary bike displayed the importance of exercise participation. At rest, though the 2 groups displayed similar health

markers, clustering of biochemical processes revealed multiple pathways to differ between groups both at rest (TCAC, transport systems, AA and lipid metabolites) and in response to exercise (energy metabolism, TCAC and ATP turnover).[190] Remarkably, in comparison, sedentary men experienced regulatory changes in the opposing direction for all of these pathways.[190] Here, even with similar health status, the metabolome in an active population did not appear compatible with that of a sedentary one, at rest or in response to exercise. Further, the difference between groups seemed to be amplified by the stressor of exercise.

Exercise as a Stressor. Application of a metabolomics platform was employed in an investigation of the response to various stressors, including physical exercise.[191] Two major findings were that inter-individual differences seen at rest were accordingly intensified in response to a stressor, and the metabolomic panel was able to differentiate between stressors. The phenomenon of exercise (or other stressors) exacerbating observed differences in metabotype is not unique, as it has been observed for cold stress,[191] dietary stressors,[191,192] sleep deprivation,[179,180] and both short and high intensity,[193,194] and prolonged aerobic exercise.[186,188,190,192,195-198] Differences in training background and sex that might go undetected at rest may be uncovered with exercise exposure. Potentially, using 2 different exercise stressors could further elucidate differences. A final observation from the aforementioned study gave insight into the time-course of the acute exercise response. Changes from rest were greatest immediately following exercise and generally returned to baseline by 2-hours.[191] This has been confirmed in other studies,[181,198] and findings suggest a 30-90-minutes post-exercise window to see the largest effect on metabolites.

ACUTE AEROBIC VERSUS ANAEROBIC EXERCISE

Aerobic Exercise. Papers specifically targeting aerobic exercise make up the bulk of the exercise metabolomics literature, but few of these examine the acute exercise response. For

those that do, variation in protocols and metabolite selection act to limit consolidation of findings. Further, most research has involved the use of exercise in a clinical setting to mitigate some condition (i.e. cardiovascular disease), rendering results dubious for the general population, and almost certainly fallacious for an active one. Relatively few studies have employed an experimental design to characterize a healthy metabolomic response to exercise.

Peake et al. (2014) had trained cyclists complete "high" and "moderate"-intensity aerobic exercise sessions and found 24 and 16 of 49 metabolites to change, respectively, post-exercise.[199] Pathways represented were monounsaturated FA, AA and TCAC metabolism.[199] Similar markers related to substrate processing clearly differentiated pre from post-90-minutes cycling in a group of 24 trained males,[196] while a pilot study found evidence of substantial escalations in glycolysis along with elevated acylcarnitines, metabolites of the gut microflora, steroid metabolism and the specific appearance of mainly ketogenic AAs in the urine following exercise.[198] Confidence in these results must be tempered by the fact that diet and physical activity leading up to the testing were not controlled, nor was the acute bout itself, outside of duration. A recent report characterizing the metabolomic response to a marathon in 31 males identified perturbations in carbohydrate, FA, AA, TCAC, ketone and microbiome metabolism as well as evidence of alternative energy-producing mechanisms.[187]

Interestingly a study of an exhaustive aerobic cycling bout offered insight into the time-course of metabolite trajectories post-exercise.[200] Here the authors reported alterations of metabolites in response to the cycling bout to be more distinguishable from rest at the moment of fatigue (cessation of exercise) than those assessed 20-minutes into the post-exercise recovery.[200] Actual metabolomic perturbations were similar to those reported elsewhere. In all, acute aerobic exercise results in a metabolic fingerprint indicative of increased overall metabolism, characterized by elevations in TCAC intermediates, purine degradation products

and substrate catabolism of the major macronutrients (carbohydrates, proteins and fats) plus ketones with some evidence of interaction with the microbiome and increased oxidative stress and antioxidant metabolism.

Supplement & Nutrition Studies. Another area metabolomics has been applied in an acute exercise setting is sports nutrition. Notably, the totality of these studies is limited to aerobic exercise. While use of a nutritional intervention can cloud results, these still provide information on the exercise response particularly in the control group. In an attempt to deplete muscle glycogen or challenge the system in some way to allow the supplement in question to have an observable effect, many of these experiments have involved particularly long and/or arduous work bouts. This has led to reports of particularly large perturbations in substrate metabolism, ketone bodies and markers of oxidative stress.[196,197,201-203]

Anaerobic Exercise. There are comparatively few explorations into the acute anaerobic metabolome. Two such investigations compared different anaerobic exercise sessions to probe intensity-driven responses.[194,195] One of these used trained, and the other untrained males. Both used a panel of only 43 metabolites and small samples sizes (n = 12 and 7, respectively). In both cases roughly half of the metabolites were observed to change within an hour post-exercise, and in both studies, changes were greater in the more strenuous of the 2 bouts, indicative of an intensity-driven dose-response. Notably, both studies found the largest changes occurring later, rather than earlier during the recovery period, an observation not made with aerobic exercise designs.[194] Pathways identified in both investigations included BCAA catabolism, glycolysis and the TCAC, with elevations of purine degradation and antioxidant activity seen in trained,[194] and lipid metabolism in untrained individuals.[195] Finally, a recent study examining the time course of metabolomic perturbations following a bout of resistance training also identified BCAA catabolites, lactate

metabolites and TCAC/urea cycle intermediates to be altered by exercise within 1-hour post-exercise.[204]

Studies within each realm (aerobic and anaerobic) seem to indicate a dose-response for exercise intensity. If intensity is the main driving factor, perhaps greater metabolic disturbances could be expected of anaerobic exercise, while if duration or total workload has a greater effect, one might suspect aerobic exercise to bring about greater changes. Perusal of the literature shows magnitudes of change to be larger in aerobic than anaerobic exercise studies, with the largest perturbations coming from the longest and most arduous aerobic sessions.[187,203] Further, a review on exercise metabolomics cited steroid biosynthesis markers as the only metabolites to respond more to anaerobic than aerobic exercise.[181] However, these comparisons are indirect and the body of literature is heavily biased towards aerobic investigations. Fundamental questions such as this are critical to our understanding of the exercise response.

Direct comparisons between acute aerobic and anaerobic exercise in a healthy cohort do not yet exist. Comparisons between individual studies seem to indicate a common theme of energy metabolism and oxidative stress with possible differences in magnitudes of change, specific substrate selection (FA with aerobic exercise, BCAA with anaerobic) and timing of the responses. [194,195, 201-203]

AEROBIC VERSUS ANAEROBIC TRAINING

Research using metabolomics has proven capable of differentiating groups by age, sex, and training background.[197] However, to date, direct comparison of different training modalities using a metabolomics panel has only been reported in 2 studies, one in a diabetic population[187] the other in healthy sedentary males,[208] and both in the rested state. There are no such comparisons of training impacts utilizing non-sedentary individuals or within the context of changes to the acute exercise response. Further, even those studies examining just 1 mode of

training have primarily used resting comparisons and rarely looked at impact on the acute exercise response. Therefore, our knowledge of training effects is limited to changes observed at rest and is largely based on individual studies examining either aerobic or anaerobic training, predominantly aerobic. Finally, for studies not utilizing a special population (athletes or diseased state), comparisons are of previously sedentary individuals in a training study or between active and sedentary cohorts. In both cases inclusion of sedentary individuals could yield results different than comparisons of habitually active individuals.

Athlete Studies. Metabolomics characterizations of athletic populations has garnered much attention in an effort to identify predictors of performance. Similar to comparisons of diseased populations, translating findings for elite athletes to the general public can be problematic. However, these studies present an opportunity for comparison by training status. Specifically, an examination of elite endurance and power athletes identified differences in steroid biosynthesis, fatty acid metabolism, oxidative stress and energy metabolism between sport classifications. Despite limitations in the design of this pilot, many of the metabolic differences reported make intuitive sense with differences between endurance and power athletes.

Aerobic Training. Characterization of the resting metabolome in aerobically-trained individuals has revealed markedly higher metabolites of cardiorespiratory, inflammatory and antioxidant capacities.[14,15,54,190] The most commonly cited changes with training are increased purine metabolism, TCAC activity, ketogenesis, β-oxidation and oxidative metabolites.[181] In response to aerobic exercise, training seems to enhance the exercise response, though this notion is often confounded by poor matching of the exercise stressor.[188,190,196]

Post-hoc analyses of aerobic fitness revealed differences in the metabolomic response to aerobic cycling bouts in "high-fit" versus "low-fit" individuals (VO_{2max} values: > 60 and < 48 ml/Kg/min, respectively).[192,196] Specifically high-fit individuals have displayed greater energy metabolism and different lipidomic and oxidative stress profiles than low-fit individuals post-exercise and into recovery.[192,196] In a study contrasting trained and sedentary age-matched individuals at rest and post-exercise, findings were similar to those listed above, however; the quantity of markers differentiating groups was substantially exaggerated with exercise (from 46 to 467), and this was partially diminished following recovery (down to 234).[190] This demonstrates both the potency of the acute exercise stressor in discriminating between groups and gives some information on the impact of training on the time-course of the metabolomic shifts.

Anaerobic Training. Anaerobic training studies are rare, and near the entirety of their findings are included in a review by Daskalaki, et. al. (2014).[181] In brief, changes are similar to those seen for aerobically trained individuals and include perturbed FA metabolites and increased purine metabolites. One major publication included comparisons of 6 resistance, endurance or mixed training protocols of differing intensity and/or duration on muscle metabolomics in pre-diabetic individuals. Resistance training alone failed to produce changes in any of the individual metabolites as did low-volume high-intensity aerobic exercise (the 2 lowest-volume protocols).[210] Accordingly, in terms of number of metabolites changed, high-volume/high intensity was the most impactful exercise, followed by resistance training + low-volume/moderate-intensity (the 2 highest volume protocols), and across all groups total workload in training was most impactful for determining metabolomic changes. This contrasts with the intensity-dependent acute changes observed elsewhere,[194,211] and intimates training volume may be more impactful on the resting metabolome. However, within the aerobic

training groups, response size was intensity-dependent. The dichotomy of total volume across training groups and intensity within the aerobic-training groups as determining factors remains unexplained.

A recently published project used a smaller metabolomics panel (70 muscle and 43 serum metabolites) to compare endurance versus high-intensity interval training in healthy sedentary males. While both programs increased markers of AA metabolism and translation processes, authors found only endurance training to result in elevated carbohydrate metabolism.[208] Specific resting metabolite changes were noted for glycerol, pyruvate/alanine, AMP, creatinine, proline and threonine following endurance training and creatinine, glycolate, lysine and phenylalanine following high-intensity interval training.[208] The comparison of these interventions is the only such study in a healthy cohort. As such little is known of the long-term effects of anaerobic training on the metabolome at rest, and even less about how this training impacts the metabolomic response to acute exercise.

MALE VERSUS FEMALE

Sex differences are widely-reported in the resting metabolome and are mostly in agreement.[66,194, 212-214] Males regularly display higher metabolite levels in most pathways, with the biggest differences typically presiding in glucose, BCAA and purine metabolism, while females consistently report elevated lipid biosynthesis.[214] Notably, the concentrations in lipid species shift with age and male/female levels may even reverse from young-to-late adulthood.[66,212]

Females have rarely been included in metabolomic investigations of the chronic or acute exercise responses. Studies including both sexes often do not comment, or point out a lack of any sex difference.[203] Direct comparisons of the sexes are rare and have been lacking in some methodological form. These include use of restricted metabolomic panels including

10's rather than 100's of metabolites;[211,215,216] evaluation of sweat[215] or urine rather than tissue;[211,215,216] and very small samples (n ≤ 6-females and n ≤ 6-males).[211,215,216] Unfortunately, the only study using a larger (n = 30 + 30 males + females) examined eccrine sweat, and was unable to find any significant differences between sexes.[215] In response to exercise, similar patterns of difference were observed as those reported at rest. Males were seen to respond to exercise with greater increases in AA, carbohydrate, and energy metabolism.[211,216]

Interestingly, an all-female study investigated the responses to acute aerobic and anaerobic exercises in untrained individuals plus aerobic and anaerobic athletes. A single Wingate sprint on the bike sufficiently perturbed the urinary metabolome 30-minutes post-exercise, while a ride to exhaustion at 75% of VO_{2MAX} did not.[211] It was conjectured that metabolites of aerobic metabolism were reabsorbed in the kidney, and so absent from the urine, though elevated urinary TCAC metabolites have been reported following exercise in males.[190,194] Information on sex differences, and the female response to acute or chronic exercise of any kind are near completely lacking in the metabolomics literature.

CONCLUSIONS

Novel Biomarker Discovery. Though not the purpose of most designed studies, another feature of metabolomics is the ability to identify unknown entities or relate some process or experimental variable to unsuspected biomolecules. Examples within the context of exercise have been reports of up-regulated microbiome metabolism in response to acute exercise,[187,198] and links between β-aminoisobutyrate and FA oxidation,[191] or pseudouridine and anabolism.[196] Such reports exemplify the power of this technology in discovery science, to expand knowledge and provide direction for mechanistic investigations.

Conclusion. The specific changes in multiple biochemical pathways are overwhelmingly voluminous. Simultaneously tracking them all through individual assays would be impossible.

Fortuitously, evaluation of the metabolome encompasses all pathways concurrently. Though often used in cross-sectional analyses, metabolomics can be applied to controlled experimental designs to quantify phenotypic changes. Alterations in specific pathways appear to relate well back to the genotype[190] and clinical outcome measures.[188] This robustness presents metabolomics as a powerful descriptive tool for exercise physiology. Moreover, metabolomics and pathway analysis serve as vital tools for discovery.

Pathways observed to respond to exercise included carbohydrate/glycolysis, BCAA, FA, ketone, TCAC and purine metabolites for aerobic exercise. The same is true for anaerobic minus the FAs or ketones and lesser responses in the TCAC. The largest changes occur immediately following aerobic exercise and within an hour of anaerobic exercise. Both exercise modes display an intensity-dependent dose-response, but there is also strong evidence for volume or work to impact the metabolome. Training backgrounds appear to recapitulate the acute exercise differences. In general training leads to elevated levels at rest and greater responses in these same pathways following exercise.

Most of these observations are based on data from very different metabolomic platforms, participant pools, exercise protocols (acute or training), sample medium and timing of collection. Moreover, the body of literature is not that extensive, and so any conclusions must be made with caution. Data on anaerobic exercise and training is particularly lacking. Lastly, sex differences in the exercise response are as yet uncharacterized, and data on females is nearly absent.

Exercise induces physiological strain on the organismal level, resulting in a holistic response that, when repeated, will net adaptations across multiple systems. It is well established that aerobic and anaerobic exercise have both disparate and analogous responses.[11,19] This has been observed in the context of acute exposures[20] and chronic training.[217,218] Based on findings from previous metabolomics studies of exercise, we sought to induce significant changes in the metabolome using acute cycling (AE) and resistance training (AN) sessions, and characterize the response to each exercise mode. Use of individuals with either endurance (END) or resistance training (RES) experience provided insight into the impact of training history on the metabolic response to either a familiar or novel exercise bout. Finally, inclusion of males (M) and females (F) allowed for comparison of sex differences and interactions with exercise modality.

Our purpose was to implement an exploratory metabolomics panel to characterize and compare metabolomes at rest and perturbations brought on by exercise between conditions (aerobic and anaerobic exercise) and groups (END versus RES and M versus F).

Aim 1: To compare the metabolome at all 3 time points, as well as change values between pre-post$_0$ and pre-post$_{60}$ following acute exposure to each exercise bout. All participants were analyzed as a single cohort (n = 40). We hypothesized that the resting metabolome would be significantly altered following each bout of exercise (AE vs AN) in a mode-dependent manner.

Aim 2: To explore the effect of training history on metabolomic signatures during exercise and at rest. Collapsing across sex created groups of 20 END and 20 RES. We hypothesized that adaptations from previous training would affect both resting and exercise metabolomes, with an interdependence on exercise mode and training history.

Aim 3: To explore effects of sex on metabolomic signatures during exercise and at rest. Collapsing across training background created groups of 20 M and 20 F. We hypothesized that sex would affect resting and exercise metabolomes, with and interact with exercise mode.

METHODS

DESIGN PARAMETERS

Approach. This study was aimed at characterizing and comparing the responses to aerobic and anaerobic exercise with a metabolomic panel. The resting metabolome of endurance- and resistance-trained individuals, as well as their exercise responses to acute bouts of both types were examined to typify the acute responses and test for differential profiles relating to training background. Additionally, sex differences were explored. To accomplish this, a mixed model, repeated measures, crossover design was implemented. Approval from the Rutgers Institutional Review Board and informed consent from all participants was obtained prior to enrollment.

Participants. N=40 participants for this study were evenly divided as follows n=10 endurance trained males, n=10 resistance-trained males, n=10 endurance-trained females and n=10 resistance-trained females. These groups were collapsed across either sex or training background to give groups of 20 endurance-trained individuals (END), resistance-trained individuals (RES), males (M) or females (F). Pre-screening involved a brief health and training history questionnaire and a short interview by the principal investigator. Inclusion criteria consisted of absence of any known cardiorespiratory, metabolic or other health issues and a minimum of 2-years of regular participation in their current physical activity regimen, limited to one primary modality. Exclusion criteria included recent illness/injury (> 1-week disruption to normal exercise routine within the past 6 months) and use of tobacco products or recreational drugs. Supplement use was permitted so long as participants had not begun use within 6-months of enrollment and continued through completion of the study.

Design. We explored time and condition-dependent responses to exercise while considering both sex- and training background-dependent effects on the metabolomic response. Targeted recruitment, balanced groupings, matched exercise conditions, vehicle selection (serum) and use of Metabolon's advanced platform allowed relative quantification of a broad range of metabolites in a controlled setting. Experiments were designed to characterize the resting metabolome of END, RES, M and F, and to describe the systemic changes for each when exposed to bouts of aerobic and anaerobic exercise on separate days. In turn, comparisons were made between these 2 disparate exercise types. Our specific design included resting values on separate days, permitting an estimation of day-to-day variation within individuals, and thus a more meaningful comparison between groups.

PROTOCOL

TESTING SESSIONS

Testing sessions were completed for assessment of training status, collection of fitness data, and exercise prescription prior to the condition sessions. All testing, including aerobic and anaerobic conditions, took place at the Center for Health and Human Performance (CHHP) in the Institute for Food, Nutrition, and Health at Rutgers University.

Visit 1. Participants reported to the CHHP following a 2-hour fast and having refrained from exercise for the previous 24-hours. Upon arrival, body composition testing with a Bod Pod (Cosmed, Concord, CA) was performed using the Brozek equation.[219] After this, participants were permitted to warm-up for 10 minutes before completing a continuous graded exercise test (**Table 1**) on a Velotron cycling ergometer (Racermate, Seattle, WA). Depending on estimated fitness level, individuals started between stages 70 and 220 Watts (W) and continued cycling, increasing wattage by 30 W every 3-minutes. For testing, participants were fitted with a Polar M-400 HR monitor (Polar Inc., Bethpage, NY) and Quark C-PET metabolic measuring system

(Cosmed, Concord, CA) for HR and breath-by-breath assessment of gas analysis with 15-second rolling averages. All participants met a minimum of 3 American College of Sports Medicine criteria for attainment of VO_{2MAX},[220] and continued until volitional fatigue or the inability to keep cadence ≥ 80 rpm.

Cycling VO_{2MAX} Protocol			
Stage	**Wattage (W)**	**Elapsed time**	**Begin**
1	**70**	**3:00**	**F END+RES**
2	**100**	**+3:00**	**M RES**
3	130	+3:00	
4	**160**	**+3:00**	**M END**
5	190	+3:00	
6	220	+3:00	
7	250	+3:00	
8	280	+3:00	
9	310	+3:00	
10	340	+3:00	
11	370	+3:00	
12	400	+3:00	

Table 1. VO_{2MAX} Protocol. Right-most column demarcates average starting workload for group indicated.

Ventilatory threshold (VT) was calculated using the graphical ventilatory equivalent method as described by Davis et al. (1980) and verified by 2 separate exercise physiologists.[220] These values were further verified using internal software on the Quark C-PET. The average of the 2 closest values was taken as VT, with a discrepancy never more than 0.1L/min O_2 between comparators. Maximal aerobic power output (PO_{MAX}) was calculated as per Naperalsky et al. (2010).[221]

PO_{MAX} =(Watts on last full stage) + (30) x (% of final stage attempted)

Seventy percent of this wattage value was used as the intensity prescription during the aerobic exercise session (AE). This percentage was chosen, as it allowed for an arduous exercise bout while still falling below average measured VT of all participants.

Visit 2. During the second visit to the CHHP participants completed 10RM testing for 7 lower-body exercises following the guidelines set forth by the National Strength and Conditioning Association (NSCA).[222] In order, exercises were back squat, leg press, Romanian deadlift, lunges, prone leg curls, leg extensions and seated calf raises. A certified strength and conditioning specialist (CSCS-NSCA) administered testing to ensure participant safety and proper execution of all lifts. The same 7 exercises, in the same order, were performed during the anaerobic exercise session (AN). Three sets of 10 repetitions were performed for each exercise using 90% of the determined 10RM weight and a 90-second recovery between all sets. This combination of load, rest interval, repetition and set count was selected as it falls within the NSCA guidelines for hypertrophy training and well within an "anaerobic" working intensity.

EXERCISE CONDITIONS

Control Factors. In accordance with findings on metabolomic variability, certain precautions were taken to minimize external variability.[62,223] Individuals reported to the CHHP for each of the 2 experimental conditions having refrained from exercise for the previous 24 hours and avoided intense or prolonged exercise for the previous 48. Prior to their first condition, participants were provided a 24-hour food log to record their diet so that it could be replicated prior to the second condition. They were encouraged to eat as normal, other than consuming a meal 2-3 hours prior to the visit, with only water after this point. All visits started between 8:00AM and 12:00PM, and exact time of day was matched between conditions for each participant. Lastly, individuals were instructed to abstain from ingestion of caffeine intake that day (none within 6-hours of the visit). Each condition was run 3-7 days removed from the previous session (either testing visit 2 or condition visit 1). In addition to relative workload, exercise sessions were matched for total duration (45 minutes), and were perceived as equally demanding by subjects during pilot testing (rating of perceived exertion (RPE) between 8 and 10 for both conditions).[224]

Aerobic Session (AE). Upon arrival, participants sat quietly for 10-15 minutes and a resting blood draw from the antecubital vein was performed (T0). This was followed by a 15-minute warm-up period before commencing with the prescribed exercise. The AE cycling bout was a 45-minute steady state ride at ≈70% of PO_{MAX} on the same ergometer as used during VO_{2MAX} testing. A trainer monitored HR and cadence during the session and participants were encouraged to keep their cadence at ≥ 90rpm. In order to maintain an aerobic effort, if HR went above 90% HR_{MAX} (as observed during VO_{2MAX} testing), or cadence below 80rpm, wattage was adjusted until HR and/or cadence normalized.

After completion of the exercise, participants were promptly escorted into an adjacent phlebotomy room where an indwelling catheter was inserted into the contralateral arm for subsequent draws, collected immediately (T1) and 1-hour post-exercise (T2). The line was flushed with 4ml saline before and after each draw, as well as halfway between them, to prevent coagulation. Nutrient intake was not permitted at any point during the session, but participants were allowed to drink water *ad libitum* throughout.

Anaerobic Session (AN). AN was identical to AE, including sample collection, except that the cycling bout was replaced with a resistance training session of the 7 exercises listed above. A CSCS oversaw all sessions to ensure participant safety and proper execution of all lifts. If the workload was too high (participant could not complete a full set of 10) they were spot-assisted to completion and weight was adjusted accordingly on any subsequent sets of that exercise. The entire exercise portion of the session took ≈45-minutes. Order of AE and AN was randomized for all 40 participants.

METABOLOMICS ANALYSIS

Quantification. Following collection and processing, serum samples were aliquoted into 2.5 ml cryovials and stored at -80^{O}C until all testing was complete. Samples were packed on dry ice

and shipped to the Metabolon® core facility in Durham, NC for analysis with Metabolon's Discovery Panel of 754 metabolites. Automated ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry was used for metabolite identification. Raw data were extracted, peak identified, quality control processed with internal and external controls, and quantified by area under the curve using Metabolon's hardware and software. Compounds were then identified by comparison to library entries of purified standards or recurrent unknown entities based on 3 criteria: retention index, mass, and the MS/MS forward and reverse scores between the experimental data and authentic standards. All samples were run in a single batch to reduce variance. *Please refer to reference 203 for a detailed description of analysis techniques.*

Values were normalized in terms of raw area counts through log transformation and rescaled to set the median equal to 1.00 for each metabolite. Then, missing values were imputed with the minimum value. All statistical procedures were carried out using these values. Data were re-run excluding any metabolites using > 25% imputed values. This did not impact the global biochemical profiles, and inclusive data is reported here. Instrument variability for UHPLC/MS (7%) was determined by median relative standard deviation of internal standards. Total process variability (9%) was determined by median relative standard deviation of all endogenous metabolites present in all 240 sample matrices, not including added standards. Both values met Metabolon's acceptance criteria.

Statistical Analyses. Unsupervised approaches to analysis were performed in Array Studio and included hierarchical clustering and principle components analysis (PCA). The hierarchical clustering method performed was complete clustering using the Euclidean distance, where each value was factored independently as a vector to all other metabolite values. Condition clusters were identified and superimposed on the output. PCA analysis was done by singular value

decomposition, where the coefficient maximizing linear variance is identified and labeled as the first principle component. This iteration is then repeated for components statistically independent to the first for all observations in the data. This procedure reduces dimensionality and generates data clusters by overall metabolomic profile for all samples run allowing identification of similar and dissimilar data points.

Pathway analysis was performed with Metaboanalyst (http://www.metaboanalyst.ca). Metabolites were entered for each condition separately, and pathway impact and significance, based on the *Homo sapiens* Kegg Pathway relations were calculated. Pathway algorithms included the Hypergeometric Test for over representation analysis and Relative-betweeness Centrality for pathway topology analysis.

AE/AN Comparisons. A 2x3 (condition x time) Repeated Measures Analysis of Variance (RMANOVA) with follow-up univariate ANOVA's for the delta values between time points were run for all metabolites using a p value of < 0.05. Due to the multiple comparisons in a metabolomics data set, the false discovery rate was determined using the Benjamin-Hochberg equation with a stringency set at a q value of 0.1. For those values determined to be statistically significant by both p and q criterions, magnitude of change was used to identify the most meaningful changes.[220] Average changes from T0-T1 and T0-T2 for each exercise were calculated and considered physiologically significant when the change reached 2-fold-of difference between the resting and post-exercise values. (with resting values set at 1.00, this means a post-exercise value ≥ 2.00 or ≤ 0.50). Unless otherwise stated, changes discussed below meet p, q and 2-fold criteria. Values from the RMANOVA analyses are given in arbitrary units or expressed as a percentage of resting level.

A 2x2x3 (training background x exercise condition x time) repeated measures analysis of variance (RMANOVA) with follow-up univariate ANOVA contrasts was run for all metabolites

using a p value of < 0.05. Additionally, change values were calculated from T0 to T1 and T0 to T2 for each group. These delta scores were compared between groups, (END vs RES) using unpaired t-tests, with an alpha level p < 0.05. Due to the multiple comparisons found in a metabolomics data set, the false discovery rate was determined using the Benjamin-Hochberg equation with a stringency set at a q value of 0.1 for all ANOVA and t-tests. All changes discussed below meet both p and q criteria. Because metabolite values are all median-normalized, log-scaled means of the group, values are in relative arbitrary units and reported as relative values or relative changes from resting.

END RES Comparisons. A 2x2x3 (sex x exercise condition x time) repeated measures analysis of variance (RMANOVA) with follow-up univariate ANOVA contrasts was run for all metabolites using a p value of < 0.05. Additionally, change values were calculated from T0 to T1 and T0 to T2 for each group. These delta scores were compared between groups, (M vs F) using unpaired t-tests, with an alpha level p < 0.05. Due to the multiple comparisons found in a metabolomics data set, the false discovery rate was determined using the Benjamin-Hochberg equation with a stringency set at a q value of 0.1 for all ANOVA and t-tests. All changes discussed below meet both p and q criteria. Because metabolite values are all median-normalized, log-scaled means of the group, values are in relative arbitrary units, and reported as relative values or relative changes from resting.

For those values determined to be statistically significant by both p and q criterions, a magnitude of 2-fold change from resting level was used to identify the most impactful metabolic perturbations.[220] With resting values set at 1.00, this means a post-exercise values ≥ 2.00 for increases or ≤ 0.50 for decreases. This more stringent listing of metabolites was used for Venn diagram comparisons and to generate lists of individual metabolites showing unique and common perturbations for the groups and/or conditions. Pathways containing these

metabolites were identified and similarly to those distinguished by pathway analysis, all metabolites within these pathways were further scrutinized. To do this, differences between groups or conditions in delta scores or relative metabolite levels and for all statistically significant ($p < 0.05$, $q < 0.1$) metabolites were considered, regardless of magnitude change.

M/F Comparisons. Statistical analysis was identical to that for END/RES comparisons, but used data grouped by sex rather than training background.

RESULTS

Chapter 4.1

The Exercise Metabolome: A comparison of Acute Aerobic and Anaerobic Exercise

INTRODUCTION

Aerobic & Anaerobic Exercise. There is a rich body of literature supporting the salutary effects of physical activity. Regular participation in exercise has been correlated to lowered risk of chronic diseases and reduced mortality rates,[1] and the efficacy of exercise has even been favorably compared to pharmaceutical treatment of disease.[2] However, despite having daily recommendations for physical activity and exercise guidelines from multiple governing bodies (American College of Sports Medicine (ACSM), American Heart Association, National Strength and Conditioning Association (NSCA)), there still exists a knowledge gap in our understanding of exercise's ability to foster good health. Categorically, there are two major exercise modalities: aerobic and anaerobic. While they both foster health benefits, the former primarily targets cardiorespiratory fitness and mitochondrial biogenesis, while the latter provides primarily neuromuscular strength gains and muscle hypertrophy. Both bring about improved health, body composition and energy levels, plus recent findings support (aerobic) cardiovascular benefits of high intensity (anaerobic) training.[3] Indeed, because of the benefits seen with exercise in general, questions regarding the specifics of optimizing exercise prescription (duration, intensity, etc.) persist even at the most fundamental level of exercise modality.

Information regarding the differences between exercise mode comes largely from studies examining aerobic and anaerobic exercise individually, while direct comparisons between the two are less common. Instead, research tends to focus on the effects of different levels of intensity within a given mode of activity. Certainly, at its most

rudimentary level, intensity is the defining characteristic separating aerobic from anaerobic exercise. And while this work has revealed much regarding the role of exercise intensity as a continuum, anaerobic exercise is more than just very high intensity aerobic exercise. Physiologic changes are non-linear in the transition from aerobic to anaerobic exercise (e.g. lactate threshold), and some metabolic adaptations are even oppositional in nature (i.e., shifting of the type IIa muscle fibers).

It is the accumulation of acute exercise-generated stressors, and subsequent management of these stressors, that leads to the beneficial adaptations (improved health and fitness metrics) from chronic exercise. Identifying the characteristic acute response will lead to a greater understanding of the potential for exercise to drive long-term changes in human physiology and biochemistry.

Metabolomics. Exercise is known to have wide-ranging effects, touching upon multiple metabolic pathways and physiological systems. One needs to cast a wide net in attempting to characterize and identify major responses to exercise while also remaining specific enough to differentiate between types of exercise. Global serum metabolomic profiling provides an extensive platform befitting the broad response to exercise with the sensitivity to discern between aerobic and anaerobic exercise.

Metabolomics is the latest iteration of high throughput biotechnology, with the distinction of providing the closest assessment to phenotype of the “omics” approaches to research (**Figure 1**). While this broad-spectrum approach is common in systems biology, it is relatively new to exercise science, with the first untargeted comprehensive metabolomic examination of the exercise response not coming until

2015.[4] Most early metabolomics investigations examined a few dozen metabolites, typically aimed at one or two metabolic pathways, yielding results that expanded little on traditional assay-based studies. Combining high-specificity assessment tools (HPLC-MS/MS) and automated measurement with stringent controls and accessible informatics (like the Metabolon® LIMS system and the Human Metabolome Database, used here) has enabled researchers to simultaneously evaluate hundreds of metabolites in a given tissue sample. This methodology has only become available in the past decade, and so there exists only a limited number of consistent findings describing the exercise metabolome. Here we seek to add to this foundation.

Results from metabolomic explorations of the exercise response have typically found the greatest disruption to the resting metabolome, by magnitude and/or number of changes, to occur immediately after exercise, with individual metabolites having various post-exercise response patterns over the following 1-2 hours (i.e., return to baseline, continued disruption, latent perturbation).[5,6] Major changes to the metabolome brought on through aerobic exercise include changes in energy balance, adenine metabolism, tricarboxylic acid cycle (TCAC) intermediates and substrate (carbohydrates, lipids, amino acids (AA's) and ketones) mobilization/catabolism, as well as in oxidative stress/antioxidation.[5-12] Other areas reflecting change are varied: vitamin/cofactor cycling,[6] insulin signaling and cardiovascular function,[5] and immunity and calcium homeostasis.[8] Studies using anaerobic exercise have similarly identified perturbations in pathways of energy balance and oxidative stress.[13-16] Typically larger

perturbations within glycolysis and an emphasized branched-chain AA (BCAA) metabolism have been reported as well.[14,15]

A review paper by Daskalaki, et al. (2014) concluded that more arduous exercise leads to greater purine metabolism, enhanced glycolytic activation, and higher oxidative stress during and/or following exercise.[17] Certainly more stress likely elicits a greater response, however, the question of how to define arduous leaves much to be clarified considering that it can be impacted by intensity, duration, and total workload. Most studies have utilized just one exercise, which was typically aerobic. Several studies, however, compared exercise of the same mode (either aerobic or anaerobic) at different intensities. There have been mixed results supporting both intensity[13,14] and duration[15] as the primary factor in driving the observed exercise response. A non-exercise-specific study characterizing the metabolomic response to various stressors (36-hour fast, oral glucose and lipid loads, cold exposure, or 30-minutes aerobic cycling) was able to differentiate between challenges by the resultant metabolomic profile.[5] Here it is posited that changes in the metabolome will be sensitive enough to capture similarities and identify differences between aerobic and anaerobic exercise. In addition to expanding upon the available body of literature on exercise and metabolomics, particularly in characterization of the anaerobic response, this paper will be the first to directly compare responses to aerobic and anaerobic exercise bouts in a single cohort of human participants.

PURPOSE AND HYPOTHESIS

The aim of the present study was to use an exploratory metabolomics platform to examine the metabolic response pattern of active, healthy individuals to acute bouts of aerobic versus anaerobic exercise. We hypothesized that the two bouts would have large areas of overlapping responses representing a generalized exercise response as well as subsets of metabolites for AE and AN, delineating the unique response to each type of exercise. Moreover, given the healthy population used, these responses would lie a foundation for defining the “normal” or healthy response to exercise. Findings from this study have the capacity to provide targeted direction for mechanistic research designed to elucidate the health benefits of physical activity. To date, the acute response to a single bout of resistance training without a concurrent nutritional intervention has not been investigated using metabolomics in humans, and no study has featured a translational design, implementing a lifting protocol similar to what is used in a real-world setting. Finally, no metabolomics study has examined both aerobic and anaerobic exercises sessions using a crossover design in humans. This study addresses these issues in the context of comparing and contrasting the aerobic and anaerobic exercise responses immediately and 1-hour into a post-exercise recovery.

METHODS

Research Design. Active, young adults were exposed to 2 different exercise bouts (matched for duration and perceived exertion) and their metabolomic profiles were characterized before and at 2 times post- exercise, immediately (T1) and 60-minutes (T2) after. This design was implemented to characterize and differentiate between the acute responses to aerobic and anaerobic exercise in healthy individuals. Active participants were targeted as research has shown notable differences in the exercise metabolome of trained and untrained individuals, with the untrained response being widely heterogeneous.[8] The applied multivariate analyses were pointed towards an integrative biology approach to further our understanding of exercise physiology.

Participants. Forty (20 males + 20 females) active, healthy young adults (age: 18-39 years) were recruited to the Rutgers Center for Health & Human Performance (CHHP) for participation in the study. Inclusion criteria consisted of absence of any known cardiorespiratory, metabolic or other health issues and a minimum of 2-years of regular participation in their current physical activity regimen. Exclusion criteria included recent illness/injury (> 1-week disruption to normal exercise routine within the past 6 months) and use of tobacco products or recreational drug use. Supplement use was permitted so long as participants had not begun use within 6-months of enrollment and continued through completion. Participants reported to the lab for 2 testing trials followed by 2 experimental conditions randomized for order. Procedures were approved by the Rutgers University Institutional Review Board, and participants signed informed consents prior to enrollment.

EXERCISE TESTING TRIALS

Visit 1. Participants reported to the CHHP following a 2-hour fast and having refrained from exercise for the previous 24-hours. Upon arrival, body composition testing with a Bod Pod (Cosmed, Concord, CA) was performed using the Brozek equation.[18] After this, participants were permitted to warm-up for 10 minutes before completing a continuous graded exercise test on a Velotron cycling ergometer (Racermate, Seattle, WA). Depending on estimated fitness level, individuals started between stages 1-6 and continued to progress 1 stage at a time. During testing, participants were fitted with a Polar M-400 HR monitor and Quark C-PET metabolic measuring system (Cosmed, Concord, CA) for HR and breath-by-breath assessment of gas analysis with 15-second rolling averages. All participants met a minimum of 3 ACSM criteria for attainment of VO_{2MAX},[19] and continued until volitional fatigue or the inability to keep cadence ≥ 80rpm.

Ventilatory threshold (VT) was calculated using the graphical ventilatory equivalent method as described by Davis, et al. (1980) and verified by 2 separate exercise physiologists.[20] These values were further verified using internal software on the Quark C-PET. The average of the 2 closest values was taken as VT, with a discrepancy never more than 0.1L/min O_2 between comparators. Maximal aerobic power output (PO_{MAX}) was calculated as per Naperalsky et al. (2010):[21]

$$PO_{MAX} = (\text{Watts of last full stage}) + 30 \times (\%\text{ of final attempted stage completed})$$

Seventy percent of this wattage value was used as the intensity prescription during the aerobic exercise session (AE). This percentage was chosen, as it allowed for an arduous exercise bout while still falling below average measured VT of all participants.

Visit 2. During the second visit to the CHHP participants completed 10-repetition max (10RM) testing for 7 lower-body exercises following the guidelines set forth by the NSCA.[22] In order, exercises were back squat, leg press, Romanian deadlift, lunges, prone leg curls, leg extensions and seated calf raises. A certified strength and conditioning specialist (CSCS) administered testing to ensure participant safety and proper execution of all lifts. The same 7 exercises, in the same order, were performed during anaerobic exercise session (AN), using 90% of the determined 10RM weight. This combination of load, rest interval, repetition and set count falls within the NSCA guideline for hypertrophy training, well within an "anaerobic" working intensity.

EXERCISE CONDITIONS

Control Factors. In accordance with findings on metabolomic variability, certain precautions were taken to minimize external variability.[23,24] Individuals reported to the CHHP for each of the 2 experimental conditions having refrained from exercise for the previous 24 hours and avoided intense or prolonged exercise for the previous 48. Prior to their first condition, participants were provided a 24-hour food log to record their diet, so that it could be replicated prior to the second condition. They were encouraged to eat as normal, other than consuming a meal 2-3 hours prior to the visit, with only water after this point. All visits started between 8:00AM and 12:00PM, and exact time of day was matched between conditions for each participant. Lastly, individuals were

instructed to abstain from ingestion of caffeine intake that day (none within 6-hours of the visit). Each condition was run 3-7 days removed from the previous session (testing 2 or condition 1). Exercise sessions are matched for total duration (45 minutes), and were perceived as equally arduous by subjects during pilot testing (rating of perceived exertion (RPE) between 8 and 10 for both conditions).

Aerobic Session (AE). Upon arrival, participants sat quietly for 10-15 minutes and a resting blood draw from the antecubital vein was performed (T0). This was followed by a 15-minute warm-up period before commencing with the prescribed exercise. The AE cycling bout was a 45-minute steady state ride at ≈70% of PO_{MAX} on the same ergometer as used during VO_{2MAX} testing. A trainer monitored HR and cadence during the session and participants were encouraged to keep their cadence at ≥ 90rpm. In order to maintain an aerobic effort, if HR went above 90% HR_{MAX} (as observed during VO2MAX testing), or cadence below 80rpm, wattage was adjusted until HR and/or cadence normalized.

After completion of the exercise, participants were promptly escorted into an adjacent phlebotomy room where an indwelling catheter was inserted into the contralateral arm for subsequent draws, collected immediately (T1) and 1-hour post-exercise (T2). The line was flushed with 4ml saline before and after each draw, as well as halfway between them, to prevent coagulation. Nutrient intake was not permitted at any point during the session, but participants were allowed to drink water *ad libitum* throughout.

Anaerobic Session (AN). AN was identical to AE, including sample collection, except that the cycling bout was replaced with a resistance training session of the 7 exercises listed above. Three sets of 10 repetitions were performed for each exercise with a 90-second recovery between all sets. A CSCS oversaw all sessions to ensure participant safety and proper execution of all lifts. If the workload was too high (participant could not complete a full set of 10) they were spot-assisted to completion and weight was adjusted accordingly on any subsequent sets of that exercise. The entire exercise portion of the session took ≈45-minutes. Order of AE and AN was randomized for all 40 participants.

Metabolomics Analysis. Following collection and processing, serum samples were aliquoted into 2.5 ml cryovials and stored at -80^{O}C until all testing was complete (up to 3½-months). Samples were packed on dry ice and shipped to the Metabolon® core facility in Durham, NC for analysis with Metabolon's Discovery Panel of 754 metabolites. Automated ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry was used for metabolite identification. Raw data were extracted, peak identified, quality control processed with internal and external controls, and quantified by AUC using Metabolon's hardware and software. Compounds were then identified by comparison to library entries of purified standards or recurrent unknown entities based on 3 criteria: retention index, mass, and the MS/MS forward and reverse scores between the experimental data and authentic standards. All samples were run in a single batch to reduce variance.

Values were normalized in terms of raw area counts through log transformation and rescaled to set the median equal to 1.00 for each metabolite. Then, missing values were imputed with the minimum value. All statistical procedures were carried out using these values. Data were re-run excluding any metabolites using > 25% imputed values. This did not impact the global biochemical profiles, and inclusive data is reported here.

Statistical Procedures & Data Analysis. Unsupervised approaches to analysis were performed in Array Studio, and included hierarchical clustering and principle components analysis (PCA). The hierarchical clustering method performed was complete clustering using the Euclidean distance, where each value was factored independently as a vector to all other metabolite values. Condition clusters were identified and superimposed on the output. PCA analysis by singular value decomposition was used to reduce dimensionality and generate data clusters for all samples showing differences between condition and time.

Pathway analysis was performed using Metaboanalyst (http://www.metaboanalyst.ca). Metabolites were entered for each condition separately, and pathway impact and significance, based on the *Homo sapiens* Kegg Pathway relations were calculated. Pathway algorithms included the Hypergeometric Test for over representation analysis and Relative-betweeness Centrality for pathway topology analysis.

A 2x3 (condition x time) Repeated Measures Analysis of Variance (RM ANOVAOA) with follow-up univariate ANOVA's for the delta values between time points were run for all metabolites using a p value of < 0.05. Due to multiple comparisons in a

metabolomics data set, the Benjamin-Hochberg false discovery rate was determined with a stringency set at a q value of 0.1. For those values determined to be statistically significant by both p and q criterions, magnitude of change was used to identify the most meaningful changes. Average changes from T0-T1 and T0-T2 for each exercise were calculated and considered physiologically significant when the change reached 2-fold-of difference between the resting and post-exercise values (with resting values set at 1.00, this means a post-exercise value ≥ 2.00 or ≤ 0.50).[25] Unless otherwise stated, all changes discussed below meet these 3 criteria. Values from the RM ANOVA analyses are given in arbitrary units or expressed as a percentage of resting level.

RESULTS

PARTICIPANT CHARACTERISTICS

Details for participant descriptive and fitness data can be found in **Table 1**. Fitness characteristics of the group displayed a healthy, fit cohort of active individuals. Males were significantly taller, heavier and leaner than females. Fitness measures reflected greater strength (squat and leg press 10RM-weight) and aerobic power (VO_{2MAX} and PO_{MAX}) but not VT for males than females in absolute terms. However, differences between male and female groupings were found to eliminated when scaled to fat free mass (FFM). This Is suggestive of a metabolic uniformity across our participant-pool in terms of muscle strength and oxygen processing capacity per unit muscle mass.

SESSION CHARACTERISTICS

The average wattage used during AE was of 70±0.6% PO_{MAX}. The average weight used across lifts during AN was 89±.05% 10RM weight. When asked to give a rating of perceived exertion at the conclusion of each session the average RPE for AE was 9.1±0.7, and for AN was 9.2±0.9 With a range of 8-10 for both exercises, indicating both exercise sessions were equally challenging for the participants.

METABOLOME-LEVEL RESULTS

Hierarchical Clustering. Clustering individual metabolites across time points yielded sporadic groupings of AE and AN data points (**Figure 2**). This intermittent pattern suggests more similarity than difference in the AE and AN responses, supporting the notion of a common or generalized exercise response, rather than distinctly unique AE and AN profiles.

Principle Components Analysis. Unsupervised PCA of the data by participant, session and time revealed distinct clustering patterns (**Figure 3**). Resting data for AE and AN showed no separation, and may partially explain why hierarchical clustering failed to show a clear AE/AN difference. Significant overlay between post-AE and post-AN data implies there was appreciable similarity between responses to the 2 exercise modalities. In comparing post-exercise values, AE appeared more displaced from T0 (leftward) than AN, intimating a greater perturbation of the resting metabolome following AE than AN. The tighter clustering of AE data indicated a more homogeneous response to AE than AN. Separation between AE and AN was more apparent for values at T1 than at T2. Further, T2 groupings are less separated from T0 than are T1. Thus, revealing recovery to a more similar-to-resting profile by T2 than was observed at T1 for both AE and AN.

Pathway Analysis. Given findings from the unsupervised analyses above, potential areas of similarity and exclusivity were probed via pathway analysis. Using an FDR-corrected p value < 0.05 and an impact factor > 0.1, for changes during exercise (T0→T1) showed more overall pathway activation immediately following AE, but this mostly resulted from greater fatty acid (FA) metabolism (**Figure 4**). AN did have larger impacts for xanthine and glycolysis, though these bioenergetic pathways, as well as the TCAC were prominent for both exercises (**Figure 4**). By T2, changes in FA metabolism were evident for both AE and AN (**Figure 4**). Additionally, there was evidence of inflammatory signaling (glucocorticoids, eicosanoids and other lipid mediators) following both exercises (**Figure 4**). This served as a cursory look at overall pathway activation. Specific metabolites and direction of change were identified using RM ANOVA results.

RM ANOVA RESULTS

Resting Differences. Initial, resting differences between visits uncovered only 1 metabolite to show a significant within-subjects difference, adenosine monophosphate (AMP): 2.72 versus 1.03 (arbitrary units) for AE-T0 versus AN-T0 respectively, $p < 0.05$. However, further examination revealed a single participant's value to drive this difference, and removal of this outlier (60-fold change T0-T1) eliminated this difference (corrected = AE: 1.26 AU versus AN: 1.03 AU, $p > 0.05$). As such, there was no detectable day-to-day variation, between-conditions, in the resting metabolome of these fit, healthy individuals, indicating good control of extrinsic variables between conditions.

Common Exercise Response. The ability of exercise to bring about changes in the metabolome was apparent in the number of metabolites altered by each exercise bout, as was the degree of similarity between the two disparate exercises (**Supplementary Figure 1**). In fact, because exercise proved such a potent and far-reaching stressor, differences between the 2 disparate exercises bouts were largely obscured without further scrutiny. As such, a Venn diagram for the changes from T0-T1 and T0-T2 were generated using only those metabolites with at least a 2-fold changes (**Figure 5**), and these metabolites were used to determine physiologically meaningful differences between AE and AN.

Similarities in the AE versus AN responses both at T1, and T2, were again apparent. In terms of metabolite count, nearly 50% of those markers changed during AE, and 67% of those changed during AN were also altered in the opposing exercise bout at T1 and > 40% and 60% at T2, respectively (**Figure 5**). Characterizing these similarly changed

metabolites at T1 (29) and T2 (14) showed a predominance of increased bioenergetcs for both exercises (**figure 6**). This was reflected by a rise in 3 TCAC intermediates, 2 glycolytic end-products, 2 short-chain acylcarnitines and 2 purine salvage markers.

Both AE and AN revealed signs of inflammatory and oxidative stress. Following each exercise, corticosterone, the eicosanoids 12-HETE and 12-HHTrE and glutathione metabolites were all elevated (**Figure 5**). Additionally, 2 polyunsaturated β-monoacyl-glycerides (MAG's), the endocannabinoid 2-arachidonoylglycerol (2AG) and 2-docosa-hexaenoylglycerol (2DHA), were decreased by AE and AN (2AG = 50% & 46%; 2DHA = 49% & 35% of baseline at AE-T1 & AN-T1 respectively). Together, these changes displayed elevated aerobic and anaerobic metabolism along with mixed inflammatory signaling and antioxidant cycling as part of a generalized exercise response.

Other generalized responses included reduced levels of the acylcholines (all 8, including 3 phospholipids with polyunsaturated moieties) and glycerophosphoinositol (**Table 2**). The changes in the acylcholines along with the β-MAG's may link inflammatory lipid mediator production to lipid membrane turnover following exercise (**Figure 7**). Also reduced following both exercises were a pair of secondary bile acids (glycohyocholate: 43% & 42% and glycoursodeoxycholate: 44% & 43% of baseline at AE-T1 & AN-T1, respectively), perhaps relating to the microbiome signature. Serum metabolite levels depict a general exercise signature of elevated energy metabolism, specifically, increased glycolysis, TCAC intermediates, (very) short-chain acylcarnitines and purine turnover along with increased inflammatory cascade signaling, glutathione cycling and disrupted circulating markers of the microbiome (**Supplementary Table 1**).

Repeating these analyses at T2 yielded a curtailed overall response in terms of number of metabolites and magnitude of change, but also an altered metabolomic signature. Bioenergetic metabolites still predominated the metabolome, but there was a shift away from carbohydrate and amino acid (AA) metabolism and continued (AE) or increased (AN) FA metabolism post-exercise (**Table 2**). Moreover, decreases in circulating bile acids and gut microbiome metabolism markers are more extensive at T2 for both AE and AN. See **Supplementary Table 1** for fold-of-change values of all significantly altered metabolites.

Microbiome. A substantial number of metabolites indicated an interaction between gut metabolism and exercise. Both AE and AN yielded similar response patterns, resulting in increased butyric acid derivatives (these markers, however, also relate to other metabolic pathways) and decreased bile acids and food/plant component xenobiotics (**Table 3**). The decreased xenobiotics were observed largely at T2, while bile acids were lowered at both time points. Though present for both exercises, AN caused a greater reduction in some of these markers than AE.

Unique to AE. More metabolites were significantly altered for AE at both T1 and T2 than were for AN (**figure 5**). Fold-changes for significantly altered metabolites were significantly higher for AE than AN at T1 ($p < 0.05$), but not T2. In addition to the TCAC intermediates listed above, the AE response included increased succinate and β-citrylglutamate, a TCAC stimulator, signifying further elevated aerobic-energy production associated with AE as compared to AN (**Table 4**). The urea cycle metabolite, argininosuccinate, was significantly elevated following both exercises, but 54% higher at

AE-T1 in comparison to AN-T1, $p < 0.05$, $q < 0.1$. This was taken to indicate greater use of AAs as an energy source during AE as compared to AN.

FA metabolites were heavily represented in the unique AE-T1 metabolomic signature (25 of the 33 altered, including 17 actual FA's, **Table 2** and **Supplementary Table 1**). Notably nearly every long chain FA (LCFA), both ketone bodies and most of the medium chain- (MC-) and polyunsaturated FA's (PUFA's) on the panel were elevated immediately following AE but not AN. This difference between exercises largely dissipated by T2 (**Table 5**).

A time course comparison shows a tendency for smaller perturbations at T2 as compared to T1, indicating a substantial degree of recovery from many of these metabolic disruptions within the 1-hour post-AE. Decreases from T1 to T2 were seen in several metabolites reflecting stress and inflammation (**Figure 8**). Moreover, FA metabolites with anti-inflammatory and/or antioxidant properties (n3-PUFA's, 3-methyladipate, 16-hydroxypalmitate, sphinganine) were more elevated following AE than AN at both time points (**Table 5**). Vanillylmandelate, the breakdown product of the catecholamine epinephrine, was 2-fold elevated at AE-T1, but not AE-T2 (166% and 89% above resting, respectively) reflecting a powerful, but transient stress response to AE. Together, these changes reflect a somewhat fleeting general stress response and a relatively fast pro- to anti-inflammatory shift post-exercise following AE.

Unique to AN. In response to AN, there was relatively limited evidence of FA metabolism at T1 with evidence of an imbalance in FA metabolism (malonate + azelate + maleate). AN displayed an increased FA metabolism during the post-exercise period,

though the imbalance persisted at T2 (**Table 5**). Interestingly, increases in citrate were 2-fold at T2 in the AN condition only, (AE-T1: 139%, AN-T1: 179%, AE-T2: 158%, AN-T2: 215%), despite other TCAC intermediates responding robustly to both exercises. This may reflect continued disruption to energy balance at AN-T2, only. Additionally, even though lactate and pyruvate showed the same response pattern to AE and AN (i.e. increased at T1 only), lactate experienced significantly greater perturbations in response to AN than AE, thus demonstrating the greater in-task reliance on glucose metabolism, and anaerobic glycolysis during AN in particular.

Similarly, though xanthosine and hypoxanthine were increased for both AE and AN (both at T1 and xanthosine at T2), they a magnitude difference (**Figure 9**). Increases were roughly 2x greater for AN than AE at T1 (1249% versus 640% for xanthosine and 495% versus 240% for hypoxanthine). Moreover, while xanthosine decreased in the hour following AE (to 483% of resting level), it continued to climb following AN (up to 1370%). Hypoxanthine was also significantly elevated at T2 for AN only. Purine salvage pathway metabolites exhibited the largest fold-of changes of all metabolites on the panel in response to either exercise at either time point. Notably, xanthosine was the only metabolite on the panel to be significantly altered > 2-fold following both exercises at both time points, hinting it may be particularly sensitive in reflecting the acute response to exercise.

It appears as though recovery from AN may be more delayed in comparison to AE. In opposition to the pro-to-anti-inflammatory shift seen following AE, there was a continued pro/anti-inflammatory response, with elevated corticosterone and both

eicosanoids at T2 following AN (**Figure 8**). Additionally, evidence for prolonged management of oxidative stress management via the glutathione mechanism was present at AN-T2 but not AE-T2 (**Supplementary Table 1**).

While overall AA and branch chain (BCAA) metabolism did not clearly differ between exercise modes, α-hydroxyisocaproate, the leucine degradation product and hydroxymethylbutyrate (HMB) precursor, was elevated immediately following AN but not AE. This is a known anti-catbolite, and in fact is sold as an anabolic supplement for body building. Though α-hydroxyisocaproate fell below the 2-fold criteria for both exercises at T2, it was still statistically elevated for AN-only ($p < 0.05$, $q < 0.1$) and greater for AN (90% increase) than AE (49%). Moreover, the related monohydroxy-FA, α-hydroxycaproate, was also significantly elevated immediately following AN-only (225% of resting level). Both molecules act as substrates for the same peroxisomal α-hydroxy-oxidase, giving the possibility for a similar metabolic regulation.[26]

Other metabolites. Phosphate appeared to show a differential response, albeit at different time points post exercise. There was a 112.5% increase from resting at AE-T1 and a 56.2% decrease at AN-T2. Upon further scrutinization, phosphate was 25% above baseline at AE-T1 ($p < 0.05$, $q < 0.1$), and returned to baseline by AE-T2, giving the same overall pattern for both exercises even though these latter changes did not reach the applied 2-fold criteria. A final perturbed metabolite related to energy metabolism was pantothenate, or vitamin B5. Circulating B5 was increased immediately following AN (136% above baseline), but not AE, suggesting its release from the tissue during AN.

DISCUSSION

AE and AN both induced a large number of perturbations in the resting metabolome, displaying the potency of exercise as a stressor. In fact, the far-reaching effects served to obfuscate some of the more meaningful differences, and so a minimum criterium of a 2-fold difference from resting was used to highlight the most meaningful changes. Even still, both exercises were remarkably similar. Hierarchical clustering and PCA revealed mostly similar responses between exercise modes, supporting the idea of a common response to exercise in general. RM ANOVA results substantiated this. As perhaps the most compelling evidence for a general response to exercise, amongst the entire panel no metabolites were significantly changed in opposing directions for the 2 exercise conditions, even without the 2-fold criterium. Between visits, resting metabolomes were indistinguishable by PCA and 0 of the 754 individual metabolites were different when compared by RM ANOVA at rest. This showed good control of extrinsic variables, and allowed confidence in the assumption that all differences observed post-exercise were resultant from the exercise bouts.

Both pathway analyses and RM-ANOVAs for the individual metabolites showed energy-related pathways to be the focal point of the exercise response, plus major activity relating to the stress response (inflammation, oxidative stress), and evidence of a link to gut microbiome activity. Observed changes within these pathways largely agreed with previous metabolomic explorations of the exercise response.[17]

Discernable differences in the PCA plot along with a count of altered metabolites and the average magnitude of change all indicated perturbation to the resting

metabolome was greater for AE than AN and at T1 than T2. Previous research also identified disruption of the metabolome to be greatest immediately post-exercise,[5,6] though it is possible that sampling at a time several hours post-exercise (allowing time for constitutive transcription, translation and subsequent shifts in the metabolite pool) may have altered the observed effect of each bout. Mixed conclusions have previously been reached in terms of the roles of intensity and duration as drivers of the metabolomic response to exercise.[13-15] Here exercises were matched for total duration, perceived difficulty and AE and AN were at set relative intensities. Still, the continuous (AE) versus intermittent (AN) nature of each bout led to greater total energy consumption in-task for AE. This may have driven the larger response for AE than AN at T1. A factor driving the more prolific changes brought on by AE could have been panel bias. Roughly half of the panel (355 of 754 metabolites) fell within lipid metabolism, which was the major area of difference between the 2 bouts. A heavily lipid-based panel is more likely to identify AE as a greater perturbator to the overall metabolome. Finally, the ability to push through the AN bout may have been limited in those lacking a familiarity with resistance training (half of the cohort) due to overcoming balance/coordination issues, which may have distracted from physiological exertion. On the contrary, riding a stationary bike for AE was familiar to everyone and offered no such challenge. This could have impacted homogeneity of responses, and in fact, AE values were observed to cluster tighter on the PCA plot, perhaps indicating more homogeneity. Further studies with more and less complex exercise movements with or without familiarization may help elucidate these issues.

Although the primary observation was that of a similar response to AE and AN, certain distinguishing characteristics in their respective metabolomic profiles were evident. Within energy balance, observed differences in purine turnover metabolites demonstrated greater ATP turnover elicited by AN. Elevated purine salvage markers have been reported in metabolomic studies of exercise for both modes,[8,12,14] but never compared. The higher levels seen with AN here likely reflect the faster instantaneous-rate of ATP turnover leading to larger negative spikes in the depletion of the ATP pool with AN. This demand on ATP turnover rate versus volume is the quintessential difference between AN and AE, the former placing strain on the high-energy phosphate pool (ATP, creatine-phosphate) and replenishment (glycolysis), and the latter on maximizing efficiency of substrate oxidation. The magnitude of response for markers in the purine salvage pathway (xanthosine in particular), along with the different trajectories of the post-exercise response points to their sensitivity in differentiating between exercise modes (AE versus AN). Notably, xanthine oxidase was not found in the serum at any time (data not reported) signifying xanthosine levels were increased from cellular release and not produced in circulation. This could reflect greater tissue (muscle) damage incurred during AN, though traditional markers of muscle damage (3-methyl-histidine, 2-aminioadipate and 5-methylthioadenosine) were only modestly changed and did not significantly differ between conditions. These markers better represent protein catabolism rather than tissue damage, aligning with our findings here.

In the cell, phosphate is thought of as a part of energy dynamics, but in the serum it reflects signaling related to bone matrix turnover. The pattern of a transient rise during

exercise with a subsequent drop to or slightly below baseline has been shown in research.[27,28] Further, both the peak and trough of this change have been shown to be exaggerated in an intensity-dependent fashion.[27] Though the intensity of AN was higher in the traditional sense of the word, the perceived difficulty of both sessions was equivalent. Surprisingly, we observed greater increases at AE-T1 and a lower drop at AN-T2 (below baseline). This seeming incongruence may relate back to the greater challenge on high-energy phosphate production during AN leading to lower release or higher uptake of circulating phosphate by the working muscle. It seems more likely, however, that the physical nature of the exercises selected played a role here. Cycling as a mode provides little to no compression of the skeletal tissue along the longitudinal axis, i.e. loading, while the resistance exercises used repeatedly placed heavy axial-loads on the long bones of the legs.

The high intensity of both conditions led to elevated pyruvate and lactate at T1, but not T2, demonstrating glycolytic flux during, but not after exercise, when a shift to a glycogen sparing mode appeared to have occurred. Significantly higher lactate levels for AN point to greater anaerobic glycolysis to meet the greater rate of ATP demand. Greater lactate levels coincided with limited FA metabolism during AN, potentially playing a role in limiting FA mobilization during AN. However, this presents a chicken-or-egg scenario where the use of FA during AE may have simply precluded the need for anaerobic metabolism leading to comparatively lower lactate levels with AE.

AAs, like carbohydrate metabolites, tended to show significantly lesser changes at T2 than T1, indicating greater use of this substrate during, rather than after exercise, again

indicating a shift from prioritizing energy production to protein sparing. Despite similar changes in overall AA/BCAA metabolites, argininosuccinate increased more during AE, suggesting elevated urea cycling from heightened deamination during AE. Coupled with the differential TCAC intermediate levels, this potentially supports greater catabolism of AA's as an energy substrate during AE. Within the BCAA metabolites, α-hydroxyisocaproate levels were higher at AN-T1 than AE-T1, associating AN with anabolic as opposed to catabolic signaling. This also presents the potential of a metabolite reflecting anabolic effects that responds during or immediately post-exercise. Further study to validate this is still necessary, but such a marker could be useful in gauging the effectiveness of various anabolic protocols within a short time-frame.

Sweeping increases of TCAC intermediates for both AE and AN clearly demonstrated the importance of this cycle in bioenergetics. Previous reports of elevated TCAC intermediates have been used to reflect energy use in response to exercise.[5-16] Differential expression in circulating levels of these metabolites (AE > AN for succinate at T1 and T2, AN > AE for citrate at T2) may reflect differences in anaplerotic and cataplerotic reactions associated with each exercise modality as a result of disparate substrate selection. In a metabolomics study of race horses, appearance of citrate, along with creatine, was taken to indicate leakage from the mitochondria and lysis of the muscle from the strain of exercise.[29] However, a lack of 2-fold increases in creatine as well as metabolites linked with muscle damage do not support the same conclusion here. Notably, citrate and succinate are positioned before and after the α-ketoglutarate

dehydrogenase reaction, respectively. Given the importance of this step in AA entry to the TCAC, their respective differences intimate the possibility of greater AA oxidation with AE, supporting our conclusion from changes seen in the AA metabolites. However, assessment of circulating, as opposed to cellular levels of these metabolites limits this to conjecture of cellular processes. Follow-up studies using muscle tissue are necessary to determine actual flux through this cycle at each step. Still, these findings are notable in that, while elevated circulating levels of TCAC intermediates has been published following AE[5-12] and AN[13-16] differences between the 2 exercise modes has not been previously reported.

Central dogma of substrate selection during exercise dictates greater FA use with AE.[30] Though slower to oxidize, FA's offer the greatest return in volume of ATP production. Ample evidence for greater FA oxidation during AE existed here in that FAs of all kinds (LCFA, MCFA, PUFA), acyl-carnitines and ketones were all increased 2-fold immediately following AE but not AN. Ketone body metabolism goes up in times of inadequate substrate availability.[31] Circulating acetoacetate and β-hydroxybutyrate clearly show the demand on substrate metabolism with AE. Both of these increased during AE and remained elevated at T2, but were unchanged by AN, exemplifying the strain on substrate-level energy dynamics with AE.

During exercise, FAs are obtained from intra-muscular triglyceride stores plus released into the circulation for uptake and oxidation in the muscle tissue. Given the use of serum as a medium in this study, extra-muscular adipose depots likely played a significant role in determining the lipidomic profile observed. Regardless of the source

(adipocytes, intramuscular stores, and/or cellular membranes), heightened FA metabolism during AE was seen along with *en masse* increases in a variety of FAs and their metabolites: monohydroxy-FAs, dicarboxylate-FAs, FA-carnitines, amino-FAs, membrane lipids: phospholipids, acylcholines, sphingolipids, monoacylglycerides, ketone bodies and lipid mediators. In the wake of increasing FA availability, perturbations to signaling and effector molecules (like the eicosanoids, and endocannabinoids) and their precursors (PUFAs, β-MAGs) displayed exercise-mode-dependent responses seen with AE but not AN. These differences may have resulted from substrate selection for fuel optimization during exercise.

Despite the clear preference for FAs as a substrate during AE and not AN, it appeared that after both exercise bouts, the body was in an AA and glucose sparing state, relying heavily on FA metabolism for repletion of energy stores for recovery. Though many of these FA metabolites failed to reach the 2-fold criterium at the AN-T2, an increase in FA metabolism was seen with AN by AN-T2 and differences between AE and AN had largely dissipated by T2. In fact, FA metabolites tended to increase T1→T2 for AN, but not AE. It has been suggested that while AE uses more FAs in-task, AN may lead to greater FA oxidation throughout the day.[32] It is possible that extension of the time course may have shown AN to surpass AE in terms of FA metabolism at some point post-exercise, but this is impossible to determine without area-under-the-curve calculations throughout an extended post-exercise recovery period. What can be said is that despite the perception of “fat burning” zones during exercise (within the aerobic spectrum), energy expenditure during activity leading to an energy deficit appears to be

recovered through use of FAs regardless of exercise mode or substrate choice during exercise.

In addition to the trend of sharper decreases following AE (T1→T2) for metabolites of energy balance, markers of stress, inflammation, and antioxidant activity were lower for AE than AN at T2, but not T1. These largely fell within the super pathway of lipid metabolism, being robustly observed across the inflammatory eicosanoids, and the n6-PUFA's.

The post-exercise inflammatory *milieu* has been thoroughly researched showing a mixed anti- and pro-inflammatory response.[34-36] This is seen here in response to both exercises at T1. Pro-inflammatory markers, however, largely dissipated by following AE, and remained significantly elevated at T2 for AN only. Again, differences in substrate selection, mainly volume and timing of FA use, may have acted to drive this difference in recovery rate. Recovery itself is an aerobic process, and perhaps making the transition from work to recovery is streamlined with AE when FA mobilization and priming of mitochondrial enzymes is already engaged. Interestingly, the FA metabolites that did show significant perturbations at AN-T1 were those often used in diagnostic panels to identify an increased reliance on peroxisomal oxidation and metabolic disruption of mitochondrial function, β-oxidation or FA processing. It may be that the same mitochondrial activation needed to meet the energy demands during AE is not required during AN. Regardless, the delayed FA mobilization/oxidation for AN paired with a delayed metabolism of lipid mediators within the inflammatory cascade further extended recovery from AN.

Other Metabolites. Sphingosine is readily converted to/from the lipid mediator sphingosine-1-phosphate, a circulating facilitator of angiogenesis and immune trafficking.[33] Sphingosine was statistically elevated at T1 and T2 following both exercises, but reached a 2-fold increase for AE-T1 only (**Table 6**). Interestingly, a study revealed both immune functioning and angiogenesis as major biochemical + genetic responses to aerobic exercise.[7] These results suggest sphingosine as a potentially sensitive marker of the post-exercise response, particularly for AE. Other studies are needed to confirm and extend upon these observations.

While the interaction between the glut flora and exercise has been researched, most studies focus on longitudinal, rather than acute effects of exercise, and tend to select times further removed from the exercise bout itself.[37,38] Still, exercise has been found to increase markers of microbiome activity, rather than decrease them, as seen here. This paradoxical finding may be explained by the decreased blood flow to the viscera during exercise. Given the intensity of both exercise sessions, blood flow restrictions are expected to have been substantial, giving less opportunity for gut microbiota to release their products into circulation. Further research is needed to explain this finding.

Conclusion. Though much of the discussion concentrated on the differences between AE and AN, far more striking was the degree and extent of similarity in the responses to these 2 very different exercise bouts. Major activated pathways included energy metabolism and the stress-response. Perturbations to the resting metabolome elicited by AE were more abundant than AN, though this may reflect a difference in total workload as much as any inherent difference between exercise modes, or just the lipid-

heavy bias of the platform used. Most of the changes brought about by either exercise were also seen with the other. Differences between the two modes largely reflected substrate choice, during exercise, FA for AE and carbohydrate for AN.

The nature of the exercise stressor itself was connected to differences in the exercise response, ATP turnover rate for AN versus substrate availability for AE. AN relied more heavily fast-acting anaerobic glycolysis during exercise and brought elevated markers of ATP turnover (purine salvage). AE showed greater fuel flexibility, utilizing glucose, AA, FA and ketone bodies. Clearly higher FA mobilization/oxidation during AE was accompanied by extended downstream signaling within lipid metabolism (**Supplementary Figure 2**), perhaps connecting the 2. In fact, much of the difference between the responses to AE and AN relate back to fuel selection as a driver, specifically FA use with AE. After AN ended, fuel optimization for maximized ATP turnover rate was no longer the priority and substrate utilization shifted towards FA's post-exercise. Accompanying the increase in FA metabolism post-AN was increased lipid mediators and other metabolites indicating pro-inflammatory status. By T2, AE and AN both more closely resembled resting status than they did immediately after exercise, indicating substantial recovery within an hour of exercise cessation. Nonetheless, observations supported a more expedient recovery process following AE than AN.

Potentially sensitive markers to the exercise response identified here included xanthosine, relating to ATP turnover, α-hydroxyisocaproate, relating to anabolic metabolism, sphingosine, relating to the immune response and angiogenesis and phosphate, potentially relating to bone turnover and or energy dynamics. Additional

investigations of these markers and the observed metabolic response patterns in different cohorts is recommended to corroborate and expand upon the findings herein. Bile acids and xenobiotics depicting acute changes in microbiome metabolism with exercise were also evident. These results should be used to guide more targeted approaches to delineating between the aerobic and anaerobic exercise response.

Limitations. A sample of 40 participants is relatively large for a crossover design using metabolomics, however, it is too small to draw conclusions for the general population without further evidence. Given the mixed sample of recreationally active males and females, additional investigations are warranted.

Cases have been made supporting both exercise intensity[13,14] and duration,[15] usually when clamping the other. Because of the 3-way relationship between intensity, duration and total workload, it is impossible to vary only one of these at a time. In this design, translatability of findings, in terms of using realistic exercise bouts, was favored over matching total workload (by estimated workload via HR or oxygen consumption). Moreover, using matched total workload would have also caused massive discrepancies in perceived difficulty and duration of the exercise session. Still, the reported differences between AE and AN herein may largely stem from differences in total workload rather than inherent differences between them.

AN caused greater intermittent disruption of cellular energy balance (depletion of high-energy phosphates), while the challenge of AE manifested mainly through use of intra- and extra-cellular macronutrient stores, especially FAs. Comprehensive metabolomic panels, contain more metabolites related to substrate metabolism, and

are often disproportionately lipid-based. The metabolomics panel used here was no exception (355 of 754 markers were lipids). Given the dramatic difference in FA use during AE and AN exercise, this could have acted to skew whole metabolome-level comparisons and bias interpretations of amalgamated RM ANOVA results. Caution is necessary when interpreting data from any exploratory metabolomic panel.

Use of serum as a medium for sampling was chosen to capture a systemic-level response. It is impossible to determine what tissues may be involved in the uptake and release of the measured metabolites. However, massive shifts in blood flow occur during exercise, with skeletal muscle receiving ≥ 80% of total cardiac output, helping justify the assumption of serum levels largely reflecting changes in muscle metabolism. Time points selected were pre, plus 0- and 60-minutes post. While this does give a unique window into the immediate acute response, it may have missed fleeting changes in metabolite levels within the post-60 recovery period. Further, delayed responses beyond this time frame, including metabolites requiring more time for gene expression and downstream production, remain unassessed. In all, this was the first direct comparison of AE and AN in healthy individuals using a comprehensive metabolomics panel.

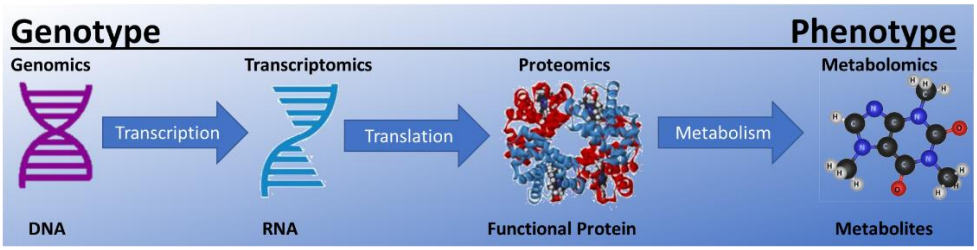


***Figure 1*.** Metabolomics offers the closest estimation of actual phenotype when compared to other "omics" approaches to research.

Category	Mean ± SD	Males	Females
Age (years)	23.0 ± 3.1	24.0 ± 3.9	22.1 ± 2.3
Height (cm)*	168.8 ± 9.3	175.2 ± 7.3	162.5 ± 6.2
Mass (kg)*	67.2 ± 9.4	72.9 ± 7.8	61.5 ± 7.2
% Body Fat*	18.1 ± 7.5%	12.8 ± 5.7%	23.3 ± 4.8%
Fat Free Mass (kg)*	55.2 ± 9.7	63.3 ± 4.9	47.1 ± 5.8
relative VO_{2MAX} (ml/kg/min)*†	46.5 ± 8.0	49.6 ± 10.1	43.3 ± 3.2
VT (% of VO_{2MAX})	72 ± 11%	73 ± 14%	71 ± 0.1%
PO_{MAX} (Watts)*†	218 ± 57	257 ± 53	178 ± 23
Squat 10RM (kg)*†	72 ± 29	88 ± 32	57 ± 17
Leg Press 10RM (kg)*†	182 ± 62	212 ± 65	153 ± 42

Table 1. Descriptive Characteristics for the entire cohort and separated by sex. SD = standard deviation; FFM = fat free mass, VO_{2MAX} = max aerobic capacity; PO_{MAX} = max aerobic power; 10RM = 10-repetition max weight.
*Males significantly different than females, $p < 0.05$
†Difference eliminated when scaled to FFM

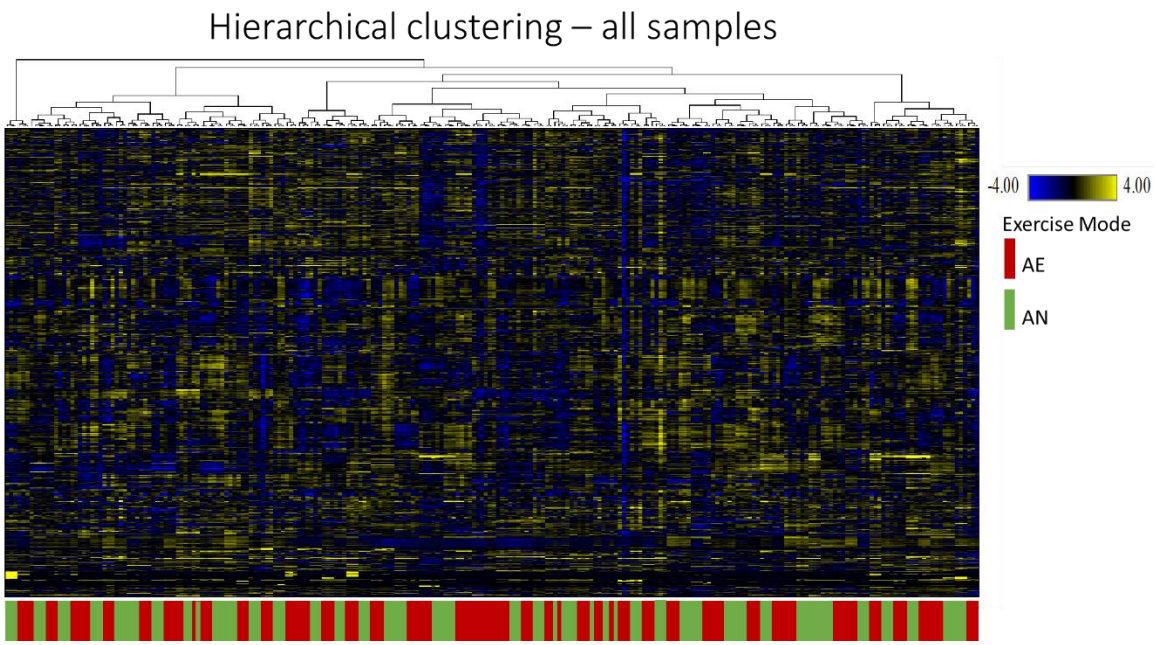


Figure 2. AE (red) and AN (green) clusters at the bottom of the display show only small contiguous groupings repeatedly broken up by one another. Substantial intermingling of AE and AN "segments" does not support patently different responses to the 2 exercise modes.

PCA Analysis Scatter Plot

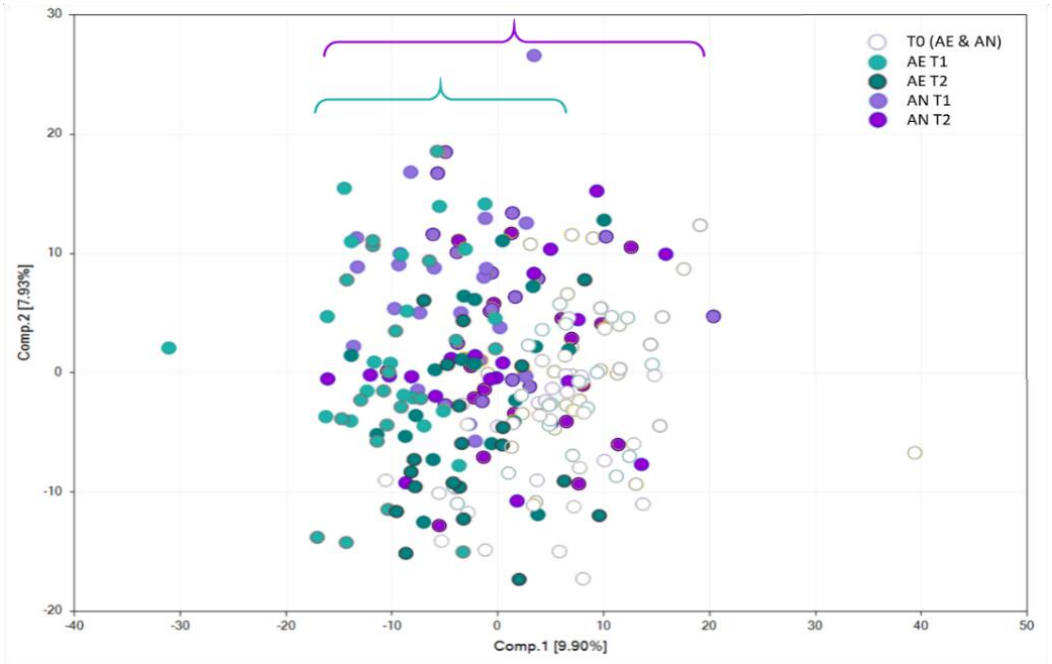


Figure 3. PCA with T0 values as white circles & gray outline; AE at T1 and T2 as lighter and darker shades of green; AN T1 and T2 as lighter and darker shades of purple, respectively. Brackets indicate spread of data along x-axis for AE (green) and AN (purple). Clustering of the resting (T0) versus post-exercise (T1 and T2) data can be seen for both exercises with a lager separation at T1 than T2 (separating resting from post-exercise as well as between AE and AN). AE = aerobic exercise session; AN = anaerobic exercise session; T0 = resting T1 =

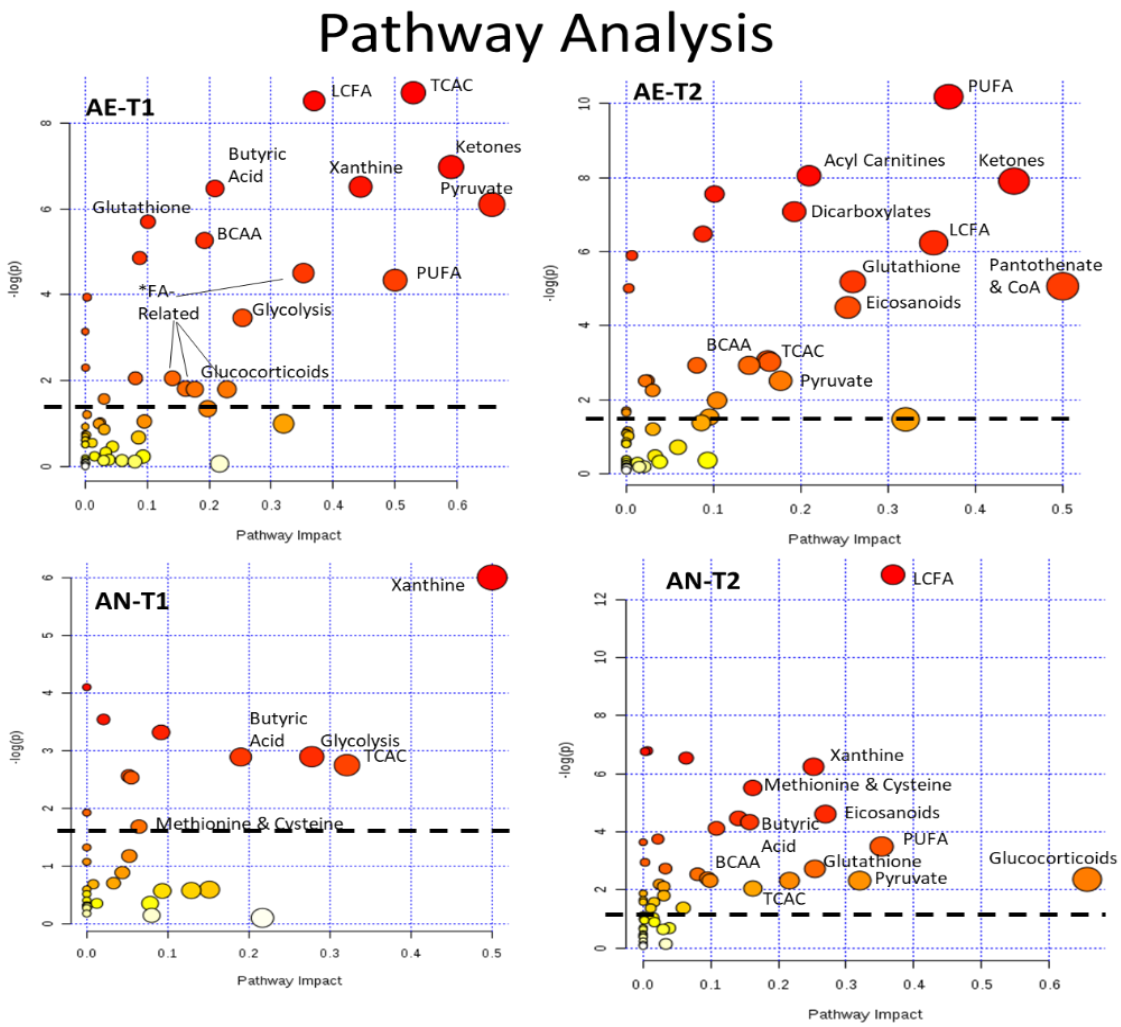
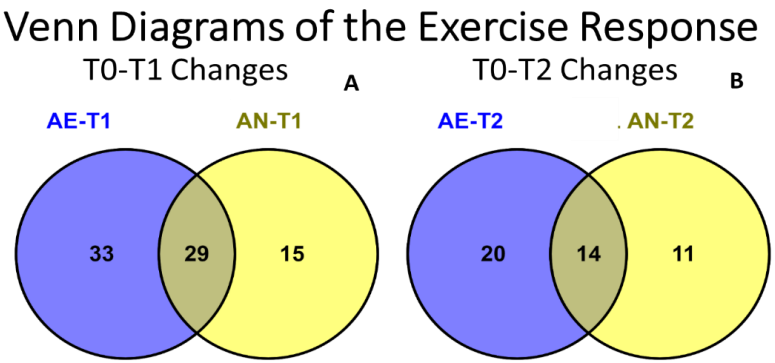


Figure 4. Pathway analysis for the immediate (T1) and 60-minute (T2) post-exercise responses to aerobic exercise (AE) and anaerobic exercise (AN). Size of circle and left-to-right placement on x-axis (impact) indicates increasing impact factor. Color of circle (white to red) and placement on y-axis (-log (p)) indicates increasing stringency for significance. Black dotted line indicates internal FDR-corrected $p < 0.05$ significance level. Analysis algorithms used: Hypergeometric Test: Over-representation Analysis; Relative betweenness centrality: Pathway topology analysis. TCAC = tricarboxylic acid cycle; LCFA = long-chain fatty acids; PUFA = polyunsaturated fatty acids; BCAA = branched-chain amino acids.
*FA-Related = mono/dihydroxy FA's, dicarboxylate FA's, lipid mediators and membrane lipids

Figure 5. Comparison of responses to aerobic exercise (AE) and Anaerobic exercise (AN) at T1 (**A**) and T2 (**B**). Indicates number of metabolites significantly altered by either exercise from baseline value at each time point. Includes a list of metabolites corresponding to Venn diagrams comparing AE to AN at T1 (left 3 columns) and T2 (right 3 columns).



33 AE only T1	29 AE & AN T1	15 AN only T1	20 AE only T2	14 AE & AN T2	11 AN only T2
beta-citrylglutamate	cysteinylglycine	alpha-hydroxyisocaproate	hexanoylglutamine	2,3-dihydroxy-2-methylbutyrate	cysteine-glutathione disulfide
3-(3-hydroxyphenyl)propionate sulfate	N-acetylmethionine	2-hydroxy(iso)butyrate	3-hydroxybutyrylcarnitine (2)	3-hydroxyisobutyrate	3-(4-hydroxyphenyl)lactate
vanillylmandelate (VMA)	4-hydroxyphenylpyruvate	cysteine sulfinic acid	suberoylcarnitine (C8-DC)	2-hydroxy(iso)butyrate	phosphate
N-acetyltryptophan	lactate	pantothenate	hexadecanedioate	alpha-ketobutyrate	citrate
xanthurenate	pyruvate	decanoylcarnitine (C10)	tetradecanedioate	vanillic alcohol sulfate	12-HETE
argininosuccinate	alpha-ketoglutarate	malonate	16-hydroxypalmitate	3-hydroxybutyrylcarnitine (1)	12-HHTrE
phosphate	fumarate	azelate (nonanedioate)	3-hydroxysebacate	myristoleate (14:1n5)	malonate
succinate	malate	maleate	3-hydroxybutyrate (BHBA)	palmitoleate (16:1n7)	glycohyocholate
propionylcarnitine (C3)	12-HETE	undecanedioate	acetoacetate	glycocholate	corticosterone
3-methyladipate	12-HHTrE	alpha-hydroxycaproate	10-heptadecenoate (17:1n7)	taurocholate	hypoxanthine
16-hydroxypalmitate	linoleoylcholine	glycochenodeoxycholate	10-nonadecenoate (19:1n9)	glycodeoxycholate	ferulic acid 4-sulfate
3-hydroxysebacate	oleoylcholine	glycocholate	eicosenoate (20:1)	taurodeoxycholate	
9-hydroxystearate	palmitoloelycholine	tauro-beta-muricholate	myristate (14:0)	xanthosine	
3-hydroxybutyrate (BHBA)	palmitoylcholine	taurocholate	oleate/vaccenate (18:1)	4-vinylguaiacol sulfate	
acetoacetate	stearoylcholine	tauroursodeoxycholate	docosatrienoate (22:3n3)		
10-heptadecenoate (17:1n7)	3-hydroxybutyrylcarnitine (1)		linolenate [α/γ; (18:3n3 or 6)]		
10-nonadecenoate (19:1n9)	3-hydroxybutyrylcarnitine (2)		stearidonate (18:4n3)		
eicosenoate (20:1)	acetylcamitine (C2)		cholate		
margarate (17:0)	2-arachidonoylglycerol (20:4)		ursodeoxycholate		
myristate (14:0)	2-docosahexaenoylglycerol (22:6)		eugenol sulfate		
myristoleate (14:1n5)	arachidonoylcholine				
oleate/vaccenate (18:1)	dihomo-linolenoyl-choline				
palmitate (16:0)	docosahexaenoylcholine				
palmitoleate (16:1n7)	glycerophosphoinositol				
5-dodecenoate (12:1n7)	glycohyocholate				
laurate (12:0)	glycoursodeoxycholate				
dihomo-linoleate (20:2n6)	corticosterone				
docosatrienoate (22:3n3)	hypoxanthine				
linoleate (18:2n6)	xanthosine				
linolenate [α/γ; (18:3n3 or 6)]					
stearidonate (18:4n3)					
glycodeoxycholate					
sphingosine					

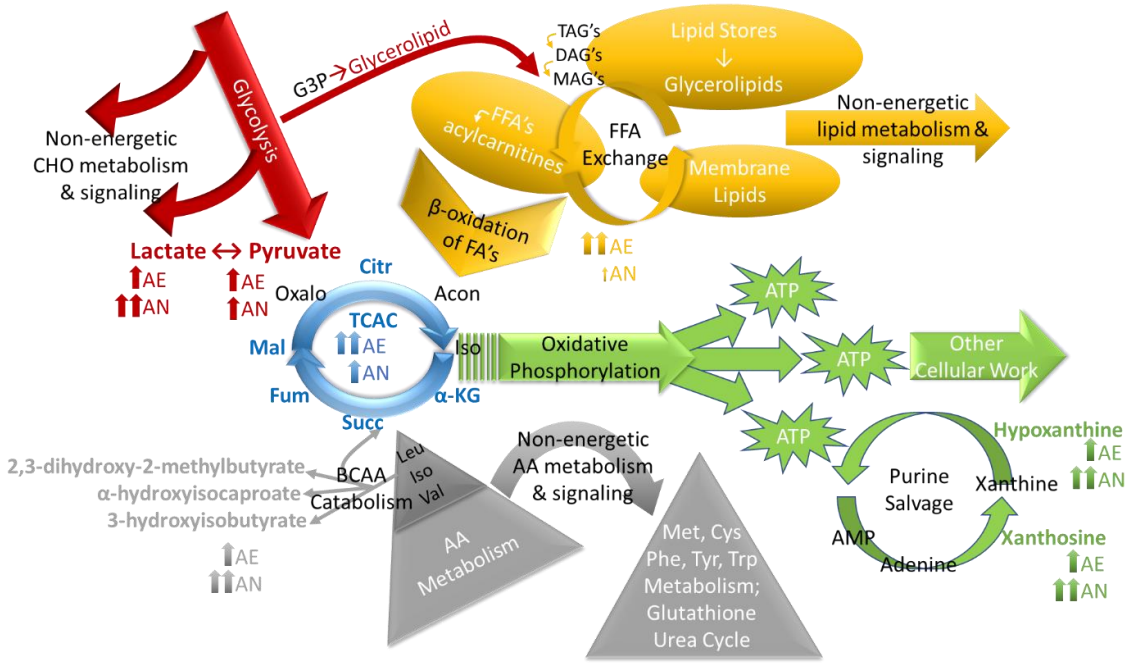


Figure 6. Major pathways of energy derivation and generalized responses to AE and AN. Arrows indicate direction of change brought on through each exercise. Two arrows = significantly greater change than 1 arrow; smaller arrow for AN lipid (FA) metabolism (yellow) indicates limited evidence for increased net metabolic response (delayed until T2). All specific metabolites written in color (red: glycolysis, blue: TCAC, grey: BCAA catabolism, green: purine salvage) significantly altered; those in black font: unchanged or not on panel. AE = aerobic exercise; AN = anaerobic exercise; G3P = glycerol-3-phosphate; TCAC = tricarboxylic acid cycle; Citr = citrate; Acon = aconitase; Iso = isocitrate; α-KG = α-ketoglutarate; Succ = succinate; Fum = fumarate, Mal = malate, Oxalo = oxaloacetate; FFA = free fatty acids; TAG = triacylglycerides; DAG = diacylglycerides; MAG = monoacylglycerides; AA = amino acid; BCAA = branched-chain AA; Leu = leucine; Iso = isoleucine; Val = valine; Met = methionine, Cys = cysteine; Phe = phenylalanine; Tyr = tyrosine Trp = tryptophan; ATP = adenosine triphosphate; AMP = adenosine monophosphate.

Sub Pathway	Metabolite	AE-T1	AN-T1	AE-T2	AN-T2
Phospholipid	glycerophosphoinositol	36%	28%	83%	73%
	dihomo-linolenoyl-choline	31%	29%	84%	93%
	arachidonoylcholine	35%	31%	79%	93%
	docosahexaenoylcholine	36%	31%	79%	90%
FA (Choline)	palmitoloelycholine	33%	45%	78%	96%
	stearoylcholine	29%	26%	78%	92%
	palmitoylcholine	32%	30%	78%	89%
	oleoylcholine	31%	30%	77%	91%
	linoleoylcholine	32%	29%	75%	86%

Table 2. Fatty-acylcholines & phospholipids significantly ($p < 0.05$, $q < 0.1$) altered > 2-fold. Vales expressed as % of resting level at each time point. Significant changes are shaded blue -darker = greater magnitude of change. AE = aerobic exercise; AN = anaerobic exercise; T1 = immediate post=exercise; T2 = 60-minutes post-exercise.

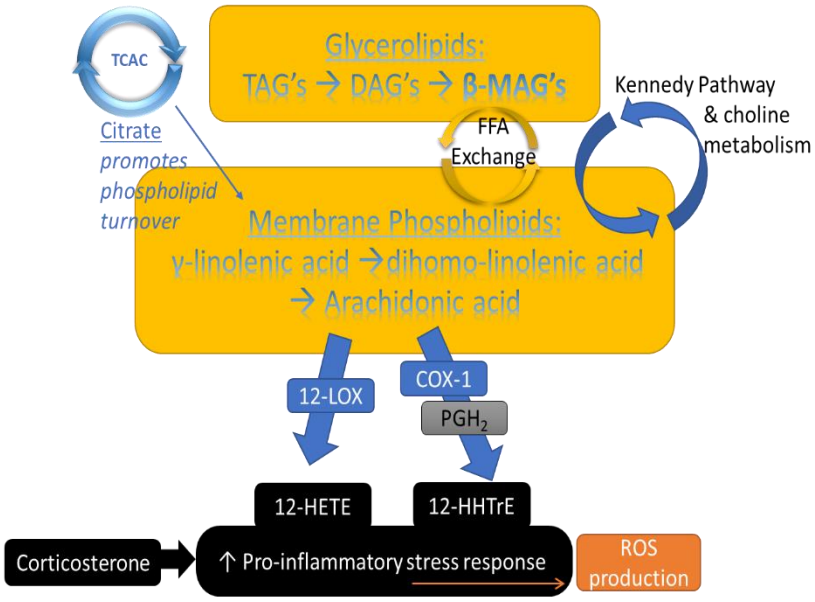


Figure 7. Metabolism of pro-inflammatory lipid mediators in the serum from phospholipids in the membrane and the glycerolipid pool released form lipid droplets. The eicosanoids 12-HETE and 12-HHTrE mediate inflammation and the stress response to exercise, including ROS production. FA mobilization and metabolism increased the pool of PUFAs, increasing available arachidonic acid for their production. FA entry into the TCAC elevated citrate production, which acted to increase phospholipid synthesis. Choline metabolism also perpetuated phospholipid production, perpetuating phospholipid metabolism. TCAC = tricarboxylic acid cycle; TAG = triacylglyceride; DAG = diacylglyceride; MAG = monoacylglyceride; FA = fatty acid; PUFA = polyunsaturated FA; LOX = lipoxygenase; COX = cyclooxygenase; PGH2 = prostaglandin H2; 12-HETE = 12-hydroxyeicosatetraenoic acid; 12-HHTrE = 12-hydroxyheptadecatrienoic acid; ROS = reactive oxygen species.

Super Pathway	Sub Pathway	Metabolite	AE-T1	AN-T1	AE-T2	AN-T2
Lipid	Primary Bile Acid	glycocholate	**55%**	**46%**	**49%**	**37%**
		taurocholate*	**73%**	**47%**	**43%**	**42%**
		glycochenodeoxycholate	**52%**	**47%**	**59%**	**54%**
		tauro-beta-muricholate	**54%**	**48%**	**54%**	**51%**
		cholate	**56%**	86%	**45%**	**47%**
	Secondary Bile Acid	glycohyocholate	**43%**	**42%**	**59%**	**42%**
		glycoursodeoxycholate	**44%**	**43%**	**62%**	**58%**
		glycodeoxycholate	**49%**	**51%**	**48%**	**46%**
		tauroursodeoxycholate*†	**68%**	**39%**	**61%**	92%
		taurodeoxycholate	**62%**	**51%**	**41%**	**46%**
		ursodeoxycholate	**63%**	**67%**	**48%**	**53%**
Xenobiotics	Food /Plant Component	ferulic acid 4-sulfate*	117%	81%	**64%**	**43%**
		4-vinylguaiacol sulfate	77%	**56%**	**35%**	**33%**
		eugenol sulfate	**78%**	**75%**	**50%**	**59%**

Table 3. Heat map of metabolites related to microbiome immediately (T1) and 1-hour (T2) post-exercise. Values expressed as % of resting level at each time point. **Bold** indicates a significant change from baseline, $p < 0.05$ & $q < 0.1$.
*AE significantly different from AN at 1
†AE significantly different from AN at T2

Super Pathway	Sub Pathway	Metabolite	AE-T1	AN-T1	AE-T2	AN-T2
Energy	TCAC	malate*	4.09	4.05	1.67	1.83
		fumarate*	3.58	3.19	1.55	1.42
		α-ketoglutarate*	2.36	2.30	1.42	1.45
		succinate**	4.21	1.78	1.60	1.01
		citrate†	1.39	1.79	1.58	2.15
		β-citrylglutamate**	2.56	1.89	1.30	0.97

Table 4. Heat map of TCAC intermediates & β-citrylglutamate levels immediately (T1) and 1-hour (T2) post-exercise. Values expressed as a % of resting level at each time point. All values statistically different from resting except: succinate and β-citrylglutamate AN-T2

*AE > AN at T1;

** AE > AN at T1 & T2;

† AN > AE at T2.

Sub Pathway	Metabolite	AE-T1	AN-T1	AE-T2	AN-T2
Ketone Bodies	acetoacetate	**210%**	**90%**	**292%**	**129%**
	3-hydroxybutyrate (BHBA)	**243%**	**109%**	**347%**	**145%**
FA (Carnitine)	3-hydroxybutyrylcarnitine (1)	275%	217%	298%	210%
	3-hydroxybutyrylcarnitine (2)	323%	272%	256%	190%
	acetylcarnitine (C2)	288%	247%	199%	178%
	propionylcarnitine (C3)	**243%**	**120%**	**123%**	**97%**
	decanoylcarnitine (C10)	**103%**	**46%**	89%	79%
	suberoylcarnitine (C8-DC)	158%	127%	**237%**	**119%**
Medium Chain FA	5-dodecenoate (12:1n7)	**213%**	**91%**	179%	189%
	laurate (12:0)	**247%**	**98%**	190%	164%
Long Chain FA	myristate (14:0)	**280%**	**114%**	203%	181%
	margarate (17:0)	**203%**	**105%**	184%	143%
	palmitate (16:0)	**201%**	**101%**	170%	144%
	palmitoleate (16:1n7)	**331%**	**107%**	243%	243%
	myristoleate (14:1n5)	**261%**	**96%**	221%	214%
	10-heptadecenoate (17:1n7)	**309%**	**108%**	237%	191%
	eicosenoate (20:1)	**229%**	**101%**	**227%**	**157%**
	10-nonadecenoate (19:1n9)	**236%**	**122%**	222%	162%
	oleate/vaccenate (18:1)	**241%**	**107%**	202%	160%
n3 PUFA's	linolenate [α/γ (18:3n3)]	**331%**	**101%**	240%	179%
	stearidonate (18:4n3)	**300%**	**120%**	227%	175%
	docosatrienoate (22:3n3)	**265%**	**111%**	225%	173%
n6 PUFAs	dihomo-linoleate (20:2n6)	**206%**	**111%**	199%	161%
	linoleate (18:2n6)	**233%**	**94%**	188%	150%
Sphingolipid	sphingosine	209%	176%	133%	125%
FA Synthesis	malonate	**161%**	**224%**	189%	271%
Dicarboxylate FA	azelate (nonanedioate)	**164%**	**305%**	81%	223%
	maleate	184%	200%	156%	163%
	hexadecanedioate	**186%**	**107%**	**221%**	**108%**
	undecanedioate	155%	201%	74%	146%
	tetradecanedioate	**166%**	**93%**	246%	143%
	3-methyladipate	**260%**	**188%**	187%	126%
Monohydroxy FA	3-hydroxysebacate	**209%**	**156%**	**307%**	**139%**
	9-hydroxystearate	**229%**	**107%**	192%	170%
	alpha-hydroxycaproate	**193%**	**225%**	149%	174%
	16-hydroxypalmitate	**237%**	**108%**	211%	161%

Table 5. Heatmap of FA metabolites in response to AE and AN. Values expressed as a percentage of resting level. Red = increase, blue = decrease from baseline. Substantially more elevation in FA metabolism in response to AE was seen at both time points. Additionally, FA metabolites showing a response at AN-T1 tended to be markers of distressed or inadequate mitochondrial FA processing (malonate and most dicarboxylate- and monohydroxy-FA's). The split in PUFA metabolism between n3 and n6 metabolites following exercise was evident. Also, FA metabolites with antioxidant properties (3-methyladipate and 16-hydroxypalmitate) were greater in AE > AN. Values in **bold** (AE-AN pairings) are significantly different from one another at that time point.

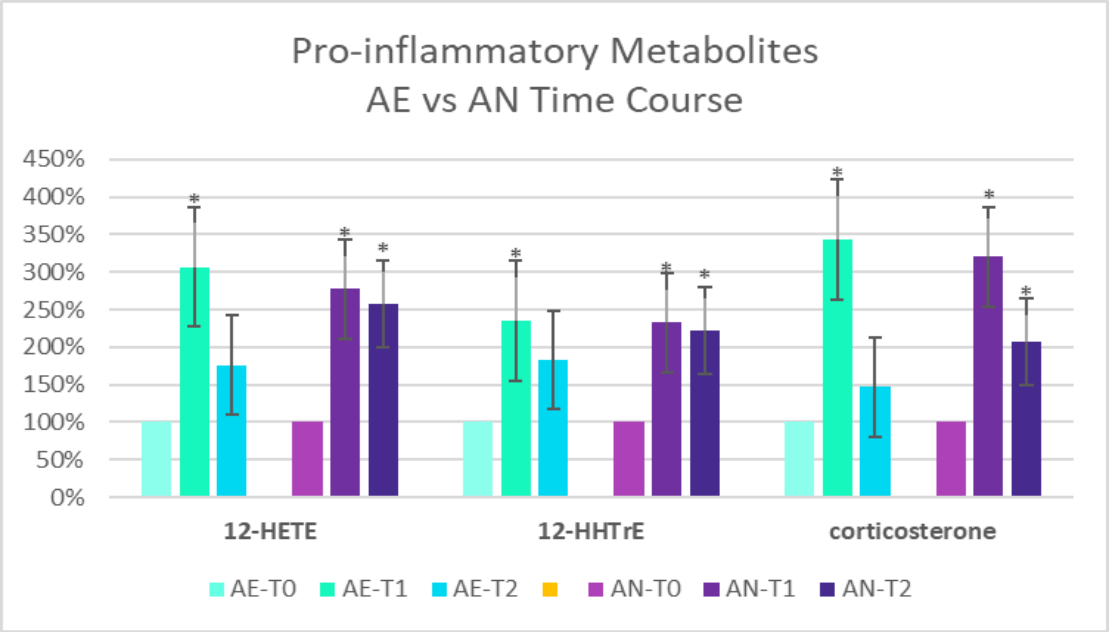


Figure 8. Pro-inflammatory markers are elevated immediately following exercise (T1) for aerobic (AE) and anaerobic exercise (AN). After 1-hour of recovery (T2) the pro-inflammatory metabolites returned to baseline following AE, but remained elevated following AN. *Significantly different from resting (T0).

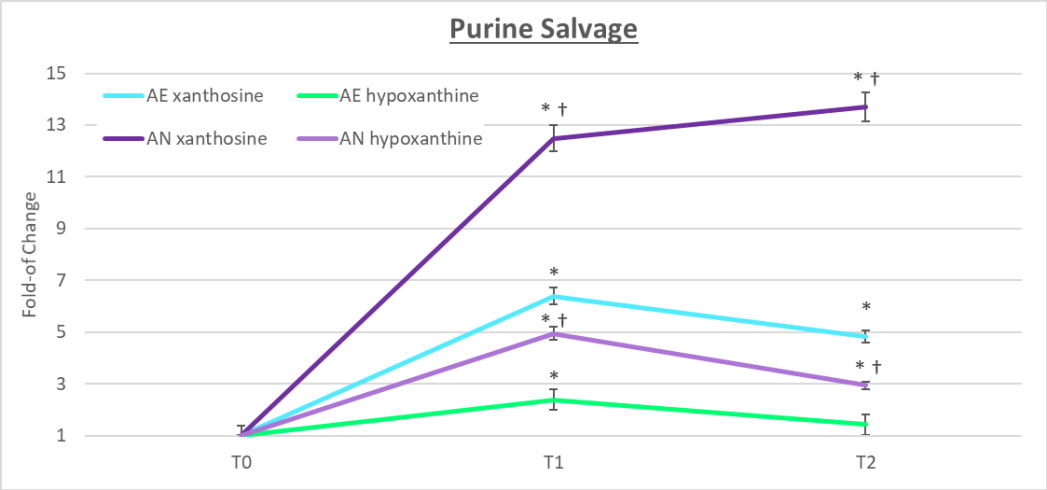


Figure 9. Responses of purine salvage markers (xanthosine & hypoxanthine) to aerobic (AE) and anaerobic exercise (AN) expressed as fold-of change form resting value. T0 = resting, T1 = immediately post-exercise, T2 = 1-hour post-exercise.
* Significant change from resting value, $p < 0.05$, $q < 0.1$, magnitude > 2-fold.
† Significantly greater than response in AE, $p < 0.05$, $q < 0.1$, magnitude > 2-fold.

Super Pathway	Sub Pathway	Metabolite	AE-T1	AN-T1	AE-T2	AN-T2
Nucleotide	Purine , (Hypo)Xanthine/Inosine	xanthosine * **	6.40	12.49	4.83	13.70
		hypoxanthine* **	2.40	4.95	1.44	2.95
Carbohydrate	Glucose	lactate *	3.40	4.44	1.48	1.74
		pyruvate	3.28	3.57	1.39	1.68
Vitamins	Pantothenate	pantothenate *	1.78	2.36	1.25	1.44
Energy	Oxidative Phosphorylation	phosphate * **	2.12	1.25	1.08	0.44
	TCAC	malate	4.09	4.05	1.67	1.83
		fumarate	3.58	3.19	1.55	1.42
		alpha-ketoglutarate	2.36	2.30	1.42	1.45
		succinate * **	4.21	1.78	1.60	1.01
		citrate **	1.39	1.79	1.58	2.15
	TCAC & Glutamate	beta-citrylglutamate * **	2.56	1.89	1.30	0.97
Amino Acid	Glutathione	cysteinylglycine	3.11	3.10	1.83	1.88
		cysteine-glutathione disulfide	1.67	1.31	1.45	2.01
	Glutathione & Energy	alpha-ketobutyrate	1.79	1.46	2.38	2.30
		2-hydroxy(iso)butyrate *	1.75	2.04	2.34	2.37
	Methionine & Cysteine	cysteine sulfinic acid *	0.68	0.48	0.87	0.85
		N-acetylmethionine	2.21	2.12	1.53	1.61
	Phenylalanine and Tyrosine	3-(3-hydroxyphenyl)propionate sulfate *	2.41	1.41	1.09	0.89
		4-hydroxyphenylpyruvate *	3.36	2.87	1.31	1.32
		vanillylmandelate (VMA) * **	2.66	1.55	1.89	1.33
		vanillic alcohol sulfate	0.61	0.62	0.30	0.37
		3-(4-hydroxyphenyl)lactate	1.68	1.83	1.68	2.00
	Tryptophan	N-acetyltryptophan	2.12	1.60	1.01	1.11
		xanthurenate * **	2.08	1.21	1.41	0.93
	Urea cycle & Arginine	argininosuccinate	2.34	2.00	1.57	1.31
	BCAA	2,3-dihydroxy-2-methylbutyrate	1.71	1.84	2.09	2.37
		alpha-hydroxyisocaproate *	1.92	2.36	1.53	1.90
		3-hydroxyisobutyrate	1.92	1.93	2.02	2.08

Supplementary Table 1.

Significantly altered from resting ($p < 0.05$, $q < 0.1$, fold change ratio >2.0 or < 0.5 up (red) or down (blue)

* Significant difference between conditions at T1 ($p < 0.05$, $q < 0.1$)

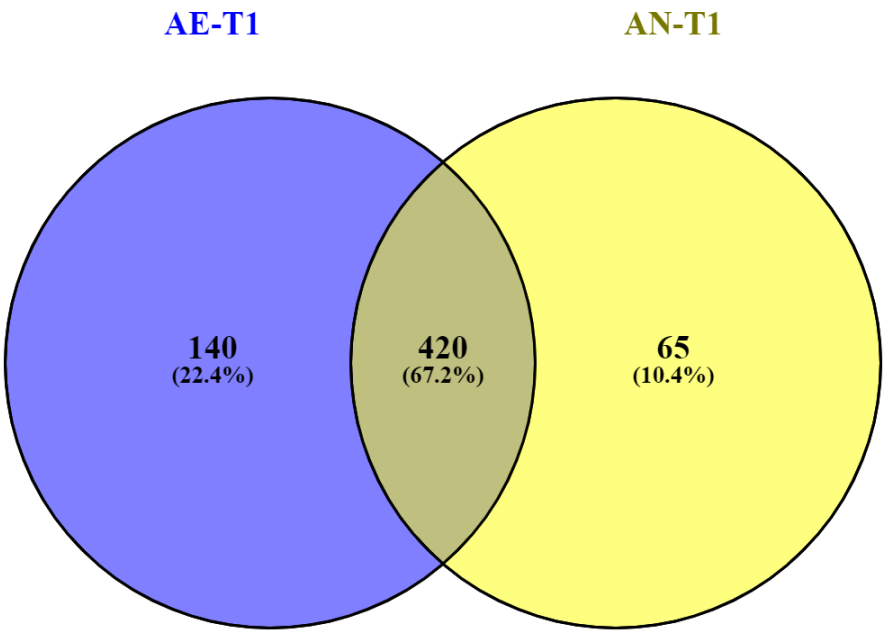
** Significant difference between conditions at T2 ($p < 0.05$, $q < 0.1$)

Supplementary Table 1 continued…

Super Pathway	Sub Pathway	Metabolite	AE-T1	AN-T1	AE-T2	AN-T2
Lipid	FA (BCAA)	propionylcarnitine (C3) * **	**2.43**	**1.20**	**1.23**	**0.97**
	Ketone Bodies	acetoacetate * **	**2.10**	**0.90**	**2.92**	**1.29**
		3-hydroxybutyrate (BHBA) * **	**2.43**	**1.09**	**3.47**	**1.45**
	FA (Carnitine)	3-hydroxybutyrylcarnitine (1)	2.75	2.17	2.98	2.10
		3-hydroxybutyrylcarnitine (2)	3.23	2.72	2.56	1.90
		acetylcarnitine (C2)	2.88	2.47	1.99	1.78
		propionylcarnitine (C3) * **	**2.43**	**1.20**	**1.23**	**0.97**
		decanoylcarnitine (C10) *	**1.03**	**0.46**	0.89	0.79
		suberoylcarnitine (C8-DC) **	1.58	1.27	**2.37**	**1.19**
	FA (Glutamine)	hexanoylglutamine **	1.84	1.35	**2.72**	**1.32**
	FA Synthesis	malonate *	**1.61**	**2.24**	1.89	2.71
	Steroid	corticosterone **	3.43	3.21	**1.47**	**2.07**
	Eicosanoid	12-HHTrE **	2.35	2.33	**1.83**	**2.23**
		12-HETE **	3.07	2.77	**1.76**	**2.57**
	FA, Dicarboxylate	azelate (nonanedioate) *	**1.64**	**3.05**	0.81	2.23
		maleate	1.84	2.00	1.56	1.63
		hexadecanedioate * **	**1.86**	**1.07**	**2.21**	**1.08**
		undecanedioate	1.55	2.01	0.74	1.46
		tetradecanedioate *	**1.66**	**0.93**	2.46	1.43
		3-methyladipate *	**2.60**	**1.88**	1.87	1.26
	FA, Monohydroxy	3-hydroxysebacate * **	**2.09**	**1.56**	**3.07**	**1.39**
		9-hydroxystearate *	**2.29**	**1.07**	1.92	1.70
		alpha-hydroxycaproate *	**1.93**	**2.25**	1.49	1.74
		16-hydroxypalmitate *	**2.37**	**1.08**	2.11	1.61
	Long Chain FA	myristate (14:0) *	**2.80**	**1.14**	2.03	1.81
		margarate (17:0) *	**2.03**	**1.05**	1.84	1.43
		palmitate (16:0) *	**2.01**	**1.01**	1.70	1.44
		palmitoleate (16:1n7) *	**3.31**	**1.07**	2.43	2.43
		myristoleate (14:1n5) *	**2.61**	**0.96**	2.21	2.14
		10-heptadecenoate (17:1n7) *	**3.09**	**1.08**	2.37	1.91
		eicosenoate (20:1) * **	**2.29**	**1.01**	**2.27**	**1.57**
		10-nonadecenoate (19:1n9) *	**2.36**	**1.22**	2.22	1.62
		oleate/vaccenate (18:1) *	**2.41**	**1.07**	2.02	1.60
	Medium Chain FA	5-dodecenoate (12:1n7) *	**2.13**	**0.91**	1.79	1.89
		laurate (12:0) *	**2.47**	**0.98**	1.90	1.64
	Monoacylglycerol	2-docosahexaenoylglycerol (22:6)	0.49	0.35	0.82	0.98
		2-arachidonoylglycerol (20:4)	0.50	0.46	0.92	1.03
	Phospholipid	glycerophosphoinositol	0.36	0.28	0.83	0.73
		dihomo-linolenoyl-choline	0.31	0.29	0.84	0.93
		arachidonoylcholine	0.35	0.31	0.79	0.93
		docosahexaenoylcholine	0.36	0.31	0.79	0.90

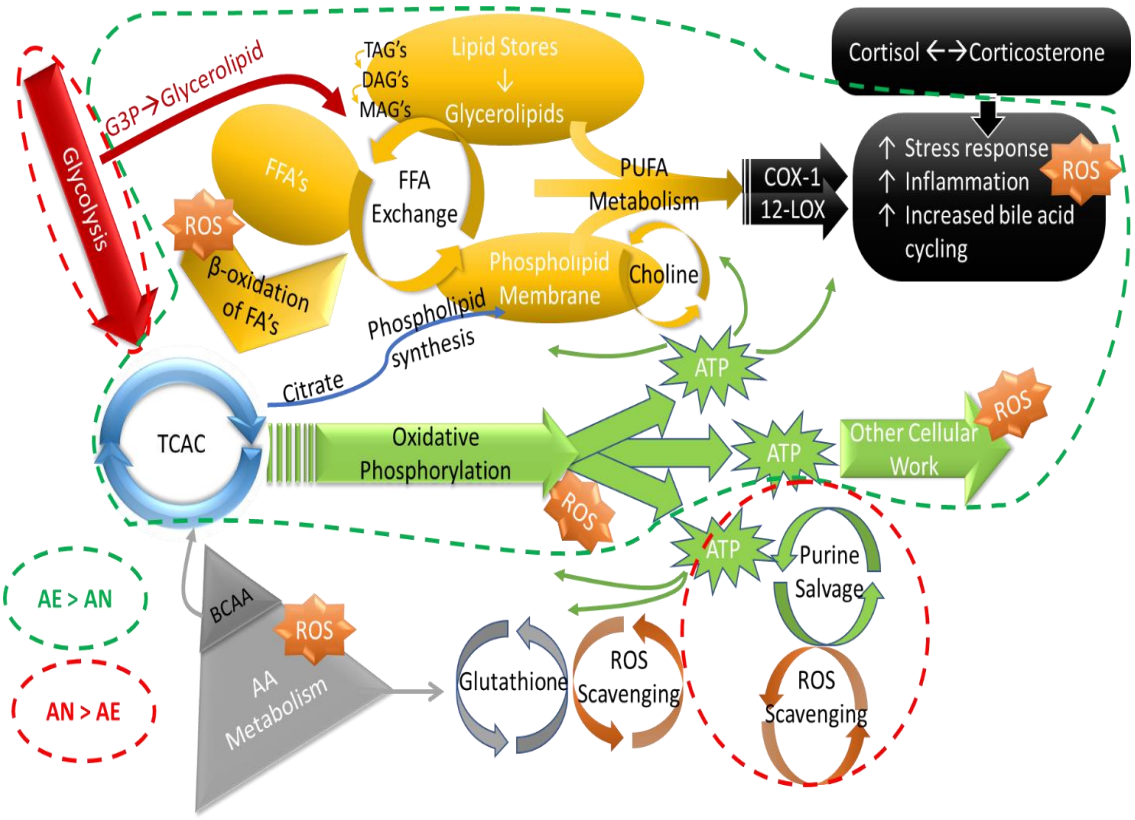
Supplementary Table 1 continued…

Super Pathway	Sub Pathway	Metabolite	AE-T1	AN-T1	AE-T2	AN-T2
	Polyunsaturated FA (n3 and n6)	linolenate [α/γ (18:3n3 or 6)] *	**3.31**	**1.01**	2.40	1.79
		stearidonate (18:4n3) *	**3.00**	**1.20**	2.27	1.75
		docosatrienoate (22:3n3) *	**2.65**	**1.11**	2.25	1.73
		dihomo-linoleate (20:2n6) *	**2.06**	**1.11**	1.99	1.61
		linoleate (18:2n6) *	**2.33**	**0.94**	1.88	1.50
	FA (Choline)	palmitoloelycholine **	0.33	0.45	**0.78**	**0.96**
		stearoylcholine **	0.29	0.26	**0.78**	**0.92**
		palmitoylcholine **	0.32	0.30	**0.78**	**0.89**
		oleoylcholine **	0.31	0.30	**0.77**	**0.91**
		linoleoylcholine	0.32	0.29	0.75	0.86
	Sphingolipid	sphingosine	2.09	1.76	1.33	1.25
	Primary Bile Acid	glycocholate	0.55	0.46	0.49	0.37
		taurocholate *	**0.73**	**0.47**	0.43	0.42
		glycochenodeoxycholate	0.52	0.47	0.59	0.54
		tauro-beta-muricholate	0.54	0.48	0.54	0.51
		cholate *	**0.56**	**0.86**	0.45	0.47
	Secondary Bile Acid	glycohyocholate	0.43	0.42	0.59	0.42
		glycoursodeoxycholate	0.44	0.43	0.62	0.58
		glycodeoxycholate	0.49	0.51	0.48	0.46
		tauroursodeoxycholate * **	**0.68**	**0.39**	**0.61**	**0.92**
		taurodeoxycholate	0.62	0.51	0.41	0.46
		ursodeoxycholate	0.63	0.67	0.48	0.53
Xenobiotics	Food Component/Plant	ferulic acid 4-sulfate *	**1.17**	**0.81**	0.64	0.43
		4-vinylguaiacol sulfate	0.77	0.56	0.35	0.33
		eugenol sulfate	0.78	0.75	0.50	0.59



Supplementary Figure 1. Venn Diagram of all significantly altered metabolites (from resting, T0) at T1 regardless of magnitude of change for AE and AN.

AE = aerobic exercise; AN = anaerobic exercise; T1 = 0-minutes post-exercise



Supplementary Figure 2. Outline of major metabolic pathways activated by exercise. AE elicited greater lipid metabolism due to greater FA substrate selection relating to a metabolomic profile indicating more membrane turnover and downstream lipid mediator signaling. AN elicited greater anaerobic glycolysis and purine salvage due to a greater demand on rate of energy production. This highlights the fundamental difference between AE and AN as a volume- versus intensity-dependent stressor, respectively.

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Chapter 4.2

The Exercise Metabolome: A comparison of Endurance- and Resistance-Trained Individuals

INTRODUCTION

The acute stress of exercise acts as a global effector influencing myriad biochemical pathways and physiological phenomena. This fact is exploited in medicine and sport to engender positive health and fitness changes. Different modes of exercise, i.e. aerobic and anaerobic, can be used to stimulate vastly different and even oppositional responses on both the intracellular and systemic levels.[1] While data on the nature of these disparities are not completely lacking, a full understanding is far from clear at present.[2,3] Further, phenotypic adaptations to each exercise manifests in accordance with the acute stressor. Biochemical and metabolic changes in the body lead to the prototypical aerobic endurance trained or anaerobic power/strength trained body types.

Aerobic & Anaerobic Exercise. Much has been made of the differential responses to aerobic versus anaerobic exercise.[1-5] Aerobic exercise is most commonly associated with improved cardiovascular fitness and its downstream health benefits. Meanwhile, anaerobic exercise tends to be related to increased muscle mass and the associated health and functional benefits. Still, overall many of the benefits of exercise arise from both aerobic and anaerobic modes, including weight loss and body composition changes,[6] improved insulin sensitivity,[7] and cardiovascular improvements.[8] Because exercise bestows these benefits while simultaneously altering the exerciser's ability to tolerate exercise, there exists a dynamic relationship between the acute and longitudinal responses to exercise. Traditionally, studies examining this have been limited to matching the acute exercise exposure to the training background, i.e. endurance trained with aerobic exercise; resistance trained with anaerobic exercise. But of equal importance and interest is the crossover between these adaptations. Namely, how does endurance training effect the acute response to anaerobic exercise and resistance training effect the acute

response to aerobic exercise? These effects are especially important in the context of determination of an as yet undetermined entity, characterization of the exercise metabolome.

Metabolomics. Because exercise is a profound stressor that spawns an intricate holistic response with effects found across miscellaneous systems and cell types in the body, isolating a single pathway or marker fails to consider cross-systemic responses that involve a multitude of reactions. This can give rise to a fragmented picture and a piecemeal understanding of the issue at hand. With something so globally pervasive as the exercise response, a systems biology approach is warranted. Metabolomics provides a means to accomplish this. A metabolomic analysis of the serum allows for high-throughput analysis of a holistic response across systems. Employing this approach offers a simultaneous look at a diversity of biomarkers and insight into a variety of pathways.

Previous findings in studies using a metabolomic platform have shown exercise to be a potent stressor with marked disruption throughout many biochemical pathways.[9,10] The greatest perturbations have been observed to occur within 2-hours of exercise.[9,10] Further, metabolomic response profiles have been observed to match the acute stressor,[9] giving reason to believe aerobic and anaerobic exercise would differ in their effect on the metabolome. Indeed, the only investigation to examine different exercise modes, did so in a type-2 diabetic population and found metabolomics capable to resolving between exercise modalities.[11] Data for the general population is non-existent.

Training intervention studies have generally only examined one form of exercise or the other, typically aerobic. Most designs used short-term (6-week-6-month) training interventions in previously sedentary individuals, and may therefore be biased towards the initial effect of exercise on sedentary individuals, missing any unique responses elicited through a specific training modality. It is imperative to our understanding of the continued impact of long-term

participation in exercise to look beyond sedentary comparisons. Studies examining long-term training (years) tend to be cross-sectional utilizing elite athletic populations,[12] or *post-hoc* training groups based solely on VO_{2MAX}.[13,14] While this tells us something about the long-term effects of exercise training, an exploration of long-term training exposure using the general population is necessary to reveal any differences in training mode. Moreover, reports are generally restricted to examination of the resting metabolome, and do not offer information on how the trained individual responds to acute exercise, nor how training background impacts this response.

Though limited in number, acute studies have linked aerobic and anaerobic exercise stressors with disruptions in energy balance and increased oxidative stress,[15-17] with a greater breadth of perturbed metabolites including more abundant oxidative metabolism: tricarboxylic-acid cycle (TCAC), β-oxidation, and ketogenesis intermediates reported following aerobic exercise, and typically larger perturbations in purine and branched-chain amino acid (BCAA) metabolism with anaerobic exercise.[17] Notably, while metabolomics investigations have been carried out for acute aerobic and anaerobic exercise bouts individually, direct contrast of these modalities is again lacking.

Despite a lack of direct comparisons of differently trained individuals, the body of literature has reported enhanced cardiorespiratory, inflammatory and antioxidant capacities in more aerobically fit individuals.[17,20] Highly opposing acute exercise responses have been found in sedentary versus active individuals in their metabolomic profile and validated with upstream transcriptomic responses.[19] Further, a study of the Chinese crew team found a metabolomics panel capable of differentiation between training status of team members where traditional biomarkers lacked the sensitivity.[20] All of this serves as evidence of the prudence in employing a metabolomics panel in examination of the aerobic versus anaerobic exercise response.

Characterization of the acute metabolomic response to exercise in trained, rather than sedentary individuals is vital to understand the context through which exercise can have continued health benefits for those participating in daily physical activity. Knowledge attained here will allow for targeted workout prescriptions and provide a better understanding of recovery needs. With these distinctions, exercise prescription can be better paired with its biochemical outcomes for use of exercise as medicine. Looking at the exercise response on a grand scale, such as that accomplished with metabolomics, presents an opportunity to separate aerobic from anaerobic exercise and endurance from resistance training background.

PURPOSE AND HYPOTHESIS

The aim of the present study was to use an exploratory, comprehensive metabolomics platform to examine the metabolic response pattern of both endurance and resistance trained individuals to acute bouts of aerobic and anaerobic exercise. We hypothesized that changes in the metabolome would reflect both convergence and divergence in the responses to specific exercise modes across training backgrounds. Metabolomic characterization of the acute anaerobic exercise response is currently lacking and comparisons between exercise modes has yet to be carried out in a healthy cohort. Furthermore, endurance and resistance trained individuals are seldomly used together in research designs, and metabolomics has never been used to study the impact of differential training background on the exercise response. This study accomplishes all of this using a cross-over design in an attempt to characterize the fit/healthy metabolome, specifically in response to varied exercise stressors.

METHODS

Research Design. Active, young adults were exposed to 2 different exercise bouts (matched for duration and perceived exertion) and their metabolomic profiles were characterized before and at 2 times post- exercise: immediately (T1) and 60-minutes after(T2). This design was implemented to characterize and differentiate between the acute responses to aerobic and anaerobic exercise in healthy individuals. Active participants engaging in principally either endurance (END) or resistance (RES) training were targeted for recruitment. Physiological parameters were measured to assess aerobic fitness (VO_{2MAX}) and anaerobic strength (10-repitition max (RM)), and prescribe equivalent relative workloads during the experimental exercise conditions. Metabolomic profile and individual metabolite differences were assessed.

Participants. Volunteers were recruited to the Rutgers Center for Health & Human Performance (CHHP) for participation in the study. Forty (20 END + 20 RES) active, healthy young adults (age: 18-39 years) completed all testing and were used for analysis. Inclusion criteria consisted of absence of any known cardiorespiratory, metabolic or other health issues and a minimum of 2-years of regular participation in their current physical activity regimen, limited to one primary modality. For END, the regimen included participation in distance cycling and/or running. For RES, this meant use of some mode of high-intensity resistance training (power-lifting, weight-training or cross-fit). Exclusion criteria included recent illness/injury (> 1-week disruption to normal exercise routine within the past 6 months) and use of tobacco products or recreational drugs. Supplement use was permitted so long as participants had not begun use within 6-months of enrollment and continued through completion of the study. Participants reported to the lab for 2 testing trials followed by 2 experimental conditions randomized for order. Procedures were approved by the Rutgers University Institutional Review Board, and participants signed informed consents prior to enrollment.

EXERCISE TESTING TRIALS

Visit 1. Participants reported to the CHHP following a 2-hour fast and having refrained from exercise for the previous 24-hours. Upon arrival, body composition testing with a Bod Pod (Cosmed, Concord, CA) was performed using the Brozek equation.[21] After this, participants were permitted to warm-up for 10 minutes before completing a continuous graded exercise test on a Velotron cycling ergometer (Racermate, Seattle, WA). Depending on estimated fitness level, individuals started between stages 70 and 220 Watts (W) and continued cycling, increasing wattage by 30 W every 3-minutes. For testing, participants were fitted with a Polar M-400 HR monitor (Polar Inc., Bethpage, NY) and Quark C-PET metabolic measuring system (Cosmed, Concord, CA) for HR and breath-by-breath assessment of gas analysis with 15-second rolling averages. All participants met a minimum of 3 American College of Sports Medicine criteria for attainment of VO_{2MAX},[22] and continued until volitional fatigue or the inability to keep cadence ≥ 80 rpm.

Ventilatory threshold (VT) was calculated using the graphical ventilatory equivalent method as described by Davis et al. (1980) and verified by 2 separate exercise physiologists.[23] These values were further verified using internal software on the Quark C-PET. The average of the 2 closest values was taken as VT, with a discrepancy never more than 0.1L/min O_2 between comparators. Maximal aerobic power output (PO_{MAX}) was calculated as per Naperalsky et al. (2010).[24]

$$PO_{MAX} = (\text{Watts on last stage completed}) + (30\ W) \times (\%\ \text{of final attempted stage completed})$$

Seventy percent of this wattage value was used as the intensity prescription during the aerobic exercise session (AE). This percentage was chosen, as it allowed for an arduous exercise bout while still falling below average measured VT of all participants.

Visit 2. During the second visit to the CHHP participants completed 10RM testing for 7 lower-body exercises following the guidelines set forth by the National Strength and Conditioning Association (NSCA).[25] In order, exercises were back squat, leg press, Romanian deadlift, lunges, prone leg curls, leg extensions and seated calf raises. A certified strength and conditioning specialist (CSCS-NSCA) administered testing to ensure participant safety and proper execution of all lifts. The same 7 exercises, in the same order, were performed during the anaerobic exercise session (AN). Three sets of 10 repetitions were performed for each exercise using 90% of the determined 10RM weight and a 90-second recovery between all sets. This combination of load, rest interval, repetition and set count was selected as it falls within the NSCA guidelines for hypertrophy training and well within an "anaerobic" working intensity.

EXERCISE CONDITIONS

Control Factors. In accordance with findings on metabolomic variability, certain precautions were taken to minimize external variability.[9,26] Individuals reported to the CHHP for each of the 2 experimental conditions having refrained from exercise for the previous 24 hours and avoided intense or prolonged exercise for the previous 48. Prior to their first condition, participants were provided a 24-hour food log to record their diet so that it could be replicated prior to the second condition. They were encouraged to eat as normal, other than consuming a meal 2-3 hours prior to the visit, with only water after this point. All visits started between 8:00AM and 12:00PM, and exact time of day was matched between conditions for each participant. Lastly, individuals were instructed to abstain from ingestion of caffeine intake that day (none within 6-hours of the visit). Each condition was run 3-7 days removed from the previous session (either testing visit 2 or condition visit 1). In addition to relative workload, exercise sessions were matched for total duration (45 minutes), and were perceived as equally demanding by subjects during pilot testing (rating of perceived exertion (RPE) between 8 and 10 for both conditions).[27]

Aerobic Session (AE). Upon arrival, participants sat quietly for 10-15 minutes and a resting blood draw from the antecubital vein was performed (T0). This was followed by a 15-minute warm-up period before commencing with the prescribed exercise. The AE cycling bout was a 45-minute steady state ride at ≈70% of PO_{MAX} on the same ergometer as used during VO_{2MAX} testing. A trainer monitored HR and cadence during the session and participants were encouraged to keep their cadence at ≥ 90rpm. In order to maintain an aerobic effort, if HR went above 90% HR_{MAX} (as observed during VO_{2MAX} testing), or cadence below 80rpm, wattage was adjusted until HR and/or cadence normalized.

After completion of the exercise, participants were promptly escorted into an adjacent phlebotomy room where an indwelling catheter was inserted into the contralateral arm for subsequent draws, collected immediately (T1) and 1-hour post-exercise (T2). The line was flushed with 4ml saline before and after each draw, as well as halfway between them, to prevent coagulation. Nutrient intake was not permitted at any point during the session, but participants were allowed to drink water *ad libitum* throughout.

Anaerobic Session (AN). AN was identical to AE, including sample collection, except that the cycling bout was replaced with a resistance training session of the 7 exercises listed above. A CSCS oversaw all sessions to ensure participant safety and proper execution of all lifts. If the workload was too high (participant could not complete a full set of 10) they were spot-assisted to completion and weight was adjusted accordingly on any subsequent sets of that exercise. The entire exercise portion of the session took ≈45-minutes. Order of AE and AN was randomized for all 40 participants.

Metabolomics Analysis. Following collection and processing, serum samples were aliquoted into 2.5 ml cryovials and stored at -80^{O}C until all testing was complete. Samples were packed on dry ice and shipped to the Metabolon® core facility in Durham, NC for analysis with Metabolon's

Discovery Panel of 754 metabolites. Automated ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry was used for metabolite identification. Raw data were extracted, peak identified, quality control processed with internal and external controls, and quantified by area under the curve using Metabolon's hardware and software. Compounds were then identified by comparison to library entries of purified standards or recurrent unknown entities based on 3 criteria: retention index, mass, and the MS/MS forward and reverse scores between the experimental data and authentic standards. All samples were run in a single batch to reduce variance.

Values were normalized in terms of raw area counts through log transformation and rescaled to set the median equal to 1.00 for each metabolite. Then, missing values were imputed with the minimum value. All statistical procedures were carried out using these values. Data were re-run excluding any metabolites using > 25% imputed values. This did not impact the global biochemical profiles, and inclusive data is reported here.

Statistical Procedures. Unsupervised approaches to analysis were performed in Array Studio and included hierarchical clustering and principle components analysis (PCA). The hierarchical clustering method performed was complete clustering using the Euclidean distance, where each value was factored independently as a vector to all other metabolite values. Condition clusters were identified and superimposed on the output. PCA analysis by singular value decomposition was used to reduce dimensionality and generate data clusters for all samples and select subgroupings showing differences between group, condition and/or time.

A 2x2x3 (training background x exercise condition x time) repeated measures analysis of variance (RMANOVA) with follow-up univariate ANOVA contrasts was run for all metabolites using a p value of < 0.05. Additionally, change values were calculated from T0 to T1 and T0 to T2 for each group. These delta scores were compared between groups, (END vs RES) using unpaired

t-tests, with an alpha level $p < 0.05$. Due to the multiple comparisons found in a metabolomics data set, the false discovery rate was determined using the Benjamin-Hochberg equation with a stringency set at a q value of 0.1 for all ANOVA and t-tests. All changes discussed below meet both p and q criteria. Because metabolite values are all median-normalized, log-scaled means of the group, values are in relative arbitrary units and reported as relative values or relative changes from resting.

For those values determined to be statistically significant by both p and q criterions, magnitude of change was used to further discriminate metabolites to obtain the most impactful metabolic perturbations and reduce data volume. Average changes from T0-T1 and T0-T2 for each exercise were considered most physiologically significant when the delta score reached 2-fold-of difference between the resting and post-exercise values.[28] With resting values set at 1.00, this means a post-exercise value ≥ 2.00 for an increase or ≤ 0.50 for a decrease. This filtered listing of metabolites was used for Venn diagram comparisons, while all statistically significant metabolites were considered in the RMANOVA comparisons.

RESULTS

Participant Characteristics. Details for participant descriptive and fitness data can be found in **Table 1**. Body composition data showed all participants to be relatively lean individuals. Fitness characteristics of each group reflected their respective training background with END significantly higher for all aerobic measures and RES significantly higher for all anaerobic measures. These differences persisted as absolute values and when expressed relative to body mass and fat free mass (FFM).

Session Characteristics. The average wattage used during AE was 68±6% PO_{MAX}. The average weight used across lifts during AN was 89±.05% 10RM weight. Neither of these differed between groups. Rating of perceived exertion at the conclusion of each session was 9.1±0.7 for AE, and 9.2±0.9 for AN, with a range of 8-10 for both conditions. Values were similar for END and RES, indicating both exercise sessions were perceived as equally challenging across groups.

METABOLOMIC PROFILE RESULTS

Resting Metabolome. No metabolites were different between conditions within training background groups or in the entire cohort as a group of 40 at T0. Few metabolites differed between END and RES at rest. Of the 754 metabolites measured, only 10, or just over 1% were significantly different between groups (**Table 2**). For all 10 of these metabolites, RES was higher than END at rest. Seven of these were plasmalogens/lysoplasmalogens, revealing a proliferative difference according to training background in baseline levels of these membrane glycerophospholipids related to oxidative stress. Additionally, on this list was the primary bile acid taurochenodeoxycholate, its secondary bile acid tauroursodeoxycholate, and 2-aminoadipate, a catabolite of lysine linked to oxidative stress. All 10 of these metabolites remained different between groups at T1 and T2 for both exercises.

Hierarchical Clustering. Extended segments of END or RES clustering offered some grouping by training background, signifying characteristics of the metabolome specific to END and to RES regardless of time or exercise condition (**Figure 1A**). Lack of elongated clusters for either exercise condition indicated little differentiation between AE and AN responses, though use of all data (T0, T1 and T2) may have occluded some differences.

Principle Components Analysis. PCA of metabolomic outputs revealed clustering of T0 values and T1 values, with a greater spread for T2 data; however, the substantial overlap for these clusters across groups and conditions highlights the commonalities between training backgrounds and exercise exposures (**Figure 1B,C)**. Analysis of each group/exercise combination individually more clearly revealed T0 and T1 separation reflecting the impact of exercise on the metabolome, and the T2 markers filling the space between them, indicative of a partial reversal of the exercise shift by 60-minutes post (**Figure 1C**).

Venn Diagrams. A set of 4-way Venn diagrams displayed significant 2-fold changes during exercise and recovery for both groups and exercise modes (**Figure 2 A,B**). During AE, perturbations to the resting metabolome were mostly identical between groups, with 17 altered metabolites unique to END (solid white oval), 9 unique to RES (dashed white oval), but 51 common to both (**Figure 2.A**). At AE-T2, however, they shared only 12 in common (**Figure 2.A**). For AN, END had only 2 unique metabolites (solid black ovals) at T1, while RES had 30 (dashed black ovals), and they shared 28 in common (**Figure 2.B**). By AN-T2 there was a more even distribution between groups and fewer common perturbations (**Figure 2.B**). When displayed as bar graphs with overlaying Venn diagrams to show the time course of metabolite changes (**Figure 2 C**), it can be seen that AE brought about more changes in END at T1, but in RES at T2. AN, on the other hand, was more impactful in RES than END at T1 and T2.

<u>END versus RES Metabolite Comparisons</u>

Energy Metabolism. The effect of training background on the exercise response was prominently on display in those pathways relating to energy dynamics. Amongst these, the purine salvage pathway was highly activated in response to both exercises in both groups. In response to AE, these changes were evident, but not different between END and RES (xanthosine END = 529% & 326% above resting at AE-T1 & T2; RES = 551% & 442% respectively, $p < 0.05$, $q < 0.1$). Following AN, the changes to metabolites reflecting ATP turnover were even greater than with AE, and a group difference was observed **(Figure 3).** Larger and longer-lasting increases of these metabolites in RES than END signified greater ATP turnover in this group in response to AN**.**

Elevated TCAC intermediates were seen immediately following AE and AN (**Figure 4**). Within both groups, levels returned to or towards baseline by T2. However, there was a tendency for larger perturbations at both time points for the group with experience in the exercise mode being tested (**Figure 4**). This effect was strongest within the span-2 intermediates, reaching greater than a 2-fold difference for succinate in both cases, END for AE and RES for AN. Appearance of the span-2 intermediates suggests mobilization for energy provisions.

Glucose Metabolism. Glycolytic end-products, pyruvate and lactate, were each elevated at AE-T1 for both groups (**Figure 5**), indicating active glycolysis during this exercise. Like with AE, glycolytic end-products rose during AN; however, RES showed higher levels of both pyruvate and lactate than END at AN-T1 and AN-T2 (AN-T1: pyruvate = 43% higher for RES; lactate = 49%; AN-T2: pyruvate = 39% higher for RES lactate = 35%, $p < 0.05$, $q < 0.1$) and a greater lactate increase during AN for RES (RES increase during AN = 417%, END = 266%, $p < 0.05$, $q < 0.1$, **Figure 5**).

AA Metabolism. Markers of heightened protein degradation, dipeptides and urea cycle metabolites, were seen to differentiate between groups. It should be noted that most

perturbations in urea cycle intermediates were fairly small (<2-fold in magnitude). Still, significant differences did exist between groups for several metabolites and changes observed tended to be exclusive to or larger in the matched-training or experienced cohort. These results imply slightly greater flux through this cycle and catabolism of AA by RES in response to AN, but END with AE (**Figure 6 A,B**).

BCAA metabolites experienced equivalent perturbations to AE in both groups. Several of these metabolites were further elevated in RES compared to END at AN-T1 and/or AN-T2 (**Figure 6 C**). Specifically, metabolites within the leucine degradation pathway leading to hydroxy-methylbutyrate (HMB) production were more elevated in RES than END, particularly at AN-T2 (**Figure 6 D**). Notably, α-hydroxycaproate, a precursor to HMB and an anabolite itself, was over 50% more elevated in RES than END at both AN-T1 and AN-T2 (**Figure 6D**). In addition to the difference in the AN response, α-hydroxycaproate responded to AE more in the RES cohort as well, 134% versus 64% above baseline for RES and END, respectively, and remained 49% higher than END at AE-T2, $p < 0.05$, $q < 0.1$.

Perhaps differences in the anabolic markers seen in response to exercise were driven by androgenic sex steroid signaling, which was predominantly more extensive in the RES cohort. When significantly altered androgenic steroid biosynthesis markers were compared between END and RES participants, it revealed that virtually none of these metabolites showed a unique response to END, but many were changed in RES only, particularly when looking at AN (**Figure 7**).

Ketone Metabolism. Ketone bodies were significantly elevated above baseline at AE-T1 and AE-T2 in both groups. They were higher for END at AE-T1 (END > RES for β-hydroxybutyrate (BHBA) = 52%; acetoacetate = 316%, $p < 0.05$, $q > 0.1$), but for RES at AE-T2 (RES > END for BHBA

= 163%; acetoacetate = 246%, $p < 0.05$, $q > 0.1$, **Figure 8**). Ketone metabolism was not altered by AN for either group.

FA metabolism. As expected, fatty acid (FA) oxidation was more prevalent during AE than AN (**Figure 9**). Less expected, RES tended to experience more, longer-lasting increases in the free FAs with AE than did END. When pooled together, the average increases in long-chain fatty acids (LCFAs) in response to AE were greater in RES than END (46% and 73% at AE-T1 and AE-T2, respectively). Interestingly, for the polyunsaturated fatty acids (PUFAs), changes in RES were only 6% and 7% higher than those in END at AE-T1 and AE-T2, respectively (**Figure 9**). In contrast, LCFAs, on average, were only minimally changed during AN (+9% and +1% above resting for END and RES, respectively at AN-T1), but increases at AN-T2 were substantial in both groups (+63% and +50% for END and RES respectively). PUFAs showed similar responses as the other LCFA's during AN, but increases in END were 2-fold higher than RES at AN-T2.

Fatty-acyl carnitines (FACs) appeared to vary by chain length as well as saturation of the acyl chain. Short-chain (< 6C) and long-chain (> 12C) FACs were universally increased across group and condition, while medium-chain (6-12C) FAC's were decreased with AN in both groups (**Figure 9**). Meanwhile, unsaturated FAC species were similarly altered by AE, but the PU-FACs in particular were further elevated in END than RES at AN-T2. These changes mirror those of the non-carnitinylated PUFAs. Together these data portrayed a picture of elevated FA mobilization and oxidation during AE and after both exercises in both groups, but higher saturated FA levels in RES and PUFA levels in END. Similarly, medium-chain FAs and dicarboxylates responses were split by acyl chain length. Short- and medium-chain dicarboxylates were further altered during exercise and post-AE in END than RES, with increases seen at T1, and decreases at T2 for the odd-numbered species (**Figure 9**). These changes signify active catabolism of FAs in the mitochondria along with alternative peroxisomal β-oxidation and α-oxidation during, but not

after, AE and during exercise and through recovery for AN. Lastly, the LC-dicarboxylates more greatly perturbed in RES (i.e. maleate, malonate) all related to mitochondrial dysfunction, or in the case of a healthy individual, an imbalance in lipid mobilization and oxidation.[29,30] This inadequacy in mitochondrial function appeared greatest in RES post-AE. Analogous results were seen throughout the varied FA metabolites (mono/di-hydroxy FA's, amino-FA's, branched-FA's and FA synthesis markers).

Membrane Lipids. In addition to this, FA metabolites connected to membrane lipid turnover, were consistently altered by exercise. While changes in groups were similar in direction, they often differed by magnitude, with bigger perturbations in the group with a training background matching the mode of exercise. The most dramatic example was seen with the acyl-cholines, where all 8 of these phospholipids were further decreased at AN-T1 and AN-T2 for RES than END. In addition to this, greater perturbations in phosphocholine at AN-T1 and glycerophosphocholine at AN-T2 for RES (66% and 25%, respectively, $p < 0.05$, $q < 0.1$) with no concomitant difference in choline further indicated a higher rate of membrane-lipid turnover for RES during AN. Similarly, changes in the glycerol-inosophosphitols, lysolipids and sphingolipids/ceramides all signified greater membrane turnover in END than RES during AE.

Lipid Mediators, Inflammation & Stress. Similar to the acyl-cholines and other membrane lipids, monoacylglycerols (MAGs) revealed a group x condition interaction. Changes in the α-MAGs aligned with the membrane lipid pattern of matching training background to exercise mode for the larger response (**Figure 10**). In contrast, the β-MAGs reversed this, tending to be more altered at T1 for each condition in the group with an opposing training background (END with AN and RES with AE). Moreover, while the α-MAGs were mostly increased with exercise (except for RES at AN-T1), the β-MAGs were decreased with exercise (except for END at AN-T2, **Figure 10**). As the α-MAGs act as signaling molecules and the β-MAGs serve as precursors to

many lipid mediator, perturbations of these metabolites could imply elevated lipid mediator signaling or synthesis. Indeed, the eicosanoids and other downstream lipid mediators were seen to change across group, condition and time (**Figure 11**). These downstream lipid metabolites consisted of both pro- and anti-inflammatory agents. In response to AN, the lipid mediators followed were trending towards baseline by AN-T2 for END, but remained elevated, and in some cases continued to rise, post-AN for RES (**Figure 11 B**).

Cortisol a potent anti-inflammant and major stress hormone of the human endocrine system, strongly inhibits eicosanoid production. Corticosteroid metabolites (21-hydroxypregnenolone-disulfate and -monosulfate, cortisol, corticosterone and cortisone) were all elevated during both exercises and mostly returned to or towards baseline by 60-min post-exercise in both END and RES. (**Figure 11 A,C**). The simultaneous rise during exercise in cortisol and the eicosanoids demonstrates just how powerful the stress of exercise is. And the continued post-AN increase in the eicosanoids for RES displays unresolved stress enacting the inflammatory system in this group for this exercise.

Another major stress hormone, epinephrine, showed more of a group x condition interaction. Catecholamine metabolites rose in response to exercise for both groups. However, circulating levels were higher, and increased significantly larger, for END in response to AE and RES in response to AN. These results imply further heightened sympathetic activity for END in response to AE and for RES following AN.

A special class of lipid mediators known as the endocannabinoids, with both anti-inflammatory and psychophysiological effects have been proposed to mediate phenomena such as the “runner’s high” and may play a role in exercise attrition.[31-33] The endocannabinoids were similar between groups at rest and had only minimal responses to AN. At both AE-T1 and AE-T2,

however, larger relative changes were seen for oleoyl ethanolamide and linoleoyl ethanolamide in RES than END (**Figure 11 D**).

Antioxidant Response & Energy. Evidence of glutathione cycle activity (including transulfuration and γ-glutamyl-AA's), plamalogen/lysoplasmalogen turnover, vitamins B3, C, E and bilirubin metabolism and various other markers of oxidative stress and antioxidation were seen in response to both exercises across groups (**Figure 12 A**). It appeared that neither exercise mode nor training background differentially influenced antioxidant activity. The transulfuration pathway provides a thiol group for glutathione production (**Figure 12 B**). However, a shunt off of this pathway can be used to supply energy provisions (propionyl-CoA) to feed into the TCAC. Metabolites reflecting diversion through this shunt were more perturbed in RES than END, particularly at the 60-minute post-exercise time point (**Figure 12 A,C**). Meanwhile, decreases in cysteine-sulfinic acid and concomitant increases in hypotaurine and taurine were observed during both exercises, indicating routing of the thiol group into taurine metabolism, but were larger immediately during AE for END and during AN and after both exercises for RES (**Figure 12 C**). Additionally, metabolites of vitamins more closely tied to energy dynamics, B5 and B6, were more elevated in RES than END as well (**Figure 12 A**). As a whole it seemed antioxidant activity was upregulated in both groups in response to both exercises, however; increases in energy metabolism appeared to be greater in RES than END, especially post-exercise.

Microbiome Metabolism. In all there were 87 altered metabolites identified as markers reflecting microbiota activity, displaying the interaction between exercise and the gut flora. This included 24 primary and secondary bile acid metabolites. The majority of these markers decreased in response to exercise. Despite the widespread response, there were only 2 bile acids and a few other metabolites that were significantly different between groups, but in all occasions, it was the group with a training background that matched the exercise mode that

experienced a larger disruption of the microbiome metabolome. Many of the perturbations were modest, with only 7 changing by more than 2-fold in one or both groups. Major (> 2-fold) changes to the xenobiotics on this panel linked both exercises to either ferulic acid or cinnamic acid metabolism by the gut microbiome.

Immune Function. Differences between END and RES extend to immune function. Sphingosine, which is readily phosphorylated to the angiogenic agent and immune trafficking mediator sphingosine-1-phosphate, was increased for END only at AE-T1 (119% above baseline) and in RES only at AN-T1 (106% above baseline), $p < 0.05$, $q < 0.1$. In both cases, sphingosine increases were seen in the group with experience in the mode of exercise being performed. Trans-urconate, an immune regulator typically found in sweat and near the skin was unchanged immediately post-exercise (AE or AN) in END, but was 2-fold higher by 60-minutes post-exercise. RES, on the other hand, saw more modest increases (50-80%) at both T1 and T2 for AE and AN.

Summary. END and RES had very similar resting metabolomes, with plasmalogen/lysoplasmalogen metabolism and a primary and secondary bile acid pair as the only pathways with multiple metabolites showing a difference between groups. Moreover, in response to both AE and AN, the extensive metabolic shifts that occurred were far more similar than different between groups. Differences that did arise, tended to be larger in the cohort with the matched-training background, END for AE and RES for AN. For both exercise modalities and both groups, the metabolomic shifts observed at T1 were substantially reduced by T2 (**Figure 2**). Furthermore, END appeared to have a faster recovery than RES, particularly for AE. While macronutrient metabolism differed greatly between exercises (FA use during AE only and higher glycolysis during AN), differences between groups were more subtle, suggesting mode trumped training status in determining substrate selection. Still, specific and impactful differences were observed. Namely, augmented purine and anaerobic metabolism, in RES and FA and aerobic

oxidation in END. The differences between groups in androgenic signaling (higher in RES) and FA selection (higher PUFAs in END) as well as the time course of the changes in these metabolites, appeared to have led to different downstream events. Resultant divergences in anabolic BCAA (leucine) catabolites, inflammatory signaling, cognitive processing and immune trafficking were all observed. In this way, the training background recapitulated and often amplified many of the metabolic differences between exercise modes. However, it must be stressed that most differences were a matter of magnitude, and not direction. In this way, END and RES metabolomes were more similar at rest, and in response to exercise, AE or AN.

DISCUSSION

Rest. Resting group comparisons revealed near identical metabotypes for END and RES. This is in agreement with a finding of altered fitness status following different training programs without concomitant differences seen in skeletal muscle metabolites.[32] However, it was still unexpected, and in contrast to previous reports comparing the same individuals pre- and post-training.[18,35-37] We did identify significant differences in the (lyso)plasmalogens as well as 2-aminoadipate and a pair of bile acids. Plasmalogens are cleaved to lysoplasmalogens through the action of phospholipases, but can also be converted by reactive oxygen species and so serve as endogenous antioxidants. Additionally, 2-aminoadipate has free-radical scavenging function. Given the lack of differences in other more potent antioxidant systems, our results may weakly suggest a difference in antioxidant status with higher resting antioxidant activity in RES.

Findings of a study characterizing the resting metabolome of a variety of elite athletes identified heightened steroid biosynthesis in endurance athletes as well as differences in fatty acid metabolism, oxidative stress, and energy-related metabolites based on power and endurance classifications of the athletes' sport.[12] Here we did not find any differences in resting markers, though it should be noted that the average concentration of steroid biosynthesis metabolites was non-significantly 50% higher in END than RES. The smaller sample size in our study (n = 40 versus 191) may explain the discrepancy in findings. Alternatively, participants in our study were healthy, recreational exercisers while Al-Khelaifi *et al.* (2014) used elite athletes.[12] The more extreme training regimens and/or genetic variants and resultant extreme physiology/metabolism in elite athletes may have led to the resting differences observed there. Regardless, we conclude that the metabotype of a physically active (healthy) individual does not differ based on type of physical activity or fitness status (strength, $VO_{2MAX,}$ etc.). This finding corroborates with mounting evidence in support of the efficacy of high-intensity-training

programs in bringing about health benefits traditionally ascribed to endurance-training.[38] Given the healthy status of our combined cohort (END + RES) and the consistency between groups, the observed resting metabolome as well as the AE and AN response profiles observed herein may represent normal, healthy metabotypes at rest and in response to each exercise respectively for reference in future studies.

Exercise Comparison. AE and AN had more in common than not. Both showed increased energy metabolism, a stress response including inflammation and oxidative stress with evidence of anti-inflammation and antioxidation and interaction with the gut microbiome. Metabolic systems and specific metabolites seen to be activated by these exercises were mostly in agreement with what has been previously published for other aerobic and anaerobic exercise bouts.[17] The most robust increases were observed in the purine metabolites, TCAC intermediates and throughout the energy-related pathways. Additionally, extensive evidence for altered metabolism of the microbiome was observed, and both AE and AN led to an initial rise in inflammatory activity along with markers of oxidative stress and concomitant antioxidative activity. Interestingly, the initial changes in the resting metabolome generally showed a tendency for recovery by 60-minutes post-exercise. Differences largely manifested in substrate selection. AE had greater FA mobilization/oxidation including stronger evidence of increased downstream lipid mediator signaling. AN was characterized by higher purine turnover, greater anaerobic glycolysis and a delayed, post-exercise rise in FA use and aerobic metabolism.

Overall, AE led to more extensive alterations of the metabolome than did AN, which may be an artifact of the large number of lipid-based metabolites included (over half of the panel), or due to the greater in-task caloric stress, or workload of AE when compared to AN. Metabolomic studies have previously shown sensitivity to workload.[16,17,39] Exercise condition differences were often more dramatic at T1 than T2, while training background differences appeared at both T1

and T2. This makes some intuitive sense, as the acute stressor gave rise to more acute differences, while the more permanent difference of training status yielded longer-lasting differences. These results intimate that while metabolism during the exercise bout may vary between exercise mode, once the exercise is stopped, recuperation from the stressor begins, and metabolism has a more generalized metabolomic signature or recovery.

Group Differences During-Exercise. Whereas the majority of studies have reported on changes in resting status of the metabolome following a training intervention in sedentary and often diseased individuals (diabetes, CVD),[11,18,20] our design fundamentally differed in two ways. First, we used previously trained and healthy, rather than sedentary individuals. Initial benefits of participation in physical activity may not accurately portray continued metabolic changes seen over a longer period of time. Adaptations to exercise are known to dissipate over time as individuals approach their genetic ceiling, i.e. law of diminishing returns. Our cohorts likely well-represented the population of physically active individuals found throughout the US in that they had been participating in their current exercise regimen for at least 2-years. Therefore, we believe our results better translate to changes seen within individuals who actually participate in exercise. Second, in addition to comparing resting metabolomes of differentially trained individuals, we demonstrated the acute responses to both AE and AN, and the impact of training status (END versus RES or matching versus opposing the condition tested) on the metabolic response pattern. This was a novel twist on models previously used in exercise metabolomics.

We had postulated that adaptations conferred through years of specific training would offer some protection from the stress of the matching exercise bout. In contrast, END experienced a greater volume and magnitude of perturbations than RES at AE-T1, while RES experienced greater disruption to the resting metabolome than END at AN-T1. This was likely the result of employing a matching relative exercise intensity. Since each group was more fit to perform

either AE or AN based-on training background, the absolute load during the conditions was greater for END than RES during AE and RES than END during AN. When viewing specific pathways, the higher absolute workload led to a greater stress on the system, which was reflected in the epinephrine response and metabolites of energy metabolism. In-task substrate metabolism, β-oxidation during AE (acyl-carnitines) and non-aerobic metabolism during AN (glycolytic end products plus ATP turnover) and TCAC activity, particularly span-2 intermediates for both, were also more impacted in the more aptly trained group. Further, disruptions to several other pathways (taurine production, microbiome activity, immune trafficking and membrane lipids) all tended to indicate absolute workload as a major factor in determining the effect of acute exercise on the resting metabolome. Though relative intensity has also shown to be impactful in workload-matched studies,[17,19] the importance of absolute workload is not without precedent,[18] and the relative impacts of each requires further clarification for consideration in future designs of exercise studies employing a metabolomic panel.

With respect to the above, improvements in fitness result in a lower stress from the same absolute-intensity, but not a matched-relative-intensity exercise bout.[38] Rather, training affords an improved ability to correct any incurred metabolic disruptions.[38] However, in response to both AE and AN, we saw a more complete recovery to the metabolome as a whole and a profile reflecting greater recovery at T2 for END than RES following both AE and AN. Given the general familiarity of riding a stationary bike and the comparable greater non-metabolic challenges associated with our AN session (balance, specific movement patterns, etc.),[39] it is plausible that END was simply unable to approach even a relative matched intensity to RES from a metabolic standpoint during AN. This may have allowed them to better recover from this exercise despite not having a training background in place to handle the specific stresses associated with it.

Still, there was ample evidence supporting a greater mitigation of incurred homeostatic imbalances in END than RES post-exercise. Energy balance appeared to be restored faster in END, as seen by their lower levels of ATP turnover and circulating glycolytic end products than in RES, particularly following AN. The sustained anaerobic metabolism and continued shuttling of glucogenic precursors suggests a prolonged energy demand in RES following AN exercise in particular, as does the shift in elevated ketone metabolism from AE-T1 (greater in END) to AE-T2 (greater in RES). Additionally, END had declining, as opposed to the increasing or sustained, inflammatory lipid mediators seen in RES 60-minutes post exercise. In our comparison, both groups were trained, just not in the same fashion. It seems equally plausible that END, with their better-trained aerobic systems, are set up to recuperate more quickly, as recovery itself is an aerobic process as evidenced here by the massive shift to oxidation of FA following both AE and AN in both END and RES (T1 versus T2).

Substrate Metabolism. As previously stated, the glycolytic end products pyruvate and lactate were both further elevated in RES than END for AN as well as AE. Purine salvage metabolites, indicative of ATP turnover, were also higher in RES than END for both conditions and at both time points. For glycolysis and purine metabolites, the differences were greater with AN than AE. Together this strongly suggests metabolic adaptations to their training allowed RES to better develop and therefore more heavily depend on these systems in the face of an exercise challenge, particularly one of higher intensity. In contrast, higher circulating LCFAs were seen in RES for AE, while higher acyl carnitines were seen in END. The difference likely reflects more efficient transport and greater β-oxidation rates for the LCFAs in END. This notion is supported by the observed changes in downstream FA metabolites indicative of an imbalance in mitochondrial FA metabolism (odd-chain FA/FACs and markers of carnitine palmitoyl transferase or medium-chain acyl dehydrogenase deficiency) being further increased in RES than END.

Interestingly, PUFAs seemed to be more elevated following AN in END than RES, hinting perhaps at directed metabolism of these functional FAs for some subsequent physiological purpose.

Along the same lines as the targeted PUFA metabolism, differences in substrate selection may extend to other areas of physiological importance. For example, changes seen in the adrenal stress hormones cortisol and epinephrine may in fact relate to their role in energy metabolism. Epinephrine increases mobilization, but not oxidation of FAs,[40] while cortisol increases gluconeogenesis and glucose use.[41] This could, in part, explain the greater epinephrine levels seen with AE than AN (an unexpected finding) and why cortisol was marginally higher in RES than END at AE-T1 and -T2 despite having a lower absolute workload.

Metabolism downstream of FAs may have been on display for the unexpected finding of greater endocannabinoid levels in RES than END at AE-T2. Increases in these anxiolytic agents mirrored FA metabolism. The response to AE was much greater than AN, and within AE, END and RES were similar at AE-T1, but RES was greater at AE-T2, precisely when FA levels were higher in each group. This finding could be impactful in determination of motivation to exercise. The endocannabinoids are known to be up-regulated with aerobic exercise,[42] and are associated with pain reduction[43] and altered emotional and cognitive processes.[32] They have also been implicated in the "runner's high" phenomenon.[33] Here we found their appearance to mirror that of metabolites depicting an overwhelmed mitochondrial system. If their appearance is dependent upon challenging, rather than just activating mitochondrial FA metabolism, it would mean development of the aerobic system would make occurrence of the runner's high less likely in highly-trained individuals. This would explain why RES had a larger response than END in response to AE.

AA metabolism again showed a pattern of more activity for the matched-training group, particularly at T1, indicating specificity of training for development of metabolic fuel flexibility

during exercise. However, though statistically significant, these differences tended to be relatively small. Larger differences between groups appeared within BCAA metabolism and more specifically downstream leucine metabolites. Interestingly, α-hydroxyisocaproate, a known anabolite rarely studied within the context of exercise, was further elevated within the RES cohort than END following both exercises. This association links α-hydroxyisocaproate to the RES phenotype and suggests its possible use in determining training status effects on the acute exercise response, specifically as it pertains to anabolism. Other markers within this metabolic pathway (leucine → HMB) were similarly further elevated in RES than END in response to AN. In addition to the anabolic metabolites, androgenic steroid biosynthesis markers were clearly more elevated in RES than END. It is possible that greater action by these steroid metabolites acts as the controlling force driving the enhanced leucine metabolism and downstream anabolic signaling.

Antioxidation. Glutathione and transulfuration pathways present a microcosm of the results of this study. Both exercises and groups were fairly similar in that glutathione cycling was increased. However, the burden of recovering the energy deficit for RES also affected this process. The α-ketobutyrate shunt connecting transulfuration to the TCAC was clearly more active in RES than END following both exercises. There was also more diversion of cysteine towards taurine/hypotaurine for AN in RES and AE in END showing a training response for taurine synthesis. Greater taurine production in the matched-training group may be due to taurine's physiological effects as an antioxidant or in enhancing glucose metabolism.[44,45]

Correspondingly, vitamins B3, C, and E, and bilirubin all displayed active antioxidant activity for both groups in response to AE and AN. However, other vitamins, particularly those related to energy metabolism seemed to show selective metabolism by group. Vitamins B6 and B5 were more elevated in RES than END for both exercise conditions. We believe these observations

reflect true metabolic differences in response to the exercise bout rather than dietary impacts. Firstly, because resting levels were not different between groups. Secondly, because these observations are based on changes from resting values and not absolute concentrations. And thirdly because of the similar depiction seen for glutathione and α-ketobutyrate metabolism. However, given our use of a free-living diet (other than a 2-hour fast leading up to the condition) it is certainly possible that dietary differences did play a role in these results.

Other Observed metabolites. Acyl-choline alterations (decreased during exercise, and more so for the correspondingly- trained exercise group) intimate the possibility that phospholipid turnover may reflect an association between exercise and training status similar to that seen for more traditional markers of exercise stress. This could relate to the greater workload and oxidative stress absorbed by membrane lipids or just upregulation of membrane turnover with higher workloads. Regardless of the cause, declines in the acyl-cholines has not been previously reported and warrants further investigation, particularly as it may relate to exercise workload and intensity.

Immune function showed limited evidence for any group differences or group by condition interaction, but the rise in trans-urconate in END post-AN may relate to its role in T-cell activation.[46] This histidine metabolite has not been studied in the context of exercise, but as T-cells trigger the release of cytokines, in addition to immune and inflammatory effects, it has implications as a player in myokine and signaling responses associated with exercise. [46] Changes in sphingosine, may relate to its functional phosphorylated form, sphingosine-1-phosphate. These functions include immunomodulatory function through T and B-cell trafficking, promoting growth and repair of capillary beds, and/or in mediating the beneficial effects of high-density lipoproteins.[47,48] Previous metabolomics investigations have revealed angiogenesis as a major pathway of activation following aerobic cycling,[19] and parallel changes in other

immunomodulatory and inflammatory metabolites would implicate these systems for relevance of this ceramide metabolite. Whatever the purpose, changes in circulating levels of this membrane lipid with exercise are novel and warrant additional research to parse out the physiological significance.

Following both exercises, a primarily decreased microbiome metabolic signature was observed. Like many other markers, perturbations were larger for the matched-training group. As a group, changes within the bile acids were one of the most prolifically altered subsets of metabolites. Xenobiotic changes were somewhat scattered as a whole, but tended to show decreases from resting. Elevated 2-oxindole-3-acetate and cinnamoylglycine at AE-T1 in RES were the only increases in products of bacterial metabolism in the gut. While exercise has been shown to have interactions with gut metabolism,[49] it is also known to reduce blood flow to the viscera, and given the short window of observation, this may explain why decreases in these metabolites were seen. Nonetheless, these results did link changes in microbiota metabolism to the exercise response.

Summary. Despite different physical characteristics, resting metabotypes and metabolomic profile shifts from exercise were mostly similar between END and RES. There were differences related to substrate use and energy dynamics, as well as lipid mediator signaling, recovery rate, anabolic and androgenic signaling and several novel metabolites including α-hydroxycaproate (anabolism), trans-urconate (immune function) and sphingosine (immune function and angiogenesis). Our findings have potential application in clinical and applied settings, sports (performance) science, and in guiding further mechanistic research.

FIGURES

Participant Characteristics

Category Mean ± SD	END	RES
Age (years)	24.8 ± 3.7	23.2 ± 2.5
Height (cm)	171 ± 8.3	166.7 ± 9.9
Mass (kg)	65.2 ± 6.6	69.2 ± 11.3
% Body Fat	16.7 ± 8.2%	19.4 ± 6.6%
Fat Free Mass (kg)	54.4 ± 8.8	55.9 ± 10.8
relative VO_{2MAX} (ml/kg/min)*	51.3 ± 8.6	41.6 ± 3.1
VT (% of VO_{2MAX})*	78.1 ± 10%	65.8 ± 7.3%
PO_{MAX} (Watts)*	241 ± 66	194 ± 35
Squat 10RM (kg)*	53 ± 13	92 ± 28
Leg Press 10RM (kg)*	147 ± 36	217 ± 63
Squat 10RM (% body mass)*	81 ± 18%	132 ± 33%
Leg Press 10RM (% body mass)*	225 ± 47%	315 ± 78%

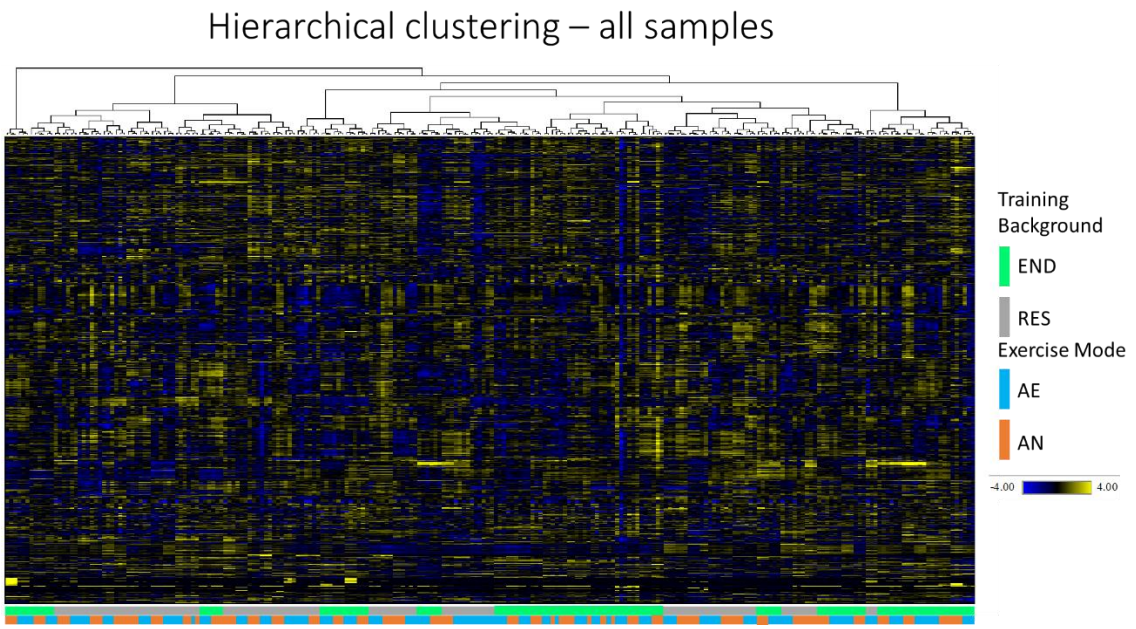
Table 1. Descriptive Characteristics for the END and RES cohorts. SD = Standard Deviation; 10RM = 10-repetition max weight.
*Significant difference between groups, $p < 0.05$

Resting Differences

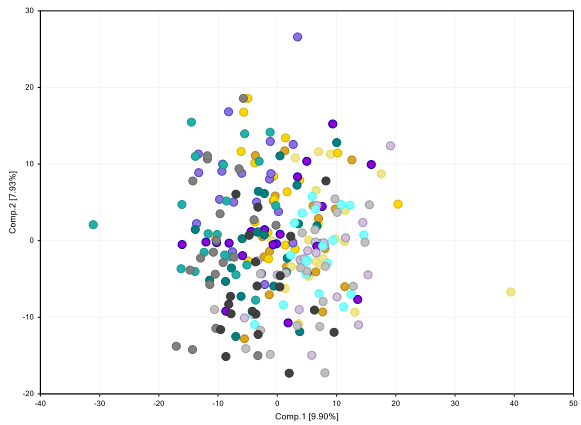
Sub-Pathway	Marker	RES > END by:
Lysine Metabolism	2-aminoadipate	79%
Lysoplasmalogens	1-(1-enyl-palmitoyl)-GPE (P-16:0)	45%
	1-(1-enyl-stearoyl)-GPE (P-18:0)	82%
Plasmalogens	1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)	52%
	1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)	69%
	1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)	56%
	1-(1-enyl-palmitoyl)-2-linoleoyl-GPE (P-16:0/18:2)	170%
	1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)	257%
Primary Bile Acid Metabolism	taurochenodeoxycholate	56%
Secondary Bile Acid Metabolism	tauroursodeoxycholate	20%

Table 2. List of metabolites that significantly differed between groups at rest. In all cases RES > END. Values give percent difference between END and RES.
END = endurance trained, RES = resistance trained

1.A



1.B



1.C

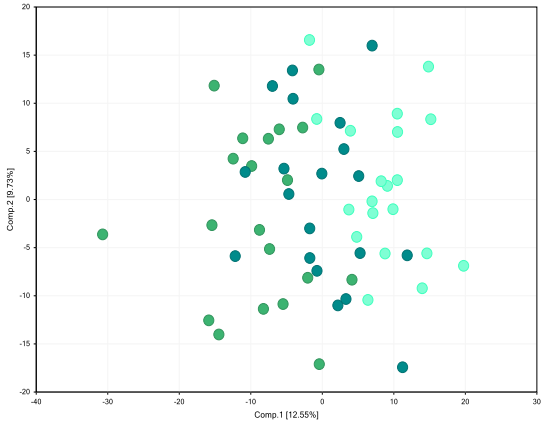


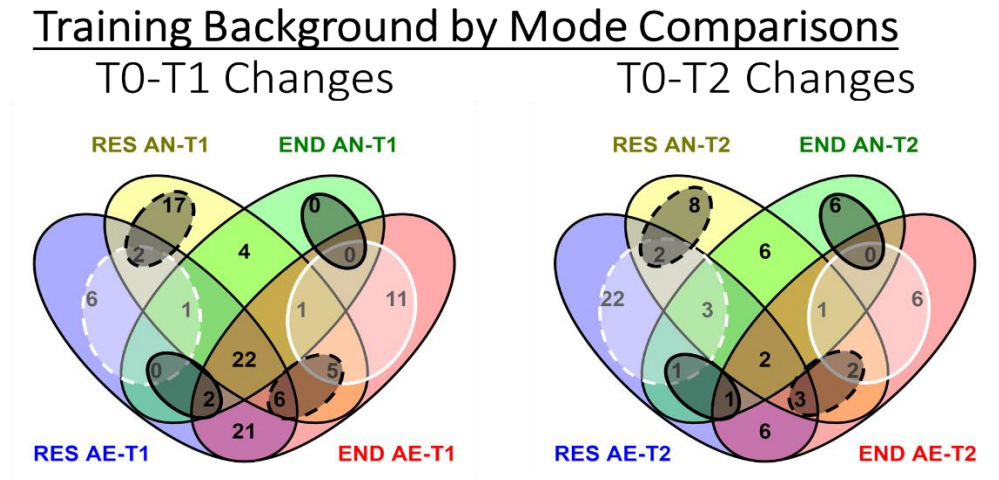
Figure 1. A) Hierarchical cluster analysis of all samples. Along bottom, clustering by exercise mode (blue & orange) and training background (green & grey). Data groups by training background more than by exercise mode, as seen by the more extensive clustering of data points.

Figure 1. B) This PCA plot color codes each exercise mode, training background and time. While some clustering is evident, it is vastly overshadowed by the overlap between different exercise conditions and groups. Dotted rings show clusters for each of the 4 condition x training data sets in corresponding colors.

Figure 1. C) PCA plot limited to just the END-AE response so that the separation by time point is more clearly seen. The same pattern is seen with other training background-exercise mode combinations. T0 and T1 data points outlined by dotted rings of corresponding color. T2 data points overlap with both T0 and T1. Interpreted as a significant shift in the metabolome by exercise and a return towards resting status with 60-minutes recovery.

END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T0 = resting, T1 = immediately post-exercise, T2 = 60-minutes post-exercise.

2.A **2.B**



2.C

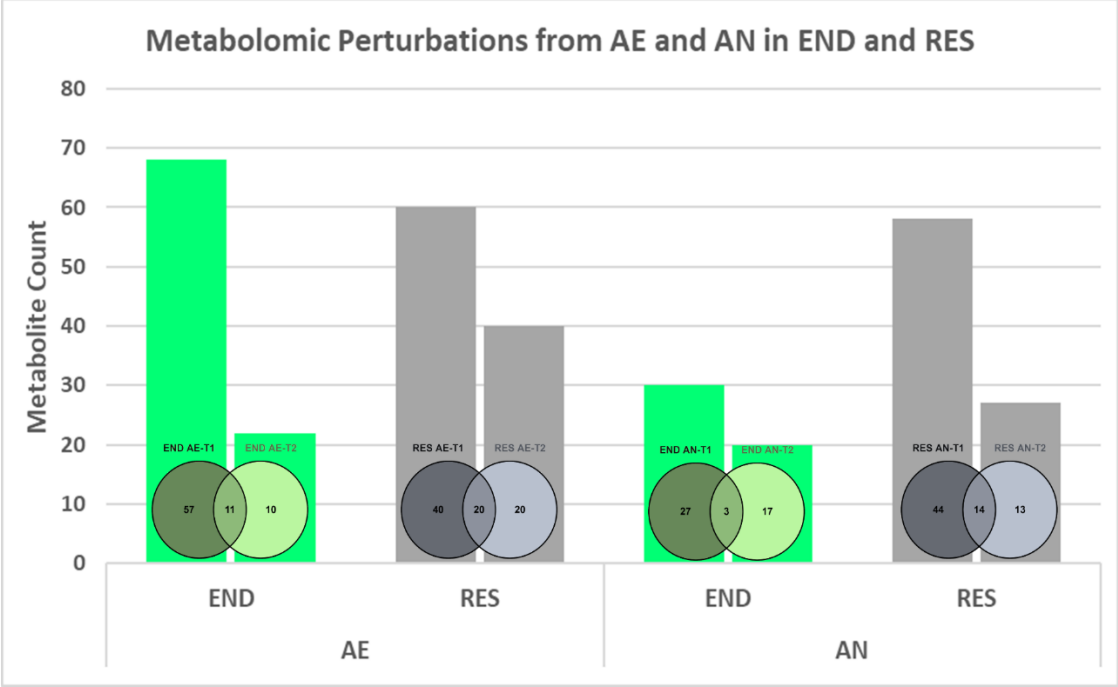
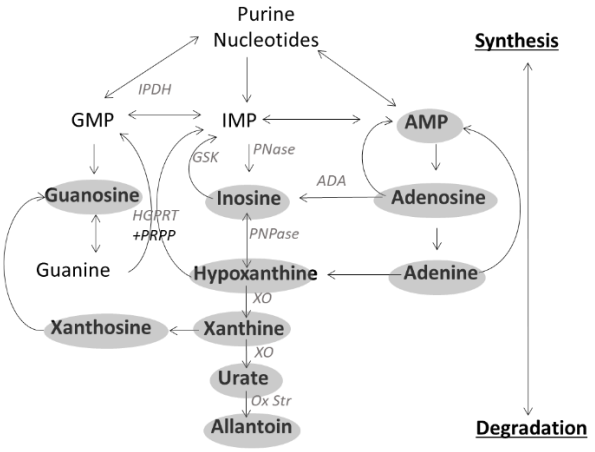


Figure 2. A&B) Four-way Venn Diagram of altered metabolites from rest at T1 (**A**) and T2 (**B**) for RES and END responses to AE and AN. Numbers generated by significantly different metabolites ($p < 0.05$, $q < 0.1$) with a minimum of a 2-fold difference from resting value. Superimposed white and black rings highlight the exclusive group responses to AE and AN respectively. Solid rings for metabolites exclusive to END and dashed for RES.

Figure 2. C) Total number of metabolites altered 2-fold or greater by exercise at each time point both groups. Overlaid with a Venn diagram showing the time course for these metabolites. END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T1 = immediately post-exercise, T2 = 60-minutes post-exercises.

3.A



3.B

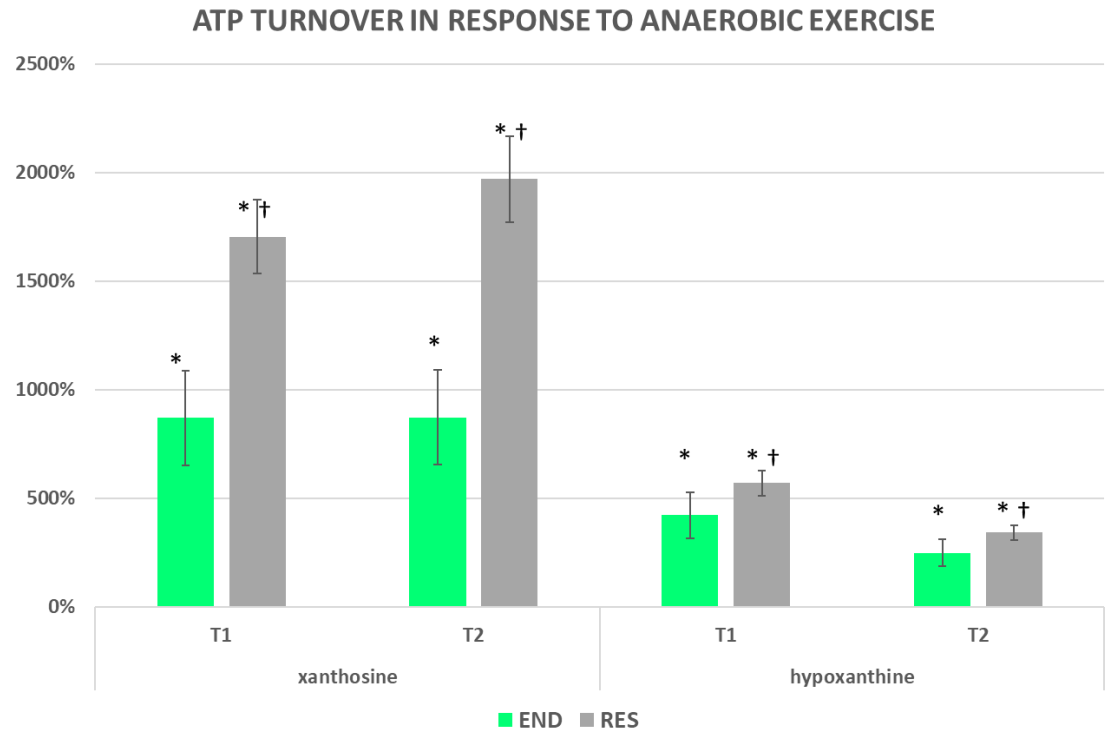


Figure 3. A) Diagram of the purine salvage pathway. Markers that were more elevated in RES than END are highlighted in grey.

Figure 3. B) Changes in purine salvage pathway markers in response to the anaerobic exercise bout. Though significantly increased in both groups, resistance trained individuals (RES) had significantly greater levels than endurance trained (END) at T1 and T2 for both markers. Values expressed as a percentage of baseline.

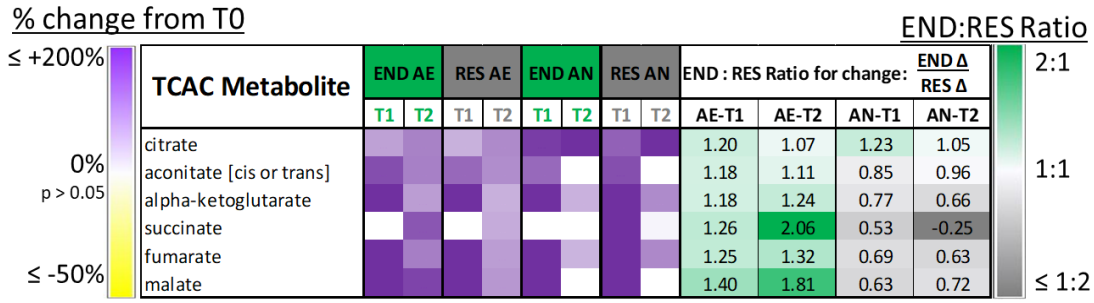
END = endurance trained, RES = resistance trained, T1 = immediately post-exercise, T2 = 60-minutes post-exercises.

* Significant difference from resting level.

† Significant difference between groups for marked exercise and time point.

Significant difference between time points for marked exercise and group.

4.



TCAC Metabolite	END AE		RES AE		END AN		RES AN		END : RES Ratio for change: END Δ / RES Δ			
	T1	T2	T1	T2	T1	T2	T1	T2	AE-T1	AE-T2	AN-T1	AN-T2
citrate									1.20	1.07	1.23	1.05
aconitate [cis or trans]									1.18	1.11	0.85	0.96
alpha-ketoglutarate									1.18	1.24	0.77	0.66
succinate									1.26	2.06	0.53	-0.25
fumarate									1.25	1.32	0.69	0.63
malate									1.40	1.81	0.63	0.72

Figure 4. Heatmap of circulating tricarboxylic acid cycle (TCAC) changes in both groups before and after each exercise. Relative change in metabolite concentration from rest at each time point (T1 & T2) highlighted in purple (increase) or yellow (decrease) by degree of change when value was significantly different ($p < 0.05$, $q < 0.1$) from rest. TCAC metabolites were all increased in excess of 2-fold in both groups for both exercises. Ratio of these values, calculated as the change in END divided by the change in RES for each exercise-time point are given and shaded when the perturbation was greater in one group versus the other (green for END> RES; grey for RES > END). Though all participants saw increases, it can be seen that TCAC intermediates were more elevated in the group with experience in the training modality (END for AE; RES for AN). END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T1 = immediately post-exercise, T2 = 60-minutes post-exercises.

5.

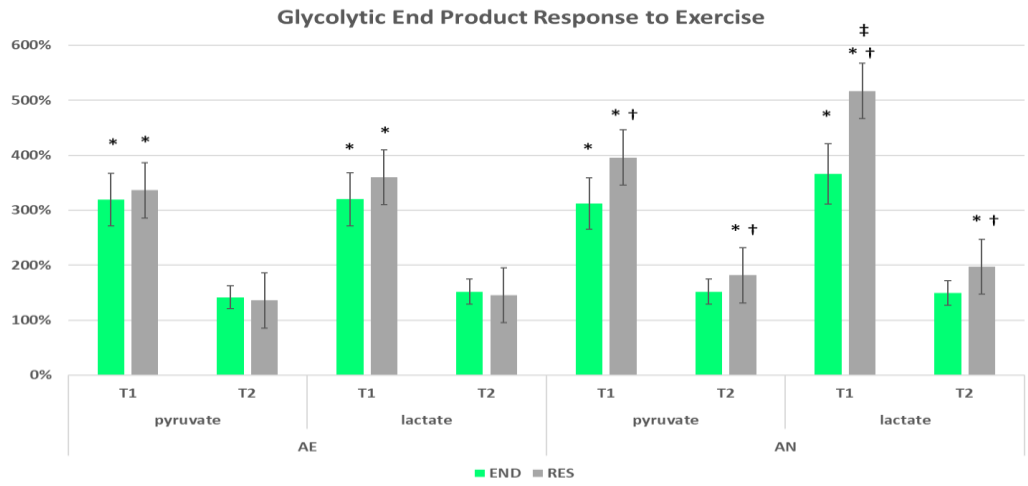


Figure 5. Changes in pyruvate and lactate in response to aerobic (AE) and anaerobic exercise (AN). Both groups were > 2-fold significantly increased at T1 following both exercises. Increases for both markers were greater in in RES than END following AN. Increases in lactate were greater at AN-T1 than AE-T1. Values expressed as a percentage of baseline.
END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T1 = immediately post-exercise, T2 = 60-minutes post-exercises.
* Significant difference from resting level.
† Significant difference between groups (END/RES) for marked exercise and time point.
‡ Significant difference between exercise conditions (AE/AN) for marked group and time point.

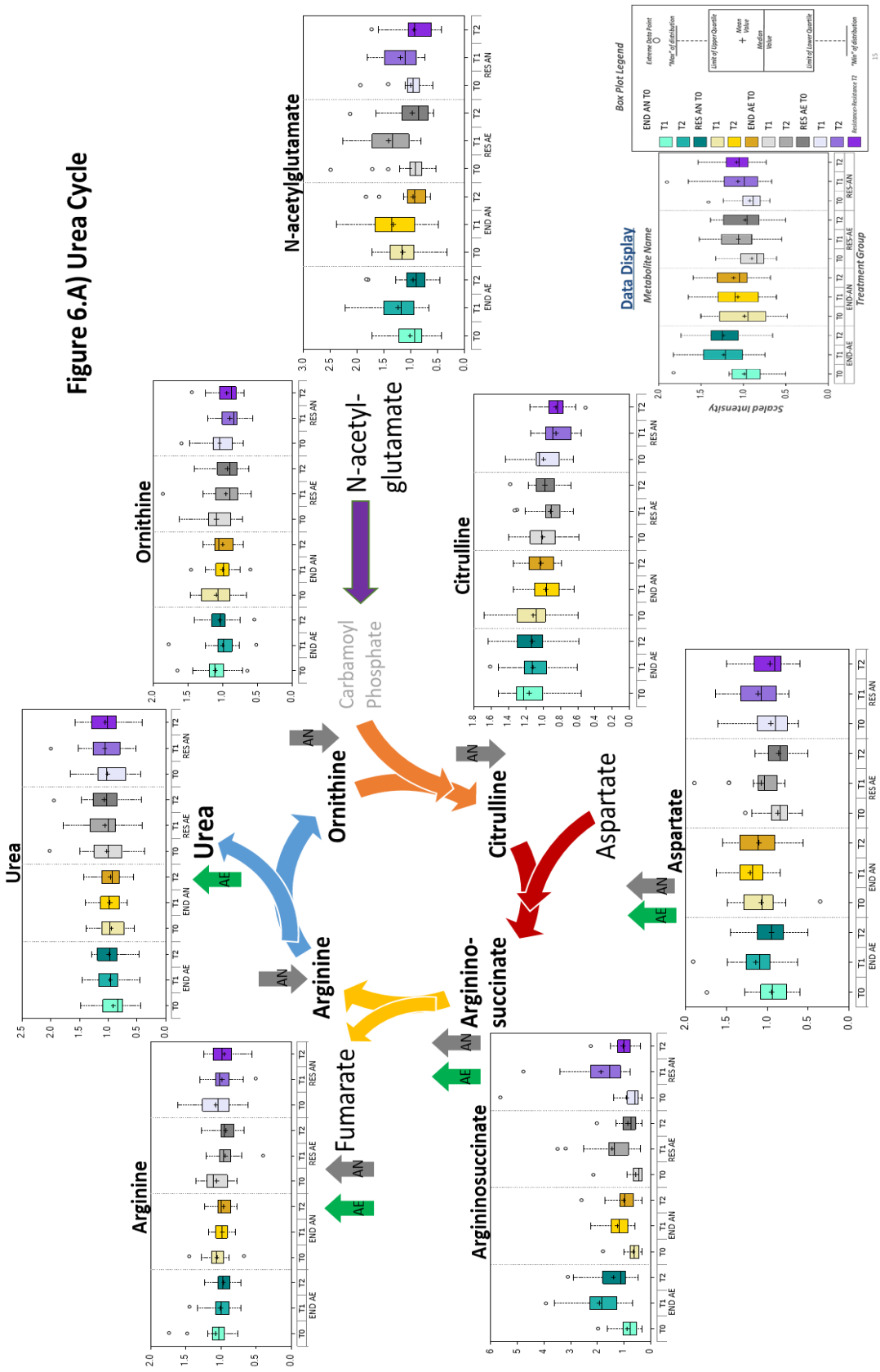
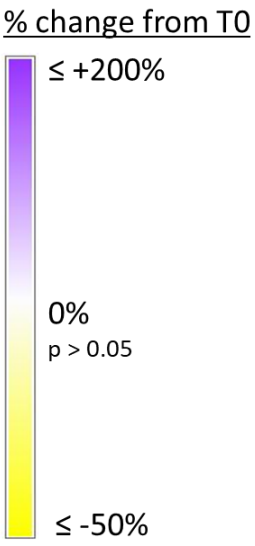


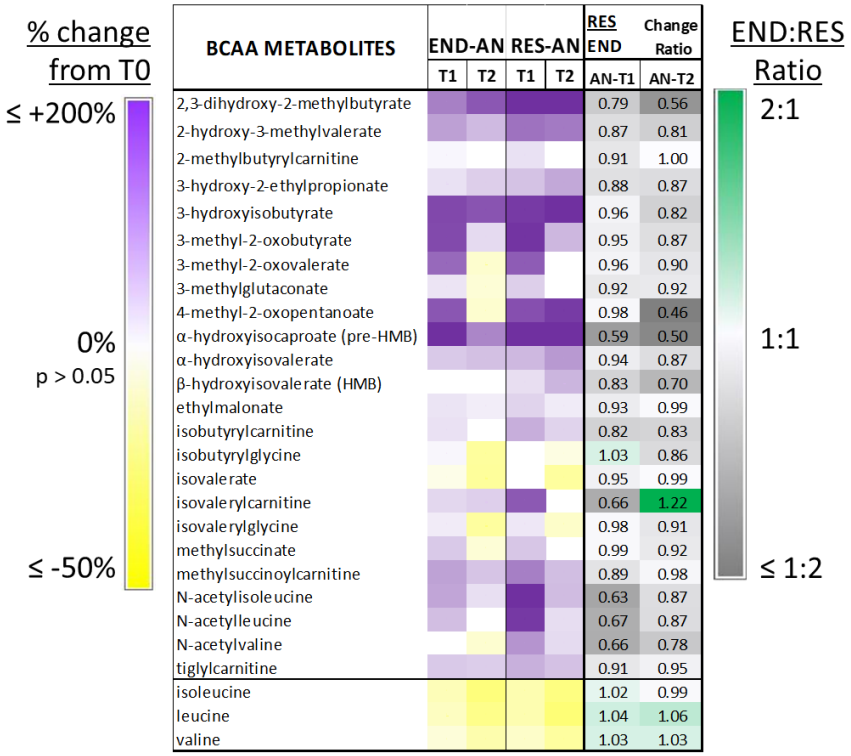
Figure 6.A) Urea Cycle
Urea
Ornithine
Citrulline
Aspartate
Argininosuccinate
Arginine
Fumarate
N-acetyl-glutamate
Carbamoyl Phosphate
AN
AE
Box Plot Legend
Data Display
Metabolite Name
Treatment Group
Scaled Intensity

Figure 6.B **Urea Cycle Heat Map**

Metabolite	END AE-T1	END AE-T2	RES AE-T1	RES AE-T2	END AN-T1	END AN-T2	RES AN-T1	RES AN-T2
ornithine	**91%**	94%	**88%**	**86%**	116%	**92%**	**86%**	**90%**
citrulline	96%	97%	92%	98%	106%	92%	**85%**	**86%**
argininosuccinate	**221%**	**161%**	**253%**	**151%**	93%	**154%**	**207%**	113%
arginine	**94%**	**90%**	**88%**	**88%**	**90%**	**92%**	**92%**	**89%**
acetylornithine	99%	96%	**90%**	**89%**	**87%**	103%	**84%**	99%
homocitrulline	119%	114%	107%	102%	99%	96%	123%	106%
N-acetylcitrulline	**174%**	**147%**	**164%**	**137%**	110%	103%	**153%**	**139%**
N-acetylarginine	**140%**	119%	123%	110%	96%	103%	127%	102%
2-oxoarginine	**137%**	115%	131%	109%	102%	102%	134%	105%
argininate	109%	110%	104%	96%	100%	102%	**109%**	100%
homoarginine	97%	102%	**91%**	**94%**	**92%**	102%	**95%**	93%
urea	106%	108%	103%	104%	103%	102%	105%	104%
N-acetylglutamate	**122%**	116%	**138%**	120%	94%	84%	95%	94%
aspartate	120%	101%	123%	99%	112%	103%	116%	101%
fumarate	**382%**	**160%**	**334%**	**150%**	**285%**	**137%**	**350%**	**147%**



6.C



Legends for Figure 6.A–C on next page

Figure 6.D

LEUCINE METABOLITES	RES / END Concentration Ratio (AN)		
	T0	T1	T2
leucine	1.05	1.05	**1.04**
4-methyl-2-oxopentanoate [2]	1.06	1.20	**1.52**
α-hydroxyisocaproate (pre-HMB) [1,2]	1.00	**1.65**	**1.56**
α-hydroxyisovalerate [2]	1.09	1.30	**1.57**
β-hydroxyisovalerate (HMB) [2]	1.03	1.24	**1.35**
isovalerylcarnitine (C5) [0,1]	**1.21**	**1.78**	1.19

Figure 6.A) Simplified Urea Cycle with box-and-whisker plots for group/exercise values at all 3 time points. Though the changes in most of these metabolites reached statistical significance, they were relatively small changes in magnitude (< 2-fold difference form resting to either T1 or T2). Most urea cycle intermediates were decreased in the circulation during exercise. Only argininosuccinate was increased at T1 for all group-exercise pairings. These changes likely reflect breakdown of AA's for mobilization of energy substrates (i.e. fumarate) to meet the energy demand of exercise. Green (END) and grey (RES) arrows depict directional response for each urea cycle metabolite when different between groups for the indicated exercise condition. Graph values are in relative arbitrary units.

Figure 6.B) Heatmap of Urea Cycle intermediates and related metabolites. Small, but mostly significant decreases in the urea and Urea Cycle intermediates were seen for ornithine, citrulline and arginine. Argininosuccinate was the only intermediate to see > 2-fold changes (increased at AE-T1 for both groups and AN-T1 for RES). Off-products of these intermediates were largely increased, indicating disruption or insufficiency of the Urea Cycle. Values expressed as a percentage of baseline. Blue indicates a decrease, red an increase from rest. Values in **bold** statistically significant ($p < 0.05$ & $q < 0.1$), white font for an increase, black for a decrease. Values underlined statistically significant ($p < 0.05$ only).
END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T1 = immediately post-exercise, T2 = 60-minutes post-exercises.

Figure 6. C) Heatmap of relative changes in the BCAA metabolites in response to AN. In the first 4 columns, shades of yellow and purple represent the fold change of each metabolite, respectively. Latter 2 columns display ratio of END:RES for these change values at AN-T1 and AN-T2. Shades of grey and green represent greater perturbation in RES and END, respectively.

Figure 6. D) Heatmap of the leucine metabolite concentrations as a ratio value between the two groups during the AN session. Shade of purple represents fold-difference between groups expressed as a ratio value (RES:END). Significant differences between groups are in **bold**.
END = endurance trained group; RES = resistance trained group; AE = aerobic exercise; AN = anaerobic exercise; T0 = resting time point; T1 = immediate post-exercise time point; T2 = 60-minutes post-exercise time point.

[0] significantly different at rest
[1] significantly different immediately post-AN
[2] significantly different 60-minutes post-AN

7

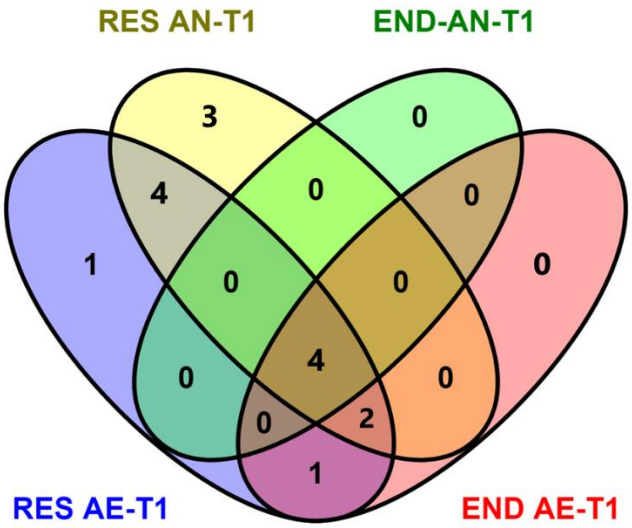


Figure 7. Four-way Venn diagram of the androgenic steroid biosynthesis metabolite changes during exercise (T0 to T1) in END and RES.
END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T0 = rest, T1 = immediately post-exercise.

8

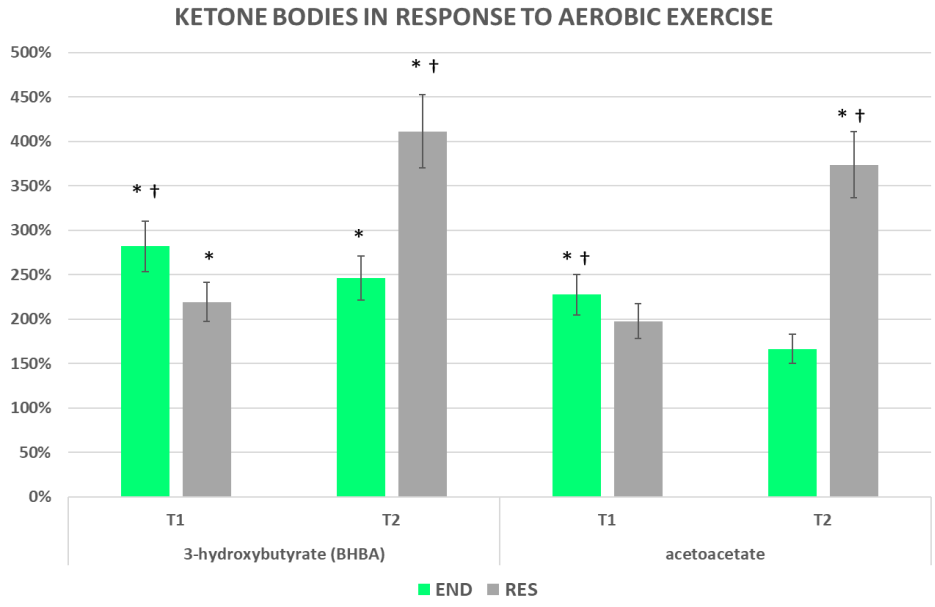


Figure 8. Changes in ketone bodies in response to the aerobic exercise bout. Endurance trained individuals (END) had significantly greater levels than resistance trained (RES) at T1 for both markers, while RES had greater levels than END at T2. Values expressed as a percentage of baseline.
AE = aerobic exercise, AN = anaerobic exercise T1 = immediately post-exercise, T2 = 60-minutes post-exercises
* Significant difference from resting level.
† Significant difference between groups for marked exercise and time point.

9

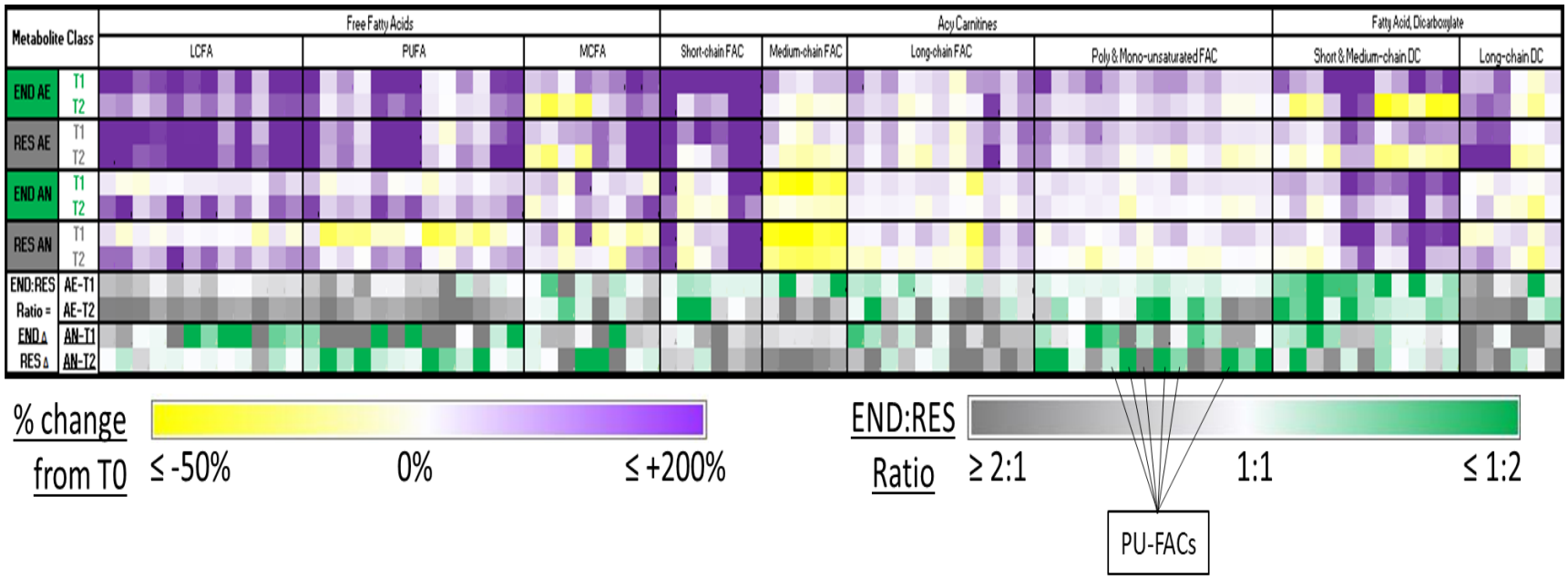


Figure 9. Heatmap of Fatty Acids (FA) and FA metabolites, including the acyl-carnitines and dicarboxylate FA's. Top 8 rows: shades of yellow and purple represent the fold change of each metabolite for given group-exercise-time, respectively. Bottom 4 rows use shades of grey and green to represent ratio for these change values (END:RES).
END = endurance trained group; RES = resistance trained group; AE = aerobic exercise; AN = anaerobic exercise; T0 = resting time point; T1 = immediate post-exercise time point; T2 = 60-minutes post-exercise time point.

10

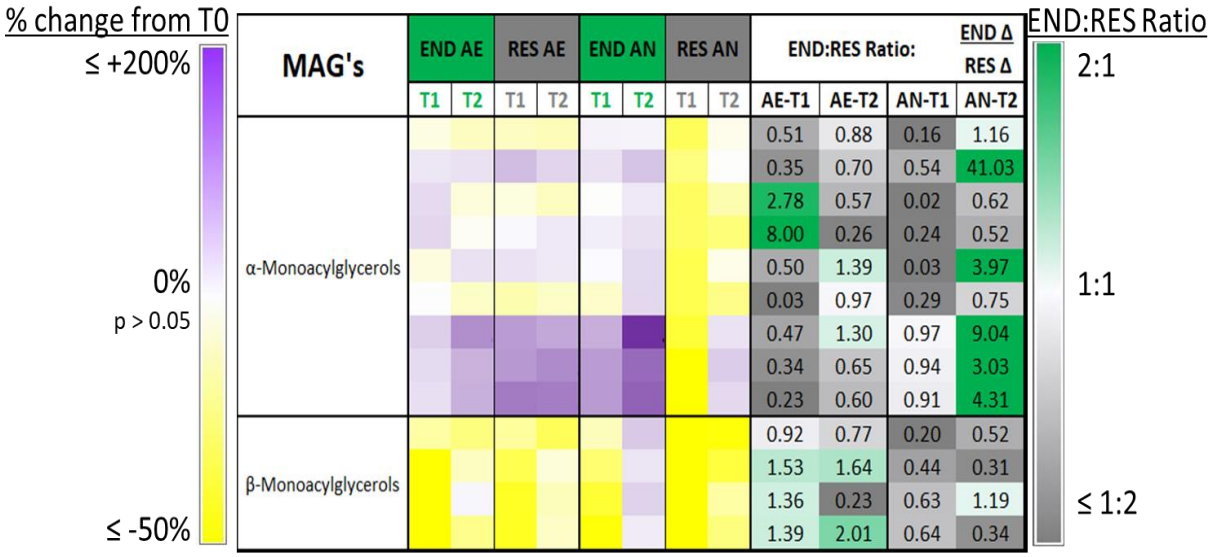
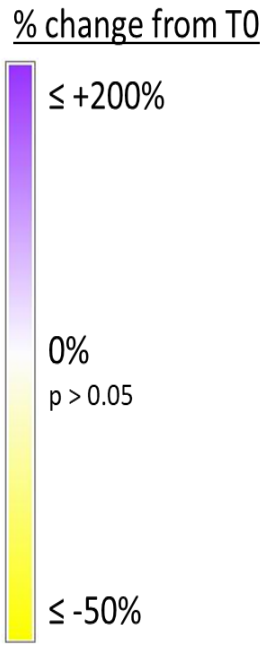


Figure 10. Heatmap of α- and β-monoacylglycerol changes across groups, condition and time. First 8 rows: shades of yellow and purple represent the fold change of each metabolite for given group-exercise-time, respectively. Latter 4 columns display ratio of END:RES for these change values for AE and AN, respectively. Shades of grey and green represent greater perturbation in RES and END, respectively.
MAG = Monoacylglycerol, END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T1 = immediately post-exercise, T2 = 60-minutes post-exercise.

11.A

Inflammatory Effect	Lipid Mediator	END AE T1	END AE T2	RES AE T1	RES AE T2	END AN T1	END AN T2	RES AN T1	RES AN T2
Pro-Inflammatory	9,10-DiHOME								
	13-HODE + 9-HODE								
	12-HETE								
	12-HHTrE								
Mixed-Inflammatory	arachidonate (20:4n6)								
	dihomo-linoleate (20:2n6)								
Anti-Inflammatory	21-hydroxypregnenolone disulfate								
	21-hydroxypregnenolone monosulfate								
	Cortisol								
	Corticosterone								
	Cortisone								
	eicosapentaenoate (EPA; 20:5n3)								
	dihomo-linolenate (20:3n3 or n6)								
	1-dihomo-linolenylglycerol (20:3)								
	1-arachidonylglycerol (20:4)								



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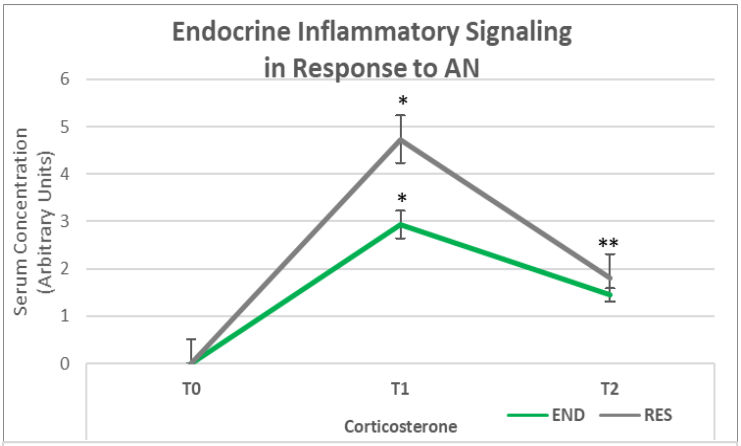

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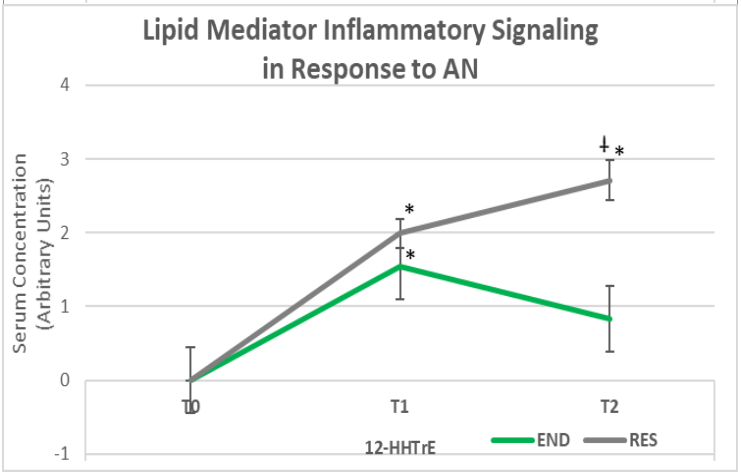


Figure 11.A-C Legend on next page

11.D

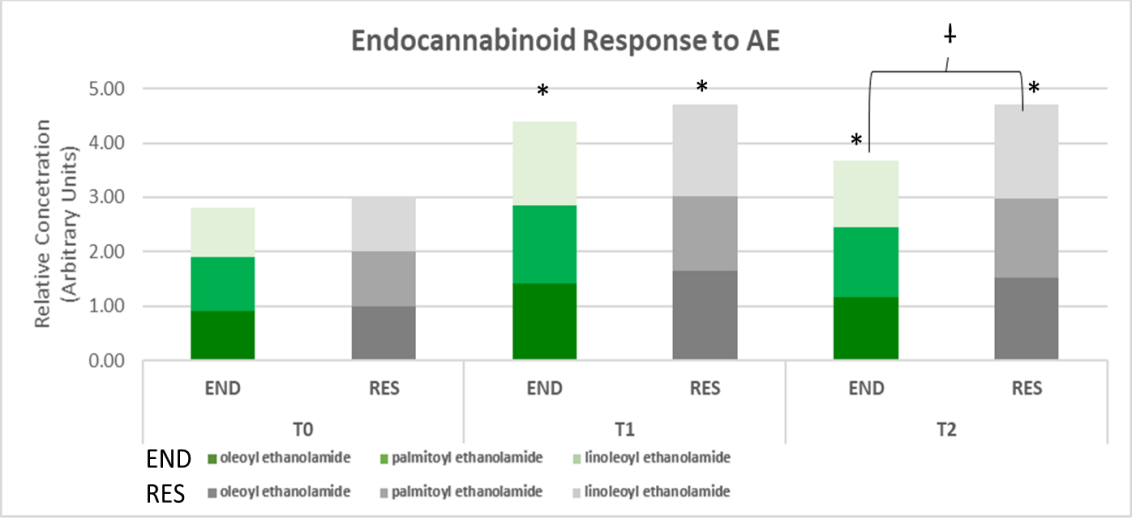
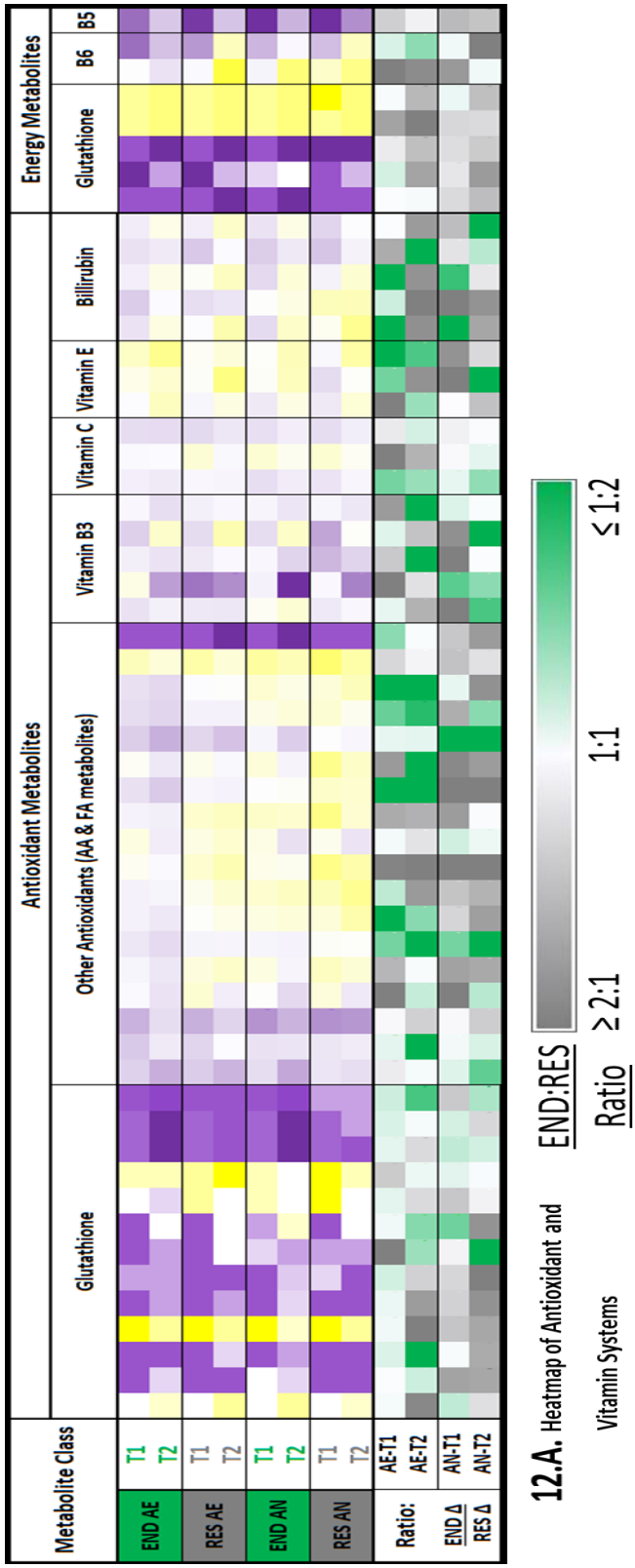
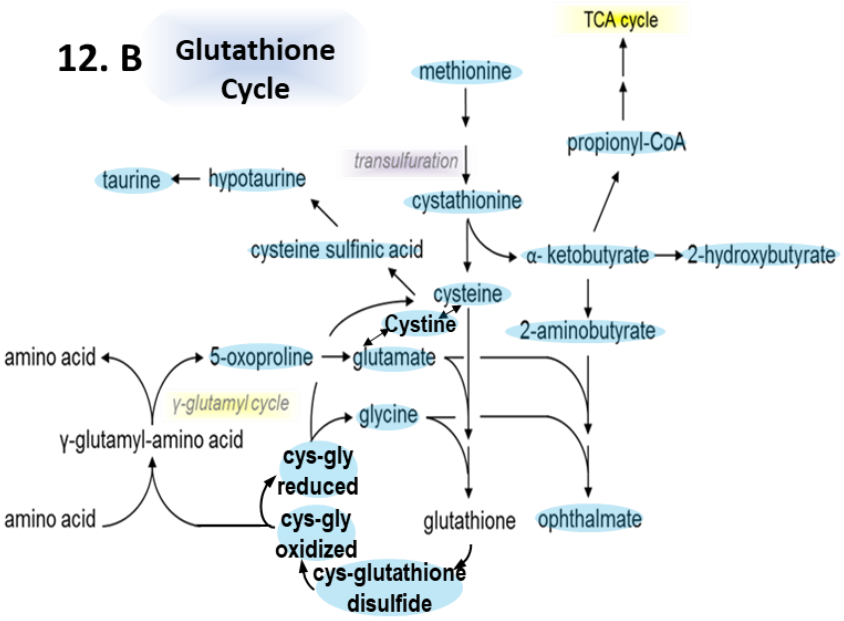


Figure 11. A) Heatmap of changes in pro/anti-inflammatory lipid mediator metabolites. Shades of yellow and purple represent the fold change of each metabolite for given group-exercise-time, respectively.
Figure 11. B) Line graph of relative corticosterone levels throughout the AN session in END and RES.
* Significantly different from resting level.
Figure 11. C) Line graph of relative 12-HHTrE levels throughout the AN session in END and RES
* Significantly different from resting level.
† Significant difference between groups
Figure 11. D) Endocannabinoid levels in END vs RES in before, immediately and 60-minutes following AE.
END = endurance trained, RES = resistance trained, AE = aerobic exercise, AN = anaerobic exercise, T1 = immediately post-exercise, T0 = rest, T2 = 60-minutes post-exercise.
* Significant difference for all 3 endocannabinioids from T0 value
† Significant difference between groups for oleoyl ethanolamide and linoleoyl ethanolamide (RES > END for both, $p < 0.05$, $q < 0.1$).



12.A. Heatmap of Antioxidant and Vitamin Systems

Legend on Next page



C Specific Heatmap of Glutathione and Transulfuration Pathway Metabolites

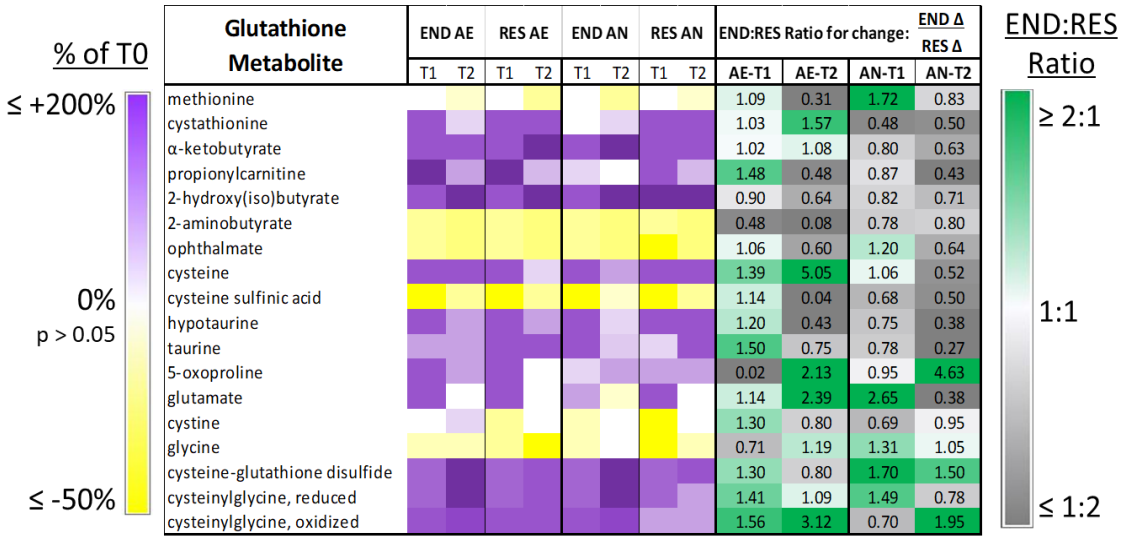


Figure 12. A) Heatmap of antioxidant and vitamin metabolites. Top 8 rows: shades of yellow and purple represent the fold change of each metabolite for given group-exercise-time, respectively. Bottom 4 rows use shades of grey and green to represent ratio for these change values (END:RES).

Figure 12. B) Glutathione pathway and transulfuration shunt. Included metabolites highlighted in blue.

Figure 12. C) Detailed heatmap of glutathione and transulfuration metabolites. First 8 rows: shades of yellow and purple represent the fold change of each metabolite for given group-exercise-time, respectively. Latter 4 columns display ratio of END:RES for these change values for AE and AN, respectively. Shades of grey and green represent greater perturbation in RES and END, respectively.

END = endurance trained group; RES = resistance trained group; AE = aerobic exercise; AN = anaerobic exercise; T0 = resting time point; T1 = immediate post-exercise time point; T2 = 60-minutes post-exercise time point.

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Chapter 4.3

The Exercise Metabolome: A Comparison of Males and Females

INTRODUCTION

Metabolomics as an investigative tool has been used to explore multiple areas of exercise science. These have included biomarker discovery for performance prediction,[1-3] supplement and ergogenic aid research,[4-9] the effects of exercise training in health and disease,[10-15] and in conjunction with other omics research in an attempt to better ascertain a holistic picture of the exercise response.[16-20] However, within the context of exercise, sex differences in the metabolome have remained largely unexplored. Metabolomics investigations of exercise including both male and female participants are limited, and are particularly lacking in the area of resistance training or anaerobic exercise.[21]

We know that there are differences between males and females that emerge in and around exercise training. There are reports showing differential responses in fitness parameters,[22-26] training response,[27,28] cognitive function[29] and multiple physiological systems (cardiovascular, endocrine, immune, etc.).[30-36] In light of the variety of systems displaying a sex difference, it would be apt to employ an encompassing measure, such as metabolomics to try and capture a snapshot of these differences in an exercise setting.

Of the biochemical sex differences identified, the most well-known are the hormones, specifically the sex steroids (estradiol and testosterone).[33-35] However, as drivers to innumerable downstream metabolic events, these hormones have required countless individual studies probing their subsequent pathways to validate or refute purported effects. A metabolomics panel has the capacity to capture differences at this level and do so over a broad range of biochemical pathways. Mechanisms underlying the training responses and how they differ between sexes also requires elucidation. Substrate selection, inflammatory signaling and other multi-systemic differences could also benefit from comprehensive metabolomic studies set up to integrate cross-systemic responses. So far, only a handful of studies have attempted this.[11]

Previous metabolomics studies examining sex differences have identified both expected and novel distinctions between males and females. More common findings include differences in sex steroid metabolites,[20,37,38] higher purine metabolism,[38,39] and amino acids (AA), especially branched-chain amino acids (BCAA),[1,38,40,41] in men, and higher fatty acids, particularly sphingolipids,[37,38,42] in females. While these results have been validated in multiple studies, they have all been done in either a diseased cohort (overweight, diabetic, etc.) or the general population, which is sedentary,[43] and most were unbalanced in the male:female ratio. By contrast, a large scale-study of "healthy" French participants included roughly equal males (n = 417) and females (n = 383), and found comparatively elevated creatinine, BCAAs and lysophosphatidylcholines in males and sphingomyelins and phosphatidylcholines in females.[41] Notably, the metabolomic panel used in this study was limited in scope. In a pilot study examining the metabolome of elite athletes, the authors identified very few metabolomic differences between male and female power athletes and none for endurance athletes.[1] However, this participant pool included 171 males and only 20 females.

All of these studies were limited to resting measurements. As such they did not probe the question of sex differences in relation to exercise. In addition to those given above, dozens of individual metabolites and myriad biochemical pathways have been found to differ by sex. More information is certainly needed to fully appreciate and understand the impact of sex differences on the metabolome at rest and in response to exercise, particularly anaerobic exercise. Moreover, use of an exploratory, rather than a targeted panel, allows for discovery of novel metabolites and specific metabolomic characteristics subject to change for each sex following exercise. Here we attempt to merge these 2 questions to examine sex differences in the response to 2 different bouts of acute exercise, aerobic (AE) and anaerobic (AN).

Purpose and Hypothesis. The aim of the present study was to use an exploratory metabolomics platform to examine the metabolic response pattern of both male and female individuals to acute bouts of aerobic and anaerobic exercise. We hypothesized that changes in the metabolome would reflect both convergence and divergence in the responses to specific exercise modes across sex. Metabolomic characterization of the acute anaerobic exercise response is currently lacking, particularly for females and comparisons between exercise modes has yet to be carried out in a healthy cohort. Furthermore, metabolomics studies rarely utilize male and female groups with an experimental design, and metabolomics has never been used to study the impact of sex on the exercise response. This study accomplishes all of this using a cross-over design in an attempt to characterize the fit/healthy metabolome of males and females, and capture the response to varied exercise stressors.

METHODS

Research Design. Active, young adults were exposed to 2 different exercise bouts (matched for duration and perceived exertion) and their metabolomic profiles were characterized before and at 2 times post- exercise: immediately (T1) and 60-minutes after (T2). This design was implemented to characterize and differentiate between the acute responses to aerobic and anaerobic exercise in healthy individuals. Active males (M) and females (F) predominantly either endurance (E) or resistance trained (R) were recruited for participation. Physiological parameters were measured to assess aerobic fitness (VO_{2MAX}) and anaerobic strength (10-repitition max (RM)), and to prescribe equivalent relative workloads during the experimental exercise conditions. Metabolomic profile and individual metabolite differences were assessed.

Participants. Volunteers were recruited to the Rutgers Center for Health & Human Performance (CHHP) for participation in the study. Forty (20 M + 20 F, each made of 10 E and 10 R) active, healthy young adults (age: 18-39 years) completed all testing and were used for analysis. Inclusion criteria consisted of absence of any known cardiorespiratory, metabolic or other health issues and a minimum of 2-years of regular participation in their current physical activity regimen, limited to one primary modality. Exclusion criteria included recent illness/injury (> 1-week disruption to normal exercise routine within the past 6 months) and use of tobacco products or recreational drugs. Supplement use was permitted so long as participants had not begun use within 6-months of enrollment and continued through completion of the study. Participants reported to the lab for 2 testing trials followed by 2 experimental conditions randomized for order. Procedures were approved by the Rutgers University Institutional Review Board, and participants signed informed consents prior to enrollment.

EXERCISE TESTING TRIALS

Visit 1. Participants reported to the CHHP following a 2-hour fast and having refrained from exercise for the previous 24-hours. Upon arrival, body composition testing with a Bod Pod (Cosmed, Concord, CA) was performed using the Brozek equation.[44] After this, participants were permitted to warm-up for 10 minutes before completing a continuous graded exercise test on a Velotron cycling ergometer (Racermate, Seattle, WA). Depending on estimated fitness level, individuals started between stages 70 and 220 Watts (W) and continued cycling, increasing wattage by 30 W every 3-minutes. For testing, participants were fitted with a Polar M-400 HR monitor (Polar Inc., Bethpage, NY) and Quark C-PET metabolic measuring system (Cosmed, Concord, CA) for HR and breath-by-breath assessment of gas analysis with 15-second rolling averages. All participants met a minimum of 3 American College of Sports Medicine criteria for attainment of VO_{2MAX},[45] and continued until volitional fatigue or the inability to keep cadence ≥ 80 rpm.

Ventilatory threshold (VT) was calculated using the graphical ventilatory equivalent method as described by Davis et al. (1980) and verified by 2 separate exercise physiologists.[46] These values were further verified using internal software on the Quark C-PET. The average of the 2 closest values was taken as VT, with a discrepancy never more than 0.1L/min O_2 between comparators. Maximal aerobic power output (PO_{MAX}) was calculated as per Naperalsky et al. (2010).[47]

$$PO_{MAX} = (\text{Watts on last completed stage}) + (30\ W) \times (\%\ \text{of final attempted stage})$$

Seventy percent of this wattage value was used as the intensity prescription during the aerobic exercise session (AE). This percentage was chosen, as it allowed for an arduous exercise bout while still falling below average measured VT of all participants.

Visit 2. During the second visit to the CHHP participants completed 10RM testing for 7 lower-body exercises following the guidelines set forth by the National Strength and Conditioning Association (NSCA).[48] In order, exercises were back squat, leg press, Romanian deadlift, lunges, prone leg curls, leg extensions and seated calf raises. A certified strength and conditioning specialist (CSCS-NSCA) administered testing to ensure participant safety and proper execution of all lifts. The same 7 exercises, in the same order, were performed during the anaerobic exercise session (AN). Three sets of 10 repetitions were performed for each exercise using 90% of the determined 10RM weight and a 90-second recovery between all sets. This combination of load, rest interval, repetition and set count was selected as it falls within the NSCA guidelines for hypertrophy training and well within an "anaerobic" working intensity.

EXERCISE CONDITIONS

Control Factors. In accordance with findings on metabolomic variability, certain precautions were taken to minimize external variability.[49,50] Individuals reported to the CHHP for each of the 2 experimental conditions having refrained from exercise for the previous 24 hours and avoided intense or prolonged exercise for the previous 48. Prior to their first condition, participants were provided a 24-hour food log to record their diet so that it could be replicated prior to the second condition. They were encouraged to eat as normal, other than consuming a meal 2-3 hours prior to the visit, with only water after this point. All visits started between 8:00AM and 12:00PM, and exact time of day was matched between conditions for each participant. Lastly, individuals were instructed to abstain from ingestion of caffeine intake that day (none within 6-hours of the visit). Each condition was run 3-7 days removed from the previous session (either testing visit 2 or condition visit 1). In addition to relative workload, exercise sessions were matched for total duration (45 minutes), and were perceived as equally demanding by subjects during pilot testing (rating of perceived exertion (RPE) between 8 and 10 for both conditions).[51]

Aerobic Session (AE). Upon arrival, participants sat quietly for 10-15 minutes and a resting blood draw from the antecubital vein was performed (T0). This was followed by a 15-minute warm-up period before commencing with the prescribed exercise. The AE cycling bout was a 45-minute steady state ride at ≈70% of PO_{MAX} on the same ergometer as used during VO_{2MAX} testing. A trainer monitored HR and cadence during the session and participants were encouraged to keep their cadence at ≥ 90rpm. In order to maintain an aerobic effort, if HR went above 90% HR_{MAX} (as observed during VO_{2MAX} testing), or cadence below 80rpm, wattage was adjusted until HR and/or cadence normalized.

After completion of the exercise, participants were promptly escorted into an adjacent phlebotomy room where an indwelling catheter was inserted into the contralateral arm for subsequent draws, collected immediately (T1) and 1-hour post-exercise (T2). The line was flushed with 4ml saline before and after each draw, as well as halfway between them, to prevent coagulation. Nutrient intake was not permitted at any point during the session, but participants were allowed to drink water *ad libitum* throughout.

Anaerobic Session (AN). AN was identical to AE, including sample collection, except that the cycling bout was replaced with a resistance training session of the 7 exercises listed above. A CSCS oversaw all sessions to ensure participant safety and proper execution of all lifts. If the workload was too high (participant could not complete a full set of 10) they were spot-assisted to completion and weight was adjusted accordingly on any subsequent sets of that exercise. The entire exercise portion of the session took ≈45-minutes. Order of AE and AN was randomized for all 40 participants.

Metabolomics Analysis. Following collection and processing, serum samples were aliquoted into 2.5 ml cryovials and stored at -80^{O}C until all testing was complete. Samples were packed on dry ice and shipped to the Metabolon® core facility in Durham, NC for analysis with Metabolon's

Discovery Panel of 754 metabolites. Automated ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry was used for metabolite identification. Raw data were extracted, peak identified, quality control processed with internal and external controls, and quantified by area under the curve using Metabolon's hardware and software. Compounds were then identified by comparison to library entries of purified standards or recurrent unknown entities based on 3 criteria: retention index, mass, and the MS/MS forward and reverse scores between the experimental data and authentic standards. All samples were run in a single batch to reduce variance.

Values were normalized in terms of raw area counts through log transformation and rescaled to set the median equal to 1.00 for each metabolite. Then, missing values were imputed with the minimum value. All statistical procedures were carried out using these values. Data were re-run excluding any metabolites using > 25% imputed values. This did not impact the global biochemical profiles, and inclusive data is reported here.

Statistical Analysis. Unsupervised approaches to analysis were performed in Array Studio and included hierarchical clustering and principle components analysis (PCA). The hierarchical clustering method performed was complete clustering using the Euclidean distance, where each value was factored independently as a vector to all other metabolite values. Condition clusters were identified and superimposed on the output. PCA analysis by singular value decomposition was used to reduce dimensionality and generate data clusters for all samples and select subgroupings showing differences between group, condition and/or time.

A 2x2x3 (sex x exercise condition x time) repeated measures analysis of variance (RMANOVA) with follow-up univariate ANOVA contrasts was run for all metabolites using a p value of < 0.05. Additionally, change values were calculated from T0 to T1 and T0 to T2 for each group. These delta scores were compared between groups, (M vs F) using unpaired t-tests, with

an alpha level $p < 0.05$. Due to the multiple comparisons found in a metabolomics data set, the false discovery rate was determined using the Benjamin-Hochberg equation with a stringency set at a q value of 0.1 for all ANOVA and t-tests. All changes discussed below meet both p and q criteria. Because metabolite values are all median-normalized, log-scaled means of the group, values are relative, and reported in arbitrary units or as relative changes from rest.

For those values determined to be statistically significant by both p and q criterions, magnitude of change was used to further discriminate metabolites to obtain the most impactful metabolic perturbations and reduce data volume. Average changes from T0-T1 and T0-T2 for each exercise were considered most physiologically significant when the delta score reached 2-fold-of difference between the resting and post-exercise values.[52] With resting values set at 1.00, this means a post-exercise value ≥ 2.00 for an increase or ≤ 0.50 for a decrease. This more stringent listing of metabolites was used for Venn diagram comparisons, while all statistically significant metabolites were considered in the RMANOVA comparisons.

RESULTS

Group Fitness Characteristics. As expected, M and F had different body composition, strength and endurance, with M being heavier, leaner, stronger and more aerobically fit (**Table 1**). Observed differences in absolute strength and endurance remained when scaled to body mass, but were eliminated when computed per unit fat free mass (**Table 1**).

Metabolomic Profile Comparisons. All 180,960 metabolite values across condition and time were compiled into a hierarchical clustering analysis. Several elongated groupings of M and F data could be seen, suggesting a discernable difference between sexes (**Figure 1.A**). Exercise mode data, on the other hand, showed much less distinction, but did not rule out sex by exercise mode interactions. Similar to the hierarchical clustering results, a PCA plot of the complete data set revealed observable clustering by sex, but included substantial overlay between M and F (**Figure 1.B**). When only the T0 values were plotted, M and F data were completely overlapping (**Figure 1.C**). This implies exercise brought about sex differences in otherwise indistinguishable M/F metabolomes. Including all time points, but isolating each condition, AE and AN each portrayed separation between sexes, but this was more evident with AE than AN (**Figure 1.D,E**).

4-way Venn Diagrams. Metabolites with greater than 2-fold change scores at T1 and T2 were put into Venn diagrams for each group and exercise condition (**Figure 2.A,B**). Markers showing between-sex similarities for both AE (white ring: 48 at T1 and 22 at T2) and AN (black ring: 28 at T1 and 12 at T2) were more abundant than those showing a sex-specific difference (AE: 26 at T1 and 11 at T2, AN: 17 at T1 and 17 at T2). Looking at the T0-T1 changes, AE disrupted more metabolites for F than M, while for AN, the number of metabolites was greater for M than F. After 1-hour of recovery, considerably fewer metabolites were different from rest than at T1 for all except the F-AN condition, which only dropped from 33 to 32 metabolites

(**Figure 2.C**). Further, unique-to-AE or unique-to-AN responses at T2 were quantitatively comparable between sexes in number of metabolites changed, supporting a notion of similarity in the M and F responses to exercise. All group-exercise pairings, except for F-AN showed a larger initial response to the exercise bout with a significant drop in perturbed metabolites after the 1-hour recovery, including some carryover from the T1 changes (**Figure 2.C**). From a time-course perspective, there was more similarity between both exercises and sexes at T1 and less at T2 (**Figure 2**). Differences in the actual metabolites comprising these counts are listed in **Figure 2.D & E**. Combined with RMANOVA results, conclusions are drawn regarding sex and sex by exercise interactions.

PATHWAY & METABOLITE COMPARISONS

Resting Differences. Within each sex, no resting differences were found in any of the metabolites between-conditions. Between sexes, M and F displayed near-identical resting metabolomes to one another (**Table 2**). In total the sexes differed in just 24 of the 754 metabolites on this panel, a near 97% similarity. Of those differences, higher resting levels of N-acetylcarnosine and creatinine were seen in M (**Table 2**), likely resulting from their higher FFM (**Table 1**). Higher M values for 2 cholesterol/bile acid metabolites and a pair of androgen biosynthesis metabolites, 5-a-androstan-3a, 17a-diol monosulfate and 5-a-androstan-3a,17b-diol monosulfate, were also seen at rest with the differences persisting through T1 and T2 following both exercise sessions for the steroid metabolites. Indeed, a lack of difference in additional androgenic metabolites was somewhat surprising. Less predictably, the branched-chain amino acid (BCAA) metabolites 2-hydroxy-3-methylvalerate and α-hydroxyisocaproate, as well as several of the γ-glutamyl AA's (GGAAs), including all 3 branched-chain GGAAs were greater in M than F at rest. Another somewhat unexpected finding was that 1,5-anhydroglucitol (1,5-AG) was lower for F at rest, plus both immediately and 1-hour after both exercises (**Table 2**

& Figure 3). While lower 1,5-AG indicates recent (≈2-weeks) hyperglycemia, actual resting glucose was similar between groups, and never more than 7% different between M and F, indicating similar control of blood glucose in response to exercise (**Figure 3**). Several other metabolites were significantly different in M and F at rest, but failed to cluster into meaningful pathways. Other than the GGAAs, no sub-pathway included more than 2 metabolites, and no differences were particularly large (<< 2-fold, **Table 2**).

MALE & FEMALE ENERGY METABOLISM

Substrate Selection. In response to exercise, M and F distinctions began to emerge. A major difference lied within substrate selection. Specifically, M appeared to have greater glucose use during/after AN and further elevated anaerobic metabolism, as depicted by higher circulating concentrations of pyruvate and lactate (**Figure 3**) and more elevated metabolites of the purine salvage pathway (**Figure 4**). In addition to these larger elevations in glycolytic end-products and signs of higher ATP turnover, M displayed evidence of greater AA metabolism than F during exercise, especially for AN. M also had larger alterations in Urea Cycle and Tricarboxylic Acid Cycle (TCAC) intermediates (**Figure 5**). Specifically, increases were greater in M than F in argininosuccinate at all post-exercise time points (M > F: 125% & 70% at AE-T1 & AE-T2 and 103% & 76% greater at AN-T1 & AN-T2, respectively) and the span-2 intermediates at AN-T1 (46%, 110% & 154% for succinate, fumarate and malate, respectively, **Figure 5**). In turn, F displayed more fatty acids (FA) use during AE and throughout the post-exercise period for both exercise conditions (**Figure 6.A**) and higher ketone metabolism during/after AE, and to a lesser extent, post-AN (**Figure 6.B**). These changes suggest more varied substrate mobilization including further increased AA and glucose use in M. Accompanying the differences in substrate selection were sex-dependent changes in anabolic and FA-derived signaling molecules.

Higher Anabolic Metabolites in M. Within the AA metabolites, BCAA metabolites proved especially susceptible to alteration by exercise in both sexes. The largest difference in response size was seen for, 2,3-dihydroxy-2-methylbutyrate, a catabolite downstream of all 3 BCAAs, with M having larger increases during and following both AE and AN. Of particular interest were responses of 2-hydroxy-3-methylvalerate and α-hydroxyisocaproate, the 2 metabolites seen to differ between sex at rest. For both metabolites, the heightened levels in M seen at rest were maintained throughout both exercise conditions. And while an increase in 2-hydroxy-3-methylvalerate were not significantly different between groups for either exercise, α-hydroxyisocaproate did show a sex by exercise by time interaction (**Figure 7**). The increases in α-hydroxyisocaproate, both immediately and 60-minutes post-exercise, were greater for M than F, particularly for AN (M increase > F increase by: 34% & 60% at AE-T1 & AE-T2 and 95% & 75% at AN-T1 & AN-T2).

Similar to α-hydroxyisocaproate, the 2 steroid metabolites observed to differ at rest also experienced larger increases in M than F in response to exercise (**Figure 8**). This yielded the response pattern for 5α-androstan-3α,17β-diol disulfate and 5α-androstan-3α,17β-diol monosulfate analogous to that observed for α-hydroxyisocaproate. Interestingly, most sex steroid biosynthesis markers including other androgenic metabolites were relatively similar between sexes, identifying these 2 as potentially impactful steroid metabolites in the expression of M/F hormonal differences at rest and related to exercise.

Downstream Lipid Signaling. As displayed in Figure 1.A, both sexes displayed elevated FAs of all kinds (long-chain (LCFA), medium-chain (MCFA) and polyunsaturated (PUFA)) and various products of FA metabolism (i.e. glycerol, acylcarnitines) during and after AE, which were mostly greater in F than M. Moreover, while changes in these markers were minimal during AN in both sexes, F experienced more significantly increased levels 60-minutes post-AN. These increases

suggest FA mobilization during AE and after AN, and to a greater extent in F than M. However, the fatty acyl carnitines (FACs), which depict β-oxidation, were markedly further elevated in F at AE-T1, but not as strongly at AE-T2 or AN-T2 (**Figure 6.A**). The difference in FACs was seen across different acyl chain species as well.

Several signaling molecules derived from lipid species also showed sex differences in the response to one or both exercises. These perturbations were again noted at AE-T1, AE-T2 and AN-T2 and were greater in F than M. Observations support the FA results above and may link differences in substrate selection to downstream lipid signaling. This pattern is seen in a variety of FA metabolites, including the monohydroxy, dihydroxy, amino- and branched FA's. Most of the dicarboxylate FA's were not significantly altered by exercise, but often showed more activity in F when changes were significant, particularly at AN-T2. When comparing M versus F changes, metabolites began to aggregate by physiological function. Metabolites observed to be more perturbed in the M cohort at AE-T1 or AN-T1 (3-hydroxysebacate, suberate, undecandioate, 3-methyladipate, 2-hydroxyadipate and malonate) are the same as those used to diagnose mitochondrial dysfunction of β-oxidation and/or the carnitine-palmitoyl transferase system. Here they likely portray an overload, rather than a dysfunction of these processes. In contrast, inflammatory-related metabolites (the eicosanoids, the endocannabinoids, 16-hydroxypalmitate, 2-hydroxypalmitate, 12,13-DiHOME, 9,10-DiHOME, 13-HODE and 9-HODE) emerged as those markers more responsive in F, and these typically showed sex differences at most times.

Between the increases and decreases in pro- and anti-inflammatory lipid mediators, respectively, a sex by condition interaction became apparent for the inflammatory response to exercise. This is most clearly depicted in the eicosanoids (12-HETE and 12-HHTrE) and cortisol metabolites. M had comparatively more elevated eicosanoids and smaller increases in cortisol

metabolites following AE showing a greater state of pro-inflammatory status than F with AE (**Figure 9**). Conversely, immediately and 60-minutes following AN, F had a larger pro-inflammatory response to AN and lesser elevated cortisol metabolites (**Figure 9**). Another class of lipid mediators showing sex differences was the endocannabinoids. In addition to anti-inflammation, these cannabinoid receptor ligands have analgesic and cognitive processing effects. Though all 3 endocannabinoids were increased at AE-T1 and AE-T2 for M and F, they were each further elevated in F than M at AE-T1 and palmitoyl-ethanolamide at AE-T2 (**Figure 9.D**). And though changes were smaller than seen with AE, linoleoyl- and oleoyl-ethanolamide were both significantly elevated at AN-T1 and AN-T2 in F, while M experienced only a nonsignificant rise and fall for these metabolites with AN (**Figure 9.D**). Perhaps more importantly, the pattern of change for all 3 endocannabinoids in response to AN displayed continued elevation at AN-T2 in F, to the extent that oleoyl-ethanolamide was significantly different between sexes at this time (**Figure 9.D**).

Corroborating with the changes in lipid mediators were observations of perturbations to the acylglycerols, particularly those with unsaturated moieties (**Figure 6.A and 10**). Both M and F had similar patterns of change in the diacylglycerols (DAG) for AE, with increases at T1 followed by a return towards baseline by T2. (**data not shown**). But the DAG response to AN differentiated between groups. M experienced a muted version of that seen for AE in that the pattern was the same, but the actual T1 increases were substantially smaller with AN. In F, on the other hand, DAGs were unchanged at AN-T1 followed by a post-exercise decrease by AN-T2 (**Figure 10**). Notably, most DAGs were altered by AE in both groups, while only about half were changed by AN in either group. These patterns of change likely reflect greater mobilization of FAs in response to AE than AN, plus more efficient uptake and/or oxidation in F than M during the AN condition. In contrast, the monoacylglycerols (MAGs) were largely unchanged in

response to exercise for either group with the polyunsaturated (PU) MAGs as the exception (**Figure 6.A**). Interestingly, the PU-α-MAGs increased in M and F post-AE and only in F post-AN. Meanwhile, the PU-β-MAGs were decreased during both exercises in both groups, but were further decreased in F at AN-T2. PU-MAGs are used as signaling molecules and β-MAGs in the production of various lipid mediators, including the endocannabinoids. Observed changes here suggest a greater shuttling of β-MAGs into this synthesis pathway for the production of lipid mediators for F in response to AN, and are consistent with sex differences seen in lipid mediator signaling and increasing endocannabinoids at AN-T2 seen for F.

OTHER METABOLIC PATHWAY CHANGES

Membrane Lipids & Sphinganine-1-Phosphate. Though there were many significantly altered membrane lipids (acyl-cholines, phospholipids, lysolipids, lysoplasmalogens/plasmalogens, ceramides and sphingolipids), actual magnitudes of the fold-change values were smaller than other noted metabolites, and only marginal differences between sexes were observed for most of these metabolites (**Figure 11.A**). However, relatively large and robust decreases were observed during both exercises in M and F across all 8 acyl-cholines on the panel. The responses to AE were similar between sexes, but average decreases in M were larger than F during AN and recovery post-AN was more complete in F (AE-T1: M = -68$\pm$3%, F = -66$\pm$4%; AE-T2: M = -18$\pm$3%, F = -14$\pm$5%; AN-T1: M = -75$\pm$7%, F = -64$\pm$4%; AN-T2: M = -33$\pm$6%, F = -1$\pm$7%, $p < 0.05$, $q < 0.1$ for all except F-AN-T2). Sphingolipid metabolites were seen to increase with exercise, and, it appeared that perturbations were more extensive in F during AE and in M during AN (**Figure 11.B**). Increases in sphinganine, sphingosine, sphinganine-1-phsophate and sphingosine-1-phosphate were much larger than changes seen for the actual sphingolipids (**Figure 11.A**), but only showed sex differences in response to AN. Both elevations at AN-T1 and actual circulating concentrations were higher in M than F.

Vitamins & Cofactors. Exercise-induced changes to serum vitamin levels reflected energy metabolism with increases in pantothenate across sex, condition, and time. Sex-specific response patterns were evident for B6 and B3 metabolites. Perturbations in pyridoxate (AA metabolism) were observed in M only at AE-T1 and AN-T1 (47±18% and 76±27%, respectively, $p < 0.05$, $q < 0.1$) aligning with the substrate results above. Nicotinamide metabolism, on the other hand, was clearly evident in F, with F-only elevations at AE-T1 for 1-methylnicotinamide (25±10%), N1-methyl-2-pyridone-5-carboxamide (10±4%), quinolate (18±10%) and trigonelline and 1-methylnicotinamide and nicotinamide at AN-T1 (39±1%, 37±11%) and AN-T2 (58±8%, 142±16%) respectively, $p < 0.05$, $q < 0.1$.

Cholesterol Metabolism. Sex-specific exercise responses were observed for cholesterol and the sterol metabolites (**Supplemental figure 1**). Despite statistical significance and large fold-differences between groups, changes from baseline in sterol metabolites elicited by exercise were relatively small in magnitude. Of note, during AE, both cholesterol and campesterol increased for F only (M: +2% & +7%, $p > 0.05$, F: +20% & +17%, $p < 0.05$, $q < 0.1$, respectively). At AE-T2 there was a decline in beta-sitosterol in F and not M (M: -5±1%, $p > 0.05$, F: -14±4%, $p < 0.05$, $q < 0.1$) and in cholesterol for M and not F (M: -10±3%, $p < 0.05$, $q < 0.1$, F: -7±2%, $p > 0.05$). In response to AN, cholesterol was elevated at AN-T1 in F only (M: +6±3%, $p > 0.05$, F: +9±2%, $p < 0.05$, $q < 0.1$), while campesterol was significantly elevated in M only (M: +16±1%, $p < 0.05$, $q < 0.1$, F: +15±2%, $p > 0.05$).

Bile Acids. Scattered primary and secondary bile acids were decreased for either or both of the sexes in response to AE. None were different between groups in change or relative concentration. In response to AN there were more total altered bile acids than AE, and 8 were further decreased in M more than F (**Supplemental Figure 2**). It should be noted, however, that resting bile acids were generally (non-significantly) higher in M which possibly impacted relative

changes of these metabolites. Increases for glycocholenate sulfate and taurocholenate sulfate opposed the trend of decreases in the bile acids, but still held to larger perturbations in M with AN. Primary bile acid synthesis was further elevated in F than M following AE as evidenced by increased 7-α-hydroxy-3-oxo-4-cholestenoate (7-Hoca) and 3-β-hydroxy-5-cholestenoate in F, but not M following AE. Following AN, 7-Hoca was elevated in both groups at AN-T1, and 3-β-hydroxy-5-cholestenoate was increased in F AN-T1 and decreased in M at AN-T2 (**Supplemental Figure 1**).

Metabolism of the Gut Microbiota. Finally, xenobiotics reflecting microbiome metabolism are illustrated in **Supplemental Figure 3**. Actual differences between sexes were scarce, and almost exclusively appeared at the T1 time point with M showing larger perturbations than F. Moreover, significant responses were more abundant in M than F, particularly in response to AN. Despite these tendencies, no statistical differences in pathway activation were observed. Xenobiotic plus bile acids changes appear to link acute exercise and microbiome metabolism, but data did not reveal a sex-dependent response pattern. Further exploration is necessary to determine whether one exists.

DISCUSSION

RESTING METABOTYPE AND GENERAL COMPARISONS

Our primary observation was that when using healthy, active males and females their metabolomes differ relatively little, especially at rest but also in response to exercise. There are less than a dozen reports on sex differences in the metabolome[17-18,20,37,40,41,42,50,53] and we are the first to use a comprehensive metabolomics panel to directly compare matched M and F groups and explore sex-dependent responses to exercise. Identification of resting differences between males and females in a mere 3.2% of the metabolites is in contrast to what has been reported elsewhere, ranging from 25%[37] to 62%.[20] The biggest difference in design between ours and these other studies was the target population. Most other investigations have examined specific "less healthy" and/or diseased populations (i.e. elderly, overweight/obese, diabetic) or used the general population, which is largely sedentary. Indeed, a study including "healthy volunteers" employing a more limited metabolomic panel found fairly similar results for male and female participants.[41] Physical activity promotes a healthy status and has been shown to impact the human metabolome.[2,18] Using active individuals may have acted to normalize sex differences otherwise observed in the resting metabolome. A pilot study of resting metabolomes for elite endurance and power athletes found no sex differences within the endurance cohort and for only 35 of 743 metabolites (4.7%) within the power athletes.[1] Participants in our study were fit, but far from elite athletes. If, however, exercise can normalize the metabolomes between sexes, given the salutary effects of exercise and healthy participants used in this study, it could be inferred that the resting metabolome observed here is an example of a healthy ideal for both sexes. It is noteworthy that individuals were trained prior to enrollment, so a variety of exercise exposures led to this common resting metabolome, which could be inferred to be a healthy metabolome. It seemed regular participation in physical activity outweighed sex in this regard.

Hierarchical clustering and PCA both portrayed much similarity in the metabolome for M and F, particularly at rest, but with at least some evidence for a discernible sex difference arising post exercise. Venn diagrams limited to specific metabolite changes of at least a 2-fold difference from rest told the same story. Comparisons of the metabolite counts and lists from the Venn diagrams were able to capture some of the major differences in the exercise response between M and F, however, intricacies of the sex-difference became clearer when the 2-fold criterion used for the Venn diagrams was eschewed, and all significant differences from the RMANOVA were considered. Generally, AE brought about a greater number of sex differences, but these largely fell within FA metabolism, which was disproportionately well-represented in our metabolomic panel (163 FA metabolites = 22% of the panel and another 128 FA membrane lipids). AN, on the other hand, resulted in sex differences over a broader variety of pathways and yielded typically larger differences when compared to those for AE. It is possible this resulted from the lack of change in F at AN-T1, thought to be driven by minimal metabolic activity within the E-F participants, due to their inexperience with this exercise mode. E-M and certainly R-participants were much more “comfortable” performing the lifts. Despite equally high ratings of perceived exertion, and observed effort put forth for all participants, differences our results may have been impacted by this factor.

Within the individual metabolite and pathway differences, 1,5-AG was 20-25% lower in F than M at all times. This has been reported in non-diabetics elsewhere.[38,54] Decreased 1,5-AG is indicative of chronically accumulated hyperglycemia (> 180mg/dl). Conversely, regular participation in exercise helps mitigate fluctuations in blood glucose and time in a hypoglycemic state. Without actual concentration values it is impossible to determine if either group was outside of the normal range for this metabolite, though it presents a potentially notable finding.

Also different at rest and following both exercises was 2-hydroxy-3-methylvalerate and α-hydroxyisocaproate, which were higher in M at all times. The former is an isoleucine ketoacid reduction product found to be elevated with BCAA metabolic disorders (i.e. Maple Syrup Urine Disease), and changes following exercise were similar across sex. The latter is an intermediate of leucine metabolism that serves as a precursor to the anabolic hydroxy-methylbutyrate (HMB) and is a known "anti-catabolic" agent itself. Further, the increases in α-hydroxyisocaproate with exercise were larger on an absolute level for the M than the F. Though α-hydroxyisocaproate has known anabolic properties and is touted as an "anti-catabolite" in supplement marketing, it is understudied within the contexts of exercise or sex differences. Higher levels and greater responsiveness to exercise, and AN in particular, for M than F may help explain findings of greater muscle mass accretion in males than females, even when on the same exercise regimen.[55] Similarly, the androgenic steroid biosynthesis markers 5α-androstan-3α,17β-diol disulfate and 5α-androstan-3α,17β-diol monosulfate differed at rest, with the difference perpetuated or enlarged by exercise. While several other androgenic steroid metabolites also increased more or only for M with exercise, it was these 2 that, by far, displayed the greatest sex difference in relative concentrations. Males have greater testosterone and androgenic steroid biosynthesis markers than females, and this difference may drive at least some of the observed sex differences observed here, like that for α-hydroxyisocaproate. Notably, there was no observable sex differences for progestin or pregnenolone steroid biosynthesis markers. That these 2 steroid biosynthesis markers were more substantially altered than the others in this pathway could implicate them as potentially sensitive to M/F differences and/or the exercise response. On the other hand, since findings are based on relative concentrations, it is also possible that very low resting levels may have artificially inflated the response size of these metabolites. Regardless, previous metabolomics studies have reported more elevated

BCAA[1,38,40] and androgens[20,37,38] in males compared to females. Data from our results extends these findings to the exercise response.

SUBSTRATE METABOLISM COMPARISONS

One of the most commonly reported changes in response to exercise is that of purine metabolites.[11] Our findings are no exception, as these were amongst the very largest changes in terms of magnitude in the entire metabolomic panel for both sexes and both exercises. Massive changes in purine salvage pathway markers indicated the high rate of ATP turnover with exercise. Even greater increases in these metabolites as well as lactate (anaerobic glycolysis) suggest greater anaerobic energy pathway upregulation by exercise in M than F. Smaller, but significant, increases in downstream metabolites (urate, allantoin) may indicate an antioxidative role in addition to energy kinetics for cycling through this pathway.[56] Again, the greater M than F levels in many of these markers suggests the potential impact for production of allantoin in response to oxidative stress. Impacts of sex differences on this pathway and its function during/after exercise require further investigation.

More elevated anaerobic metabolism and increased levels of metabolites related to insufficient mitochondrial processing of FA's can be interpreted to demonstrate a greater energy strain in M than F during exercise. M seemed to counter this through increased fuel flexibility. Evidence that M more actively engaged non-lipid energy production pathways came from bigger increases in span-2 intermediates of the TCAC as well as greater evidence of urea cycling for the production of fumarate, overall higher AA metabolism, and larger elevations in pyruvate and lactate. Correspondingly, free FAs and various metabolites of lipid metabolism were more elevated in F than M, including FA dicarboxylates, mono- and dihydroxy-FAs, branched FAs, amino-FAs, acylglycines, acyl glutamines, acylglycerols, and ketones. More robust FA metabolite perturbations in F than M at AE-T1, AE-T2 and AN-T2, suggests higher dependence on FA during

AE and in recovery from both exercises in F. However, the increased glycolytic flux in M may have been a cause rather than a result of the relatively lower FA metabolism. Because lactate attenuates lipolysis at the adipocyte and mitochondrial FA uptake via acetyl CoA sequestration of carnitine,[57] the elevated anaerobiosis in M could have hindered FA mobilization and impaired β-oxidation rates. This would explain the observation of the increased markers of mitochondrial distress in M relating to the carnitine palmitoyl-transferase system and various acyl-Co-A dehydrogenase enzymes.

Further support for sex difference in FA metabolism and effects of lactate were seen in the acylglycerol responses to AN. Only the M experienced a rise in select DAGs at AN-T1, with a return to baseline by AN-T2. F had no change in circulating DAGs at AN-T1 followed by a significant decrease by AN-T2. As males seemed to have less FA mobilization than F, the sex difference in DAGs could have resulted in leaking of DAGs from lipolyzed intramuscular lipid stores across the cell membrane, as serum DAGs have previously been shown to be representative of muscle DAGs.[58] Acylglycerol availability likely exceeded import into the cell and/or mitochondria in both sexes when mobilization was highest, i.e., during AE, and DAGs went up. Conversely, due to the difference in lactate levels seen during AN, F may have more efficiently sequestered the acylglycerols into the mitochondria than did M. In F, transport and β-oxidation were able to keep pace with acylglycerol supply during AN, and even resulted in net disappearance from the circulation by AN-T2.

A final class of FA metabolites to respond somewhat differently were the FACs, which act as indicators of actual β-oxidation. FACs were significantly higher in F than M at AE-T1, but none of the other time points. This difference suggests higher FA breakdown in F than M at AE, but not AN or for either exercise at T2. However, FA mobilization, as indicated by increasing free FAs (LCFA, MCFA, PUFA), appeared to be greater in F than M at AE-T1, AE-T2 and AN-T2. It could be

that AN led to large enough increases in lactate to blunt FA mobilization and deplete the carnitine pool in M and F at AN-T1, but as lactate decreased, FA mobilization and β-oxidation increased. For AE, the elevation in lactate was only sufficient to blunt FA processes in M. It is further possible that by 60-minutes post-exercise, both sexes had sufficiently mobilized intramuscular lactate so that no differential effect on FAC was seen, but blood-lactate was still elevated enough in the M (non-significantly higher than F at AE- and AN-T2) to see differences in mobilization, namely higher FA levels in F.

Intriguingly, Patel, *et al*., (2013)[59] reported greater long-chain FACs, but not LCFAs in males than females within an overweight population at rest. Though there have been no reports on sex differences, resting blood-lactate has been found to be higher in obese individuals.[60,61] The authors attributed the higher male than female FAC, but not LCFA levels to greater insulin resistance in their M cohort. The conflicting direction of this observation with our finding could be explained by the source of lactate. In our design the majority of lactate was almost certainly being produced in the muscle, whereas in a resting sample multiple sites likely contribute. Build-up in any one tissue would thus unlikely be sufficient to reduce FACs in a resting sample. Our FA metabolite results highlight the importance in selection of exercise intensity and timing of measurement in determination of substrate selection differences between sexes.

Markers for both ketone synthesis (acetoacetate) and degradation **(β-hydroxybutyrate**) were both higher in F than M for AE and during recovery from both exercise bouts. And though only relative concentrations were assessed, assuming normal resting levels, the magnitude of increase seen for F in response to AE (> 300% at AE-T2) likely would put values outside of the normal physiologic range. While ketones are known to increase with sustained exercise, this represents a surprisingly large response given the relatively short duration of this bout (45 minutes) and that no participants in this study were on a ketogenic diet. There is still much

controversy surrounding the use of ketones supplements or ketogenic diets as a means to bolster performance.[62] Here we show the importance of ketone both during and after a short, intense AE bout, particularly in F. Despite the popularity of this topic, the effect of sex differences on endogenous ketone metabolism is still poorly understood.[62]

Differences in substrate selection as a function of sex are commonly reported.[63-68] However, some studies have failed to show significant differences in macronutrient oxidation with exercise.[65] Because of the breadth of markers in our metabolomics panel, we showed F to engage in greater FA metabolism, including ketones, and M to involve more varied substrate selection and higher anaerobic metabolism.

Corroborating with our substrate-level observations, pyridoxate, which is a cofactor for both glycogen phosphorylase and transaminase reactions was higher in M. Meanwhile, nicotinamide metabolites, which are associated with aerobic energy production, were significantly increased in F-only. These changes in vitamin levels support greater FA selection in F and AA plus carbohydrate use in M.

Not to be lost in the observed time course of sex differences was that FA metabolism was most increased in both sexes during AE, but was also elevated across groups post-exercise. It seemed, regardless of sex, FAs were the preferred substrate for energy production in the initial (within 60-mintues) post-exercise *milieu*.

Downstream Lipid Mediators. Several lipid signaling molecules exhibited sex-dependent responses to exercise. One subset of these markers included the endocannabinoids. The endocannabinoids affect cognitive processing, improve mood state, and have been suggested to mediate some of the positive affective responses of exercise.[69,70] F had higher endocannabinoids immediately following AE. In response to AN, the endocannabinoids were less elevated, but trending up from T0-T1-T2 in F, and only experienced a non-significant rise T0-T1 and fall T1-T2

in M. It is feasible that like AE, AN may lead to increased endocannabinoids in F, just farther removed from the exercise bout. In fact, palmitoyl ethanolamide was significantly higher in F than M at AN-T2, but not AN-T1. Exercise psychology studies have shown that exercise attrition is best achieved when improvements in mood status are more closely associated with the exercise session.[71] The temporal separation for the delayed rise in endocannabinoids offers a compelling explanation as to why the majority of F gravitate towards AE rather than AN. Certainly, a complex set of sociological pressures and other factors are at play, but the endocannabinoids as an influencing factor presents an interesting line of future research.

The inflammatory lipid mediators themselves and the PUFA and PU-MAG precursors used in lipid mediator production showed differential responses by sex. Changes in inflammatory signaling indicated a relatively pro-inflammatory status for M with AE and for F with AN. It is possible that the slower FA oxidation rate for M during AE resulted in more PUFA and PU-MAG availability for eicosanoid synthesis, while the pervasive FA metabolism in F included oxidation of these precursors, precluding their use in eicosanoid production. In contrast, during AN, FA metabolism was comparatively limited in both groups, but training status could have played a role in the AN response. As a result of their more substantial prior experience with AN, M may have developed mechanisms to better manage the inflammatory response to this condition.

Another factor is the role of the glucocorticoids. Cortisol and corticosterone have been shown to inhibit 5-lipoxygenase[72] and phospholipase-A2[73] and, thus, eicosanoid production. Higher cortisol levels were found in the group with lower eicosanoids, suggesting cortisol may drive the observed sex difference. However, both the corticosteroid and eicosanoid metabolites were globally elevated across group and condition, hinting that some other factor may be at play. Training status leading to discrepancies in absolute intensity and total workload may have been a driving factor underlying this observed sex difference. Intensity and workload were

higher in M than F for both conditions, particularly for AN. However, while cortisol was higher for F than M in response to AE and vice versa with AN, metabolites of epinephrine, another major stress hormone, showed no sex difference with either exercise, indicating the same relative strain in both groups.

Membrane Lipids. Related to the FA metabolism outlined above, perturbations in circulating membrane lipids were largest during AE and after AN. Though the total number of membrane lipid metabolites observed to change was impressive, the exploratory panel used for this investigation contained a large proportion of these metabolites (128 = 17%). When expressed as an enrichment value which can be demonstrated as the ratio of (percent of total membrane lipids altered):(total percentage of the metabolome altered), the membrane lipids did not prove especially susceptible to change. Moreover, most fluctuations were not particularly large. The primary exception to this were substantial decreases in the acyl-cholines during exercise in both groups. All 8 acyl-cholines were more decreased in M than F in response to AN. Why these particular phospholipids were especially responsive to both exercise bouts or differed between sexes in response to AN is unknown and presents a novel finding here. The only previous reports of membrane lipid differences between M and F have been greater resting sphingomyelins in F at rest.[37,38,42] Overall, there was some evidence for a sex by condition interaction within the sphingolipid response to exercise. AE yielded more sphingolipid perturbations in F at AE-T1, while AN led to more extensive increases in M at AN-T1.

Another subset of sphingolipid metabolites to show a substantial sex difference were sphingosine, sphingosine-1-phosphate, sphinganine and sphinganine-1-phosphate. These 4 metabolites are readily interconverted into one another in the blood, and observed changes in one could potentially reflect changes in any. Of them, sphingosine-1-phosphate is a potent signaling molecule serving as an immune cell trafficker, angiogenic agent, and may relate to the

positive effects of high-density lipoproteins.[74,75] Given the timing and context of serum collection (immediately and 60-minutes post-exercise), the greater increase in sphingosine-1-phsophate and related metabolites for M in response to AN more likely relates to immunomodulatory rather than other purported effects of this molecule, though additional studies would be needed to verify this conjecture.

Cholesterol. There were sex differences in cholesterol, sterol, and bile acid metabolite responses. Interestingly, exercise was more effective in acutely lowering cholesterol levels in M, while phytocholesterols appeared more responsive to exercise in F. This presents an interesting observation, but given the relative health of the cohort investigated and lack of outcome measures, we cannot draw any conclusions regarding health outcomes related to cholesterol levels here.

Microbiome. The microbiome, as depicted through changes within the xenobiotic subset of metabolites showed alteration with both exercises, but no clear difference between sexes. This subset contained only about 10% of the total microbiome-related metabolites on this panel. However, markers omitted were often involved in other metabolic pathways shown to be active during exercise, i.e., lactate, ketone or vitamin-B5 metabolism. Therefore, the included metabolites may better depict the actual metabolism of the gut microbiota. Moreover, when all 186 microbiome metabolites were examined on a single heatmap, no discernible pattern beyond the scattered perturbations seen in the 17-metabolite representative sample illustrated in **Supplemental Figure 3** were identified.

LIMITATIONS & CONSIDERATIONS

Both the resting metabolome and metabolomic perturbations from exercise may have been susceptible to effects of fitness status. Half of each cohort was experienced in E and half in R. M and F reported similar exercise participation within the past 2-years, but many of the R-F had

prior experience in endurance sports while the R-M tended to have only been power/strength trained. Similarly, most of the E-M had at least some exposure to lifting weights, while none of the E-F had ever regularly lifted before. Moreover, while aerobic exercise activities varied little (cycling, running, swimming), anaerobic exercise more often took the form of true weight training in M and high-intensity training in F (i.e., cross fit). In fact, identifying females that did pure strength training was particularly difficult when compared to the other sub-groups recruited for this study. One difference between sexes appears to be the manner in which M and F exercise. This could be due to a plethora of factors far beyond the scope of this paper, but is of note in the context of the impact of training background on our results.

An observation made from exercise testing was that E-F were particularly uncomfortable during AN. The E-M may not have excelled, but were certainly better versed in the exercises used during this condition. It seems likely that limitations due to neuromuscular recruitment and coordination due to novelty of the task may have prevented these 10 F subjects from creating the same metabolic strain as the other participants in this study. This, in turn, may have given rise to some of the sex-differences with AN. There was an analogous situation with AE, via a lack of familiarity for endurance exercise seen in R-M, but not R-F. The resultant impact on AE was likely muted due to the lesser importance of balance and other non-metabolic factors on one's ability to ride a stationary bike (AE) when compared to the more complex motor skill of lifting weights (AN). Assessment of groups separated by both sex and training was evaluated, but ultimately these results were negated given the reduced sample size.[76] Though power calculations for evaluation of a metabolomics panel using RMANOVAs do not exist, it is unlikely meaningful results could be drawn from such an analysis (n = 10 per group).[76,77] Certainly, follow up in this regard is warranted.

Another result of the differences in long-term training history and exercise exposure is that the M cohort contained individuals that had been training for longer and thus may have amassed more fitness gains than their F counterparts. When M and F cohorts were further separated by training background, fitness testing results showed that for VO_{2MAX}: E-M > E-F = R-F > R-M, and for strength: R-M > E-M = R-F > E-F, whether in absolute terms or when scaled to body weight. Thus, sex differences in fitness were largely driven by the superior aerobic fitness of the E-M and strength of the R-M, plus the low strength in the E-F. Regardless of the cause, M were seen to have higher aerobic and anaerobic fitness both on an absolute level and when scaled to body weight. Interestingly, expressing either oxygen consumption, aerobic power, or strength relative to fat free mass normalized fitness data between M and F, indicating actual work output per unit muscle is similar between sexes. Still, achievement of higher fitness levels for M than F in similarly trained individuals is a common occurrence reported in the literature, even in elite athletes that have seemingly achieved their physiological peaks.[22,24,30,32] It is generally accepted that sex-differences in responsiveness to training result from hormonal differences (testosterone) in M and F,[26,27] but hormones are simply drivers of downstream metabolic actions. A comprehensive metabolomic platform is capable of identifying more direct mediators of sex differences. With that in mind, it is important to note that there was far more similarity than difference in the metabolome of M and F both at rest and post-exercise.

CONCLUSION

Here it was found that M and F resting metabotypes differed very little. This is in contrast to previous reports using sedentary or diseased comparisons between sexes. Moreover, while exercise did reveal some sex differences, M and F still displayed relatively similar metabolomes in the wake of either AE or AN. Not to be lost in the observed time course of sex differences was that FA metabolism was most increased in both sexes during AE, but was also elevated across

groups post-exercise. It seemed, regardless of sex, FAs were the preferred substrate for energy production in the initial (within 60-mintues) post-exercise *milieu*. The major difference seemed to lie within substrate preference, where F had more evidence of FA metabolism, particularly during AE and M had more glucose and AA use along with more anaerobic metabolism, particularly during AN. Additionally, the selection of FA as a fuel source seemed to increase widespread lipid metabolism giving a sex by exercise interaction for greater lipid mediators typically found for F and AE. Beyond substrate metabolism, there were differences identified between M and F for androgenic, anabolic, endocannabinoid, inflammatory and immune signaling in response to AE and/or AN including both expected and novel metabolites.

FIGURES

Table 1. Descriptive and Fitness Characteristics

Category	Mean ± SD	Males	Females
Age (years)	23.0 ± 3.1	24.0 ± 3.9	22.1 ± 2.3
Height (cm)*	168.8 ± 9.3	175.2 ± 7.3	162.5 ± 6.2
Mass (kg)*	67.2 ± 9.4	72.9 ± 7.8	61.5 ± 7.2
% Body Fat*	18.1 ± 7.5%	12.8 ± 5.7%	23.3 ± 4.8%
Fat Free Mass (kg)*	55.2 ± 9.7	63.3 ± 4.9	47.1 ± 5.8
relative VO_{2MAX} (ml/kg/min)*†	46.5 ± 8.0	49.6 ± 10.1	43.3 ± 3.2
VT (% of VO_{2MAX})	72 ± 11%	73 ± 14%	71 ± 0.1%
PO_{MAX} (Watts)*†	218 ± 57	257 ± 53	178 ± 23
Squat 10RM (kg)*†	72 ± 29	88 ± 32	57 ± 17
Leg Press 10RM (kg)*†	182 ± 62	212 ± 65	153 ± 42

Table 1. Descriptive Characteristics for the entire cohort and separated by sex.
SD = standard deviation; FFM = fat free mass, VO_{2MAX} = max aerobic capacity; PO_{MAX} = max aerobic power; 10RM = 10-repetition max weight.
*Males significantly different than females, $p < 0.05$
†Difference eliminated when scaled to FFM

Figure 1.A

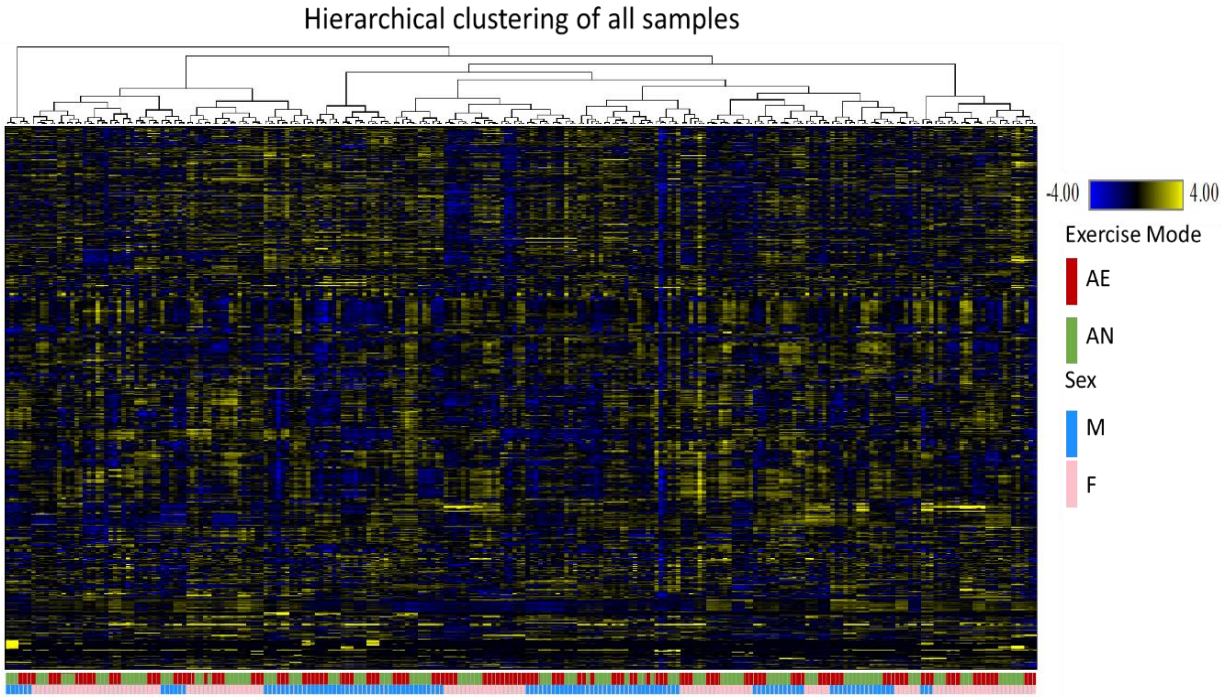
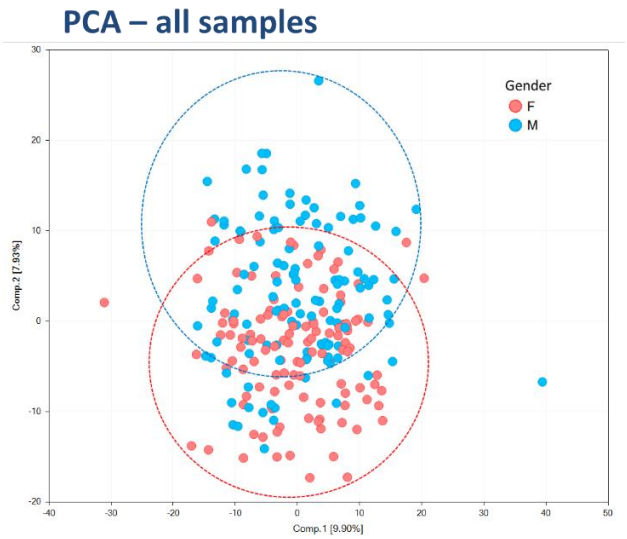


Figure 1. A) Hierarchical cluster. This analysis indiscriminately included all data points, including AE and AN at T0, T1 and T2. As can be seen along the bottom of the display, the cluster analysis revealed little AE (red) or AN (green) clustering, but substantial grouping by sex (M & F blue & pink, respectively).
M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise

Figure 1.continued

B



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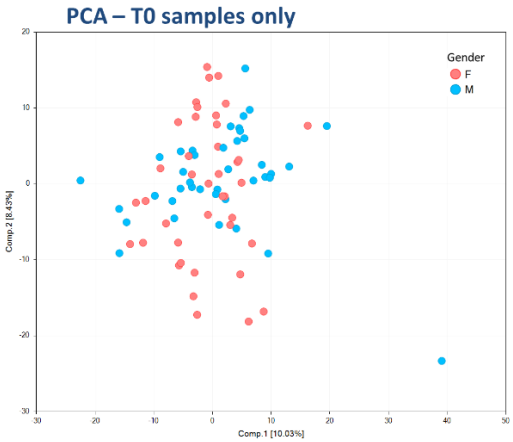

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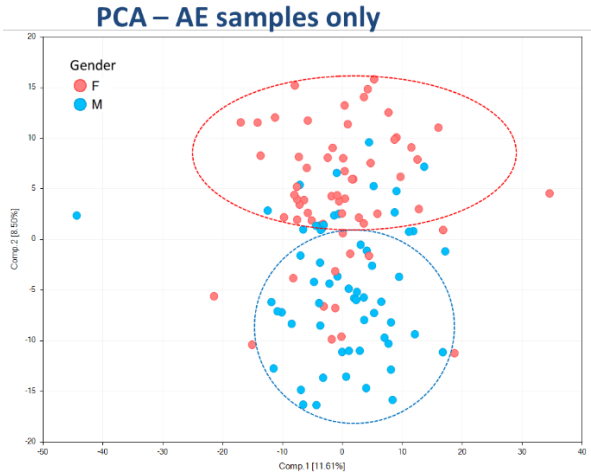

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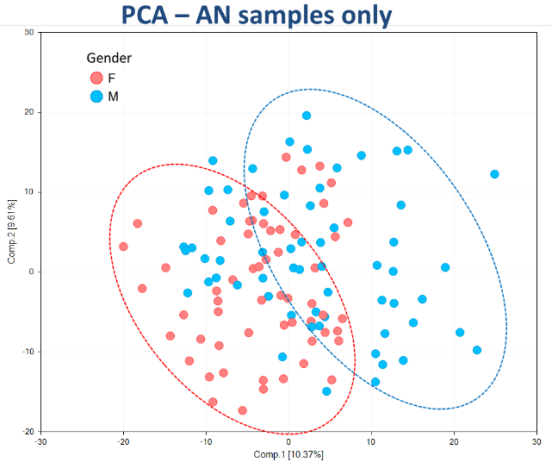
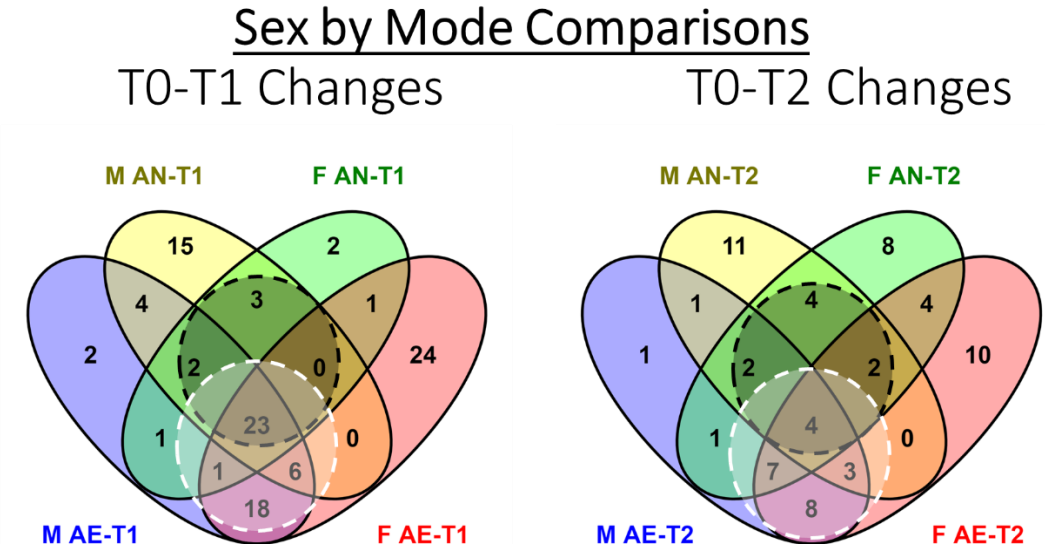


Figure 1. B) Principle Components Analysis. PCA plot indiscriminately including all data points, color-coded by sex.
Figure 1.C) PCA of just the resting (T0) data (includes both AE and AN).
Figure 1.D) PCA of just the AE condition data (includes all time points)
Figure 1.E) PCA of just the AN condition data (includes all time points)

Figure 2.A,B) 2-Fold Comparisons



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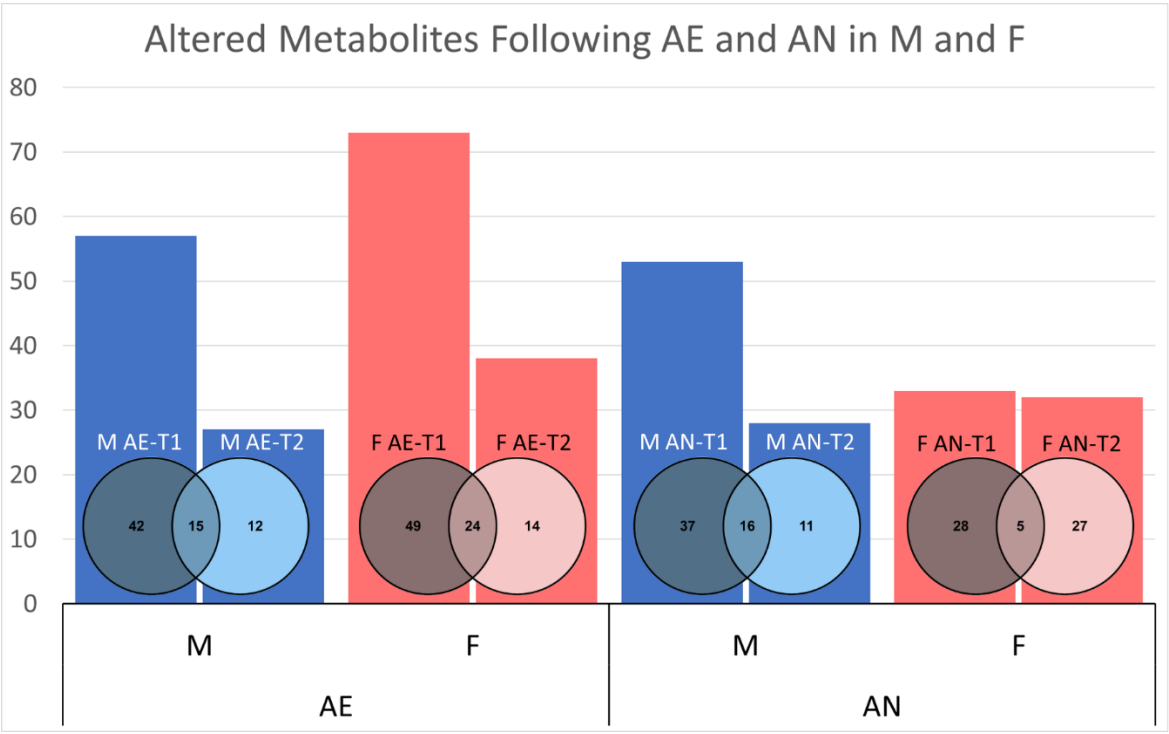


Figure 2. A,B) Venn diagrams showing major ($p < 0.05$, $q < 0.1$, magnitude > 100% increase or 50% decrease) metabolite changes (T0-T1 and T0-T2) for M and F in response to AE (A) and AN (B). White rings include common metabolomic shifts between sexes for AE. Black rings include common metabolomic shifts between sexes for AN.

Figure 2.C) Bar graph depicting total number of metabolites altered 2-fold or greater by exercise at each time point for both groups. Overlaid with Venn diagrams showing the time course for these metabolite changes.

M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.

Common to M & F AE-T1	Unique to M at AE-T1	Unique to F at AE-T1
cysteinylglycine	argininosuccinate	N-acetylphenylalanine
N-acetylmethionine	12-HHTrE	xanthurenate
3-(3-hydroxyphenyl)propionate sulfate	α-hydroxycaproate	butyrylcarnitine (C4)
4-hydroxyphenylpyruvate	2-docosahexaenoylglycerol (22:6)	palmitoleoylcarnitine (C16:1)
vanillylmandelate (VMA)	glycochenodeoxycholate	15-methylpalmitate
N-acetyltryptophan	glycocholate	hexadecanedioate
lactate	glycodeoxycholate	tetradecanedioate
pyruvate	lanthionine	16-hydroxypalmitate
phosphate	2-oxindole-3-acetate	3-hydroxylaurate
β-citrylglutamate		9-hydroxystearate
α-ketoglutarate		glycerol
fumarate		acetoacetate
malate		eicosenoate (20:1)
succinate		margarate (17:0)
12-HETE		palmitate (16:0)
linoleoylcholine		pentadecanoate (15:0)
oleoylcholine		5-dodecenoate (12:1n7)
palmitoloelycholine		caprate (10:0)
palmitoylcholine		laurate (12:0)
stearoylcholine		dihomo-linoleate (20:2n6)
propionylcarnitine (C3)		docosadienoate (22:2n6)
3-hydroxybutyrylcarnitine		docosapentaenoate (n3 DPA; 22:5n3)
acetylcarnitine (C2)		tauro-β-muricholate
3-methyladipate		17α-hydroxypregnenolone 3-sulfate
3-hydroxysebacate		cinnamoylglycine
3-hydroxybutyrate (BHBA)		
10-heptadecenoate (17:1n7)		
10-nonadecenoate (19:1n9)		
myristate (14:0)		
myristoleate (14:1n5)		
oleate/vaccenate (18:1)		
palmitoleate (16:1n7)		
2-arachidonoylglycerol (20:4)		
arachidonoylcholine		
dihomo-linolenoyl-choline		
docosahexaenoylcholine		
glycerophosphoinositol		
docosatrienoate (22:3n3)		
linoleate (18:2n6)		
linolenate [α/γ (18:3n3 or 6)]		
stearidonate (18:4n3)		
glycohyocholate		
glycoursodeoxycholate		
sphingosine		
corticosterone		
hypoxanthine		
xanthosine		

Common to M & F AE T-2	Unique to M at AE-T2	Unique to F at AE-T2
2-hydroxy(iso)butyrate	2,3-dihydroxy-2-methylbutyrate	3-hydroxyisobutyrate
α-ketobutyrate	12-HETE	acetylcarnitine (C2)
vanillic alcohol sulfate	glycocholate	suberoylcarnitine (C8-DC)
hexanoylglutamine	threonylphenylalanine	3-methyladipate
3-hydroxybutyrylcarnitine	4-vinylguaiacol sulfate	16-hydroxypalmitate
hexadecanedioate		9-hydroxystearate
tetradecanedioate		acetoacetate
3-hydroxysebacate		myristate (14:0)
3-hydroxybutyrate (BHBA)		dihomo-linoleate (20:2n6)
10-heptadecenoate (17:1n7)		tauro-β-muricholate
10-nonadecenoate (19:1n9)		glycodeoxycholate
eicosenoate (20:1)		ursodeoxycholate
myristoleate (14:1n5)		inosine
oleate/vaccenate (18:1)		guanosine
palmitoleate (16:1n7)		eugenol sulfate
docosatrienoate (22:3n3)		ferulic acid 4-sulfate
linolenate [α/γ (18:3n3 or 6)]		
stearidonate (18:4n3)		
taurocholate		
taurodeoxycholate		
xanthosine		

Figure 2.D) List of Metabolites from Venn Diagram 2.A.
M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.

Common to M & F AN-T1	Unique to M at AN-T1	Unique to F at AN-T1
cysteinylglycine	β-citrylglutamate	N-acetylisoleucine
α-hydroxyisocaproate	2,3-dihydroxy-2-methylbutyrate	12-HETE
4-hydroxyphenylpyruvate	3-methyl-2-oxobutyrate	12-HHTrE
lactate	4-methyl-2-oxopentanoate	tauro-β-muricholate
pyruvate	2-hydroxy(iso)butyrate	4-vinylguaiacol sulfate
pantothenate	cysteine sulfinic acid	
α-ketoglutarate	N-acetylmethionine	
fumarate	N-acetyltaurine	
malate	3-(4-hydroxyphenyl)lactate	
linoleoylcholine	argininosuccinate	
oleoylcholine	succinate	
palmitoloelycholine	3-hydroxybutyrylcarnitine	
palmitoylcholine	octanoylcarnitine (C8)	
stearoylcholine	malonate	
3-hydroxybutyrylcarnitine	3-methyladipate	
acetylcarnitine (C2)	maleate	
decanoylcarnitine (C10)	undecanedioate	
α-hydroxycaproate	1-palmitoyl-GPI (16:0)	
2-docosahexaenoylglycerol (22:6)	2-arachidonoylglycerol (20:4)	
arachidonoylcholine	glycochenodeoxycholate	
dihomo-linolenoyl-choline	glycocholate	
docosahexaenoylcholine	glycodeoxycholate	
glycerophosphoinositol	taurodeoxycholate	
glycohyocholate	valylleucine	
glycoursodeoxycholate	2-isopropylmalate	
corticosterone		
hypoxanthine		
xanthosine		

Common to M & F AN-T2	Unique to M at AN-T2	Unique to F at AN-T2
2-hydroxy(iso)butyrate	cysteinylglycine	3-hydroxyisobutyrate
α-ketobutyrate	2,3-dihydroxy-2-methylbutyrate	maltose
vanillic alcohol sulfate	3-(4-hydroxyphenyl)lactate	nicotinamide
phosphate	ribose	3-hydroxybutyrylcarnitine (2)
citrate	malate	3-hydroxylaurate
malonate	3-hydroxybutyrylcarnitine	9-hydroxystearate
glycocholate	glycochenodeoxycholate	α-hydroxycaproate
glycodeoxycholate	taurochenodeoxycholate	10-heptadecenoate (17:1n7)
hypoxanthine	taurocholate	myristate (14:0)
xanthosine	glycohyocholate	myristoleate (14:1n5)
4-vinylguaiacol sulfate	glycoursodeoxycholate	palmitoleate (16:1n7)
ferulic acid 4-sulfate	taurodeoxycholate	5-dodecenoate (12:1n7)
	taurolithocholate 3-sulfate	1-dihomo-linolenylglycerol (20:3)
	tauroursodeoxycholate	1,2-dilinoleoyl-GPE (18:2/18:2)
	corticosterone	docosatrienoate (22:3n3)
		linolenate [α/γ (18:3n3 or 6)]
		stearidonate (18:4n3)
		threonylphenylalanine
		lanthionine
		eugenol sulfate

Figure 2.E) List of metabolites from Venn diagram 2.B.
M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.

Table 2. Resting Differences

Pathway	Metabolite	M:F Ratio at Rest
Glycine, Serine & Threonine	sarcosine	1.07
Glutamate	pyroglutamine	1.61
BCAA	2-hydroxy-3-methylvalerate	1.34
	α-hydroxy-isocaproate	1.36
GGAA's	γ-glutamyllecuine	1.23
	γ-glutamylisolecuine	1.15
	γ-glutamylvaline	1.30
	γ-glutamylglutamate	1.30
	γ-glutamylglutamine	1.19
	γ-glutamyltyrosine	1.12
Creatine	creatinine	1.28
Dipeptide Derivative	N-acetylcarnosine	1.87
Glycolysis, Gluconeogenesis & Pyruvate	1,5-anhydroglucitol (1,5-AG)	1.19
	glucose	1.03[NS]
Carnitine	deoxycarnitine	1.27
Plasmalogen	1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)	0.94
	1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)	1.05
Sphingolipid	palmitoyl dihydrosphingomyelin (d18:0/16:0)	0.89
	sphingomyelin (d18:2/16:0, d18:1/16:1)	0.82
Sterol	cholesterol	1.09[NS]
	7-α-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	1.45
	3β-hydroxy-5-cholestenoate	1.60
Steroid	5α-androstan-3α,17β-diol disulfate	2.24
	5α-androstan-3α,17β-diol monosulfate	8.76
Primary Bile Acid	chenodeoxycholate	1.32
Hemoglobin	bilirubin (Z,Z)	1.50

Table 2. Resting Differences. List of metabolites (by Kegg sub-pathway) that were significantly different ($p < 0.05$, $q < 0.1$) between sexes at rest. Size of difference expressed as a ratio of M (male) to F (female) value. Value in blue when M > F (ratio > 1) and pink when F > M (ratio < 1). Only the 2 steroid metabolites showed a greater than 2-fold difference between sexes. Two non-significantly different metabolites glucose and cholesterol were included to give context to the 1,5-AG and sterol metabolites respectively.

[NS] No difference between sexes

M = male; F = female; BCAA = branched-chain amino acids; GGAA = γ-glutamyl amino acids.

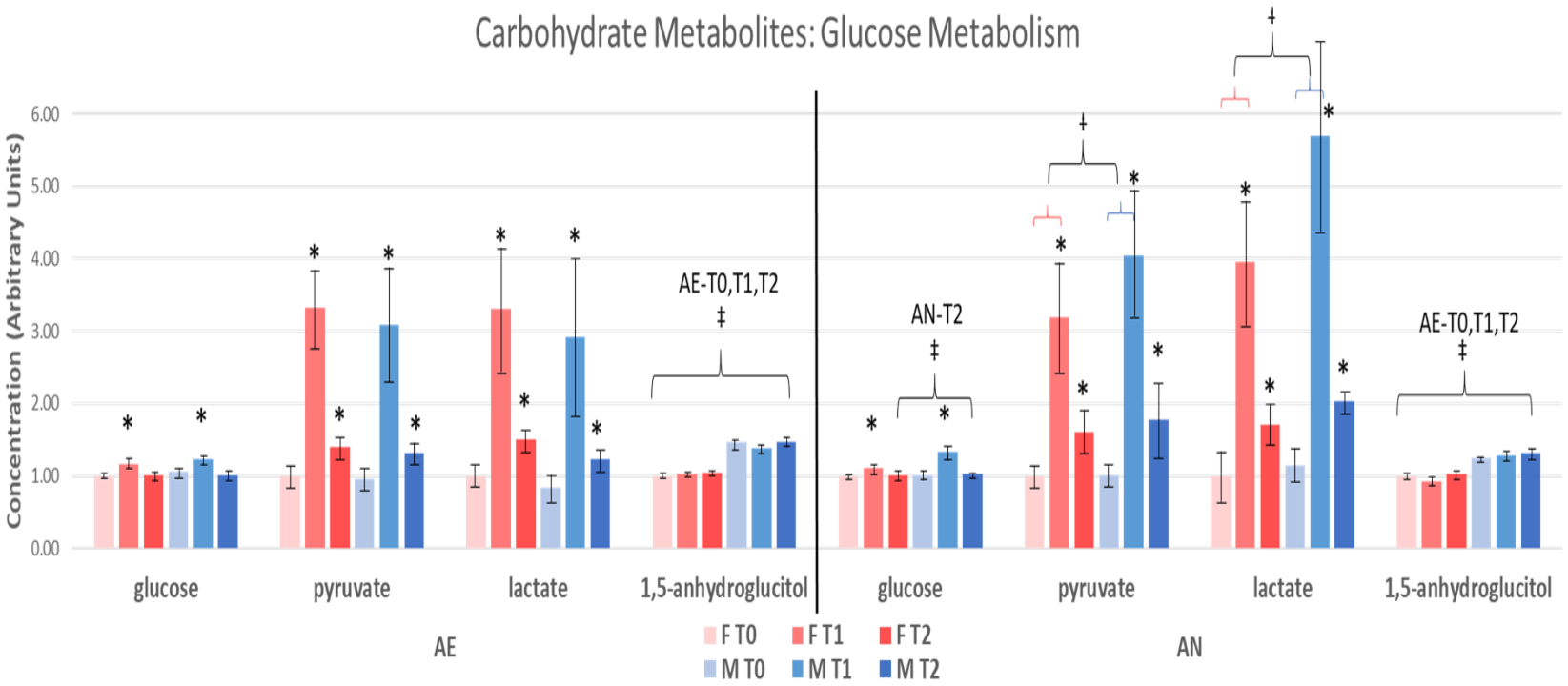
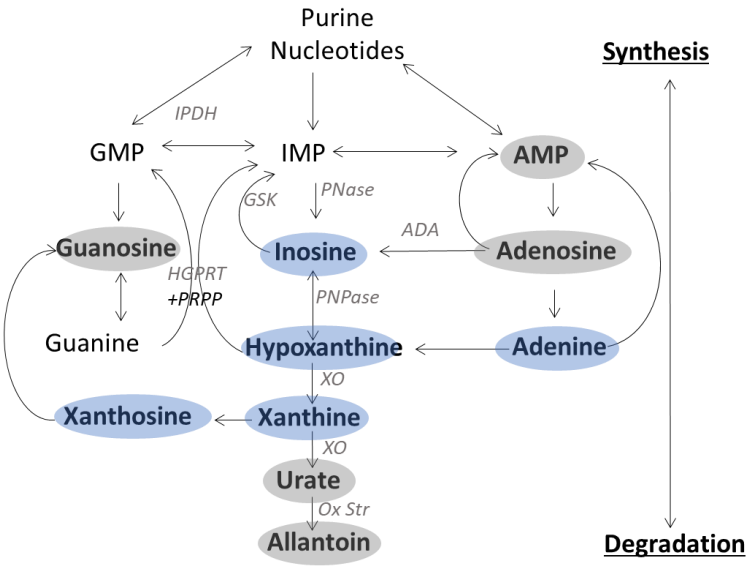


Figure 3. Relative concentrations of carbohydrate metabolites in M and F at T0, T1 and T2 for AE and AN.
M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.
* Significant difference from T0
† Significant difference between sexes for change from T0-T1
‡ Significant difference between sexes for all 3 time points

Figure 4.A Purine Salvage Pathway



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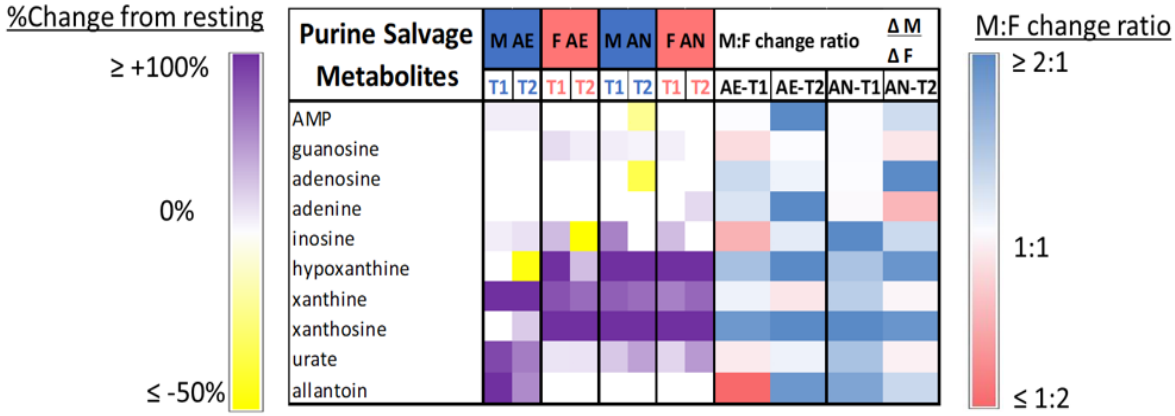
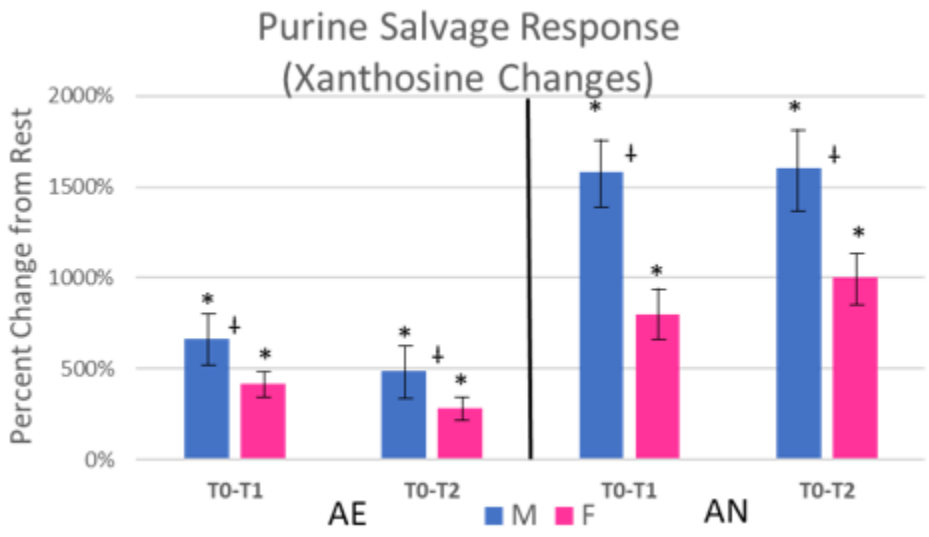


Figure 4. **A)** Diagram of the purine salvage pathway. Markers included in the panel highlighted in grey; that were more elevated in M than F are highlighted in blue.
Figure 4. B) Compound heat map for purine salvage markers. First 8 columns: shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest (T0) for the given group-exercise-time, respectively. White = no significant change from T0. Latter 4 columns use shades of blue and red to represent greater changes in M and F, respectively. Value determined as the ratio for the absolute value of percentage change values from resting level, $|\Delta M| : |\Delta F|$.
AMP = adenosine 5'-monophosphate; M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.

Figure 4.C



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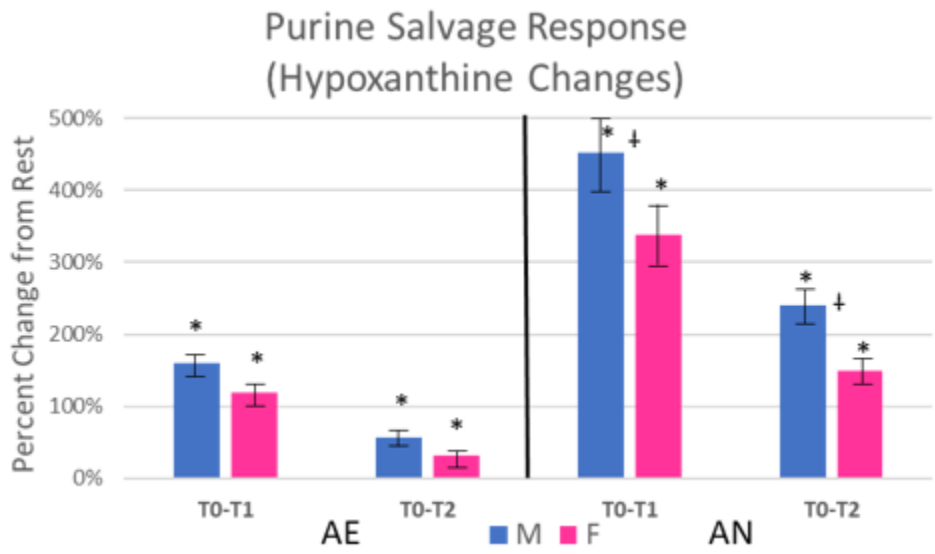


Figure 4. C,D) Graphs of changes in purine salvage pathway markers for M and F immediately and 60-minutes following AE and AN. Values expressed as percentage change from baseline.
M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.
* Significant difference from rest
† Significant difference between sexes for relative % change

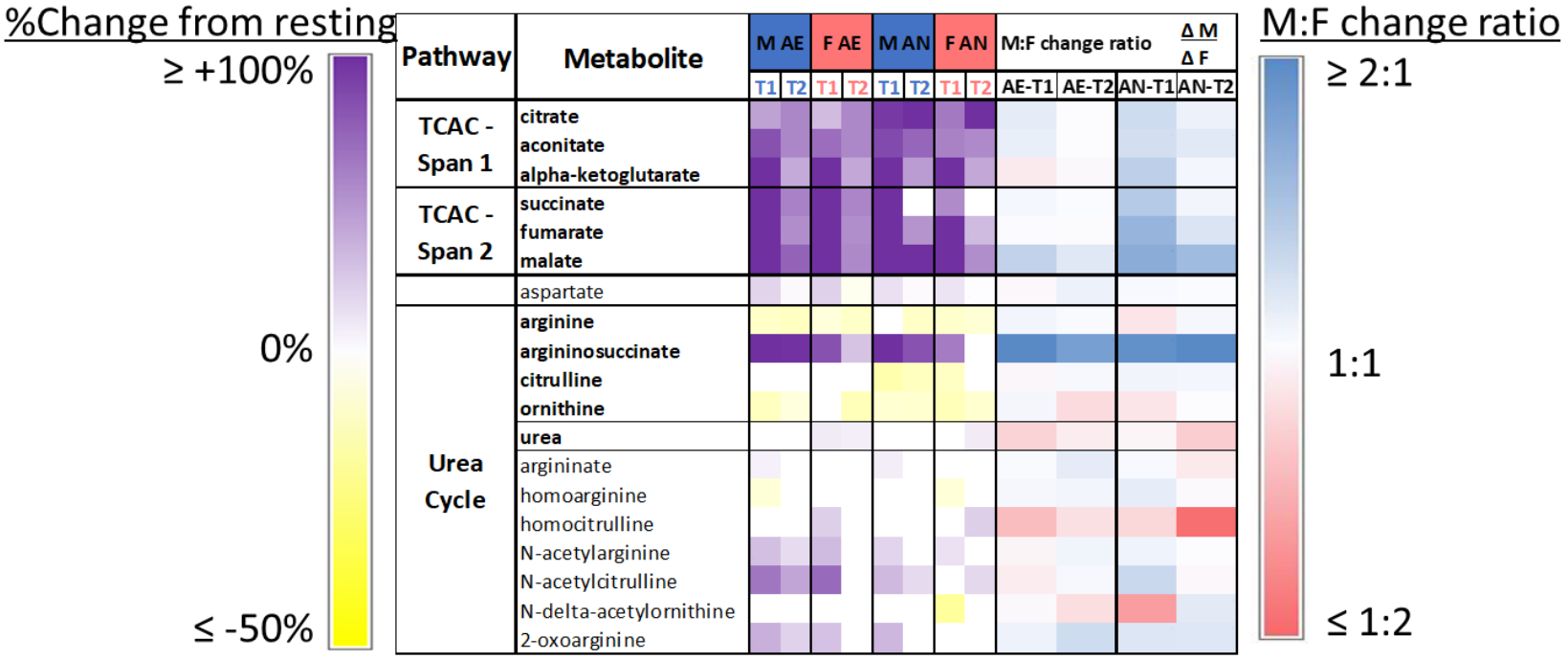


Figure 5. A) Compound heat map for TCAC and Urea Cycle metabolites. First 8 columns: shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest (T0) for the given group-exercise-time, respectively. White = no significant change from T0. Latter 4 columns use shades of blue and red to represent greater changes in M and F, respectively. Value determined as the ratio for the absolute value of percentage change values from resting level, $|\Delta M| : |\Delta F|$.
M= male; F= female; AE = aerobic exercise; AN = anaerobic exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercises; TCAC = Tricarboxylic Acid Cycle.

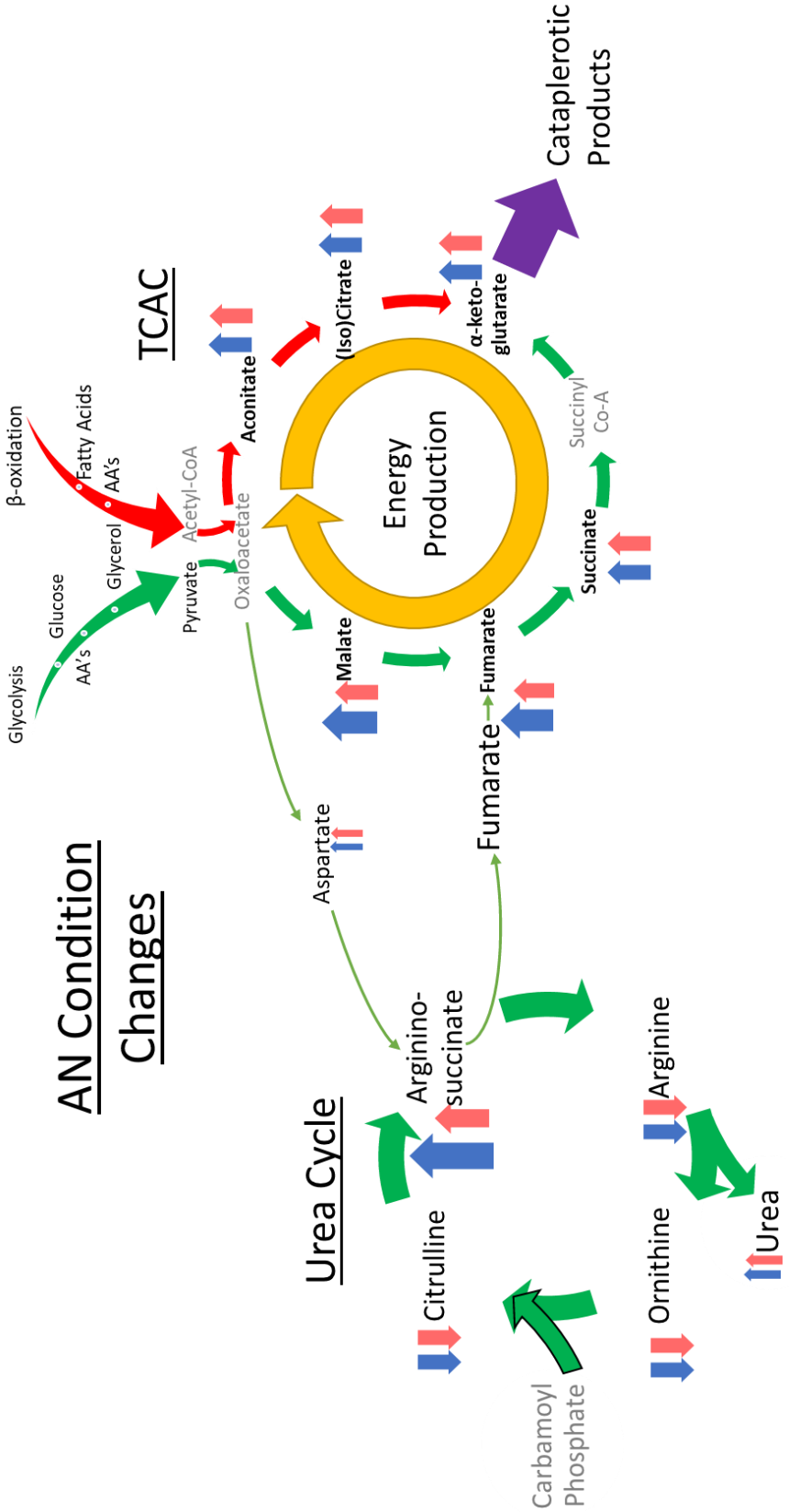


Figure 5. B) Diagram of tricarboxylic acid cycle (TCAC) and Urea Cycle, including aspartate shuttle. Blue (M) and pink (F) arrows for each metabolite depict relative size and direction of changes from rest to immediately post-exercise in response to anaerobic exercise (AN).

Span-2 Metabolism indicated by green arrow.

Figure 6.A) FA Metabolite Heat Map

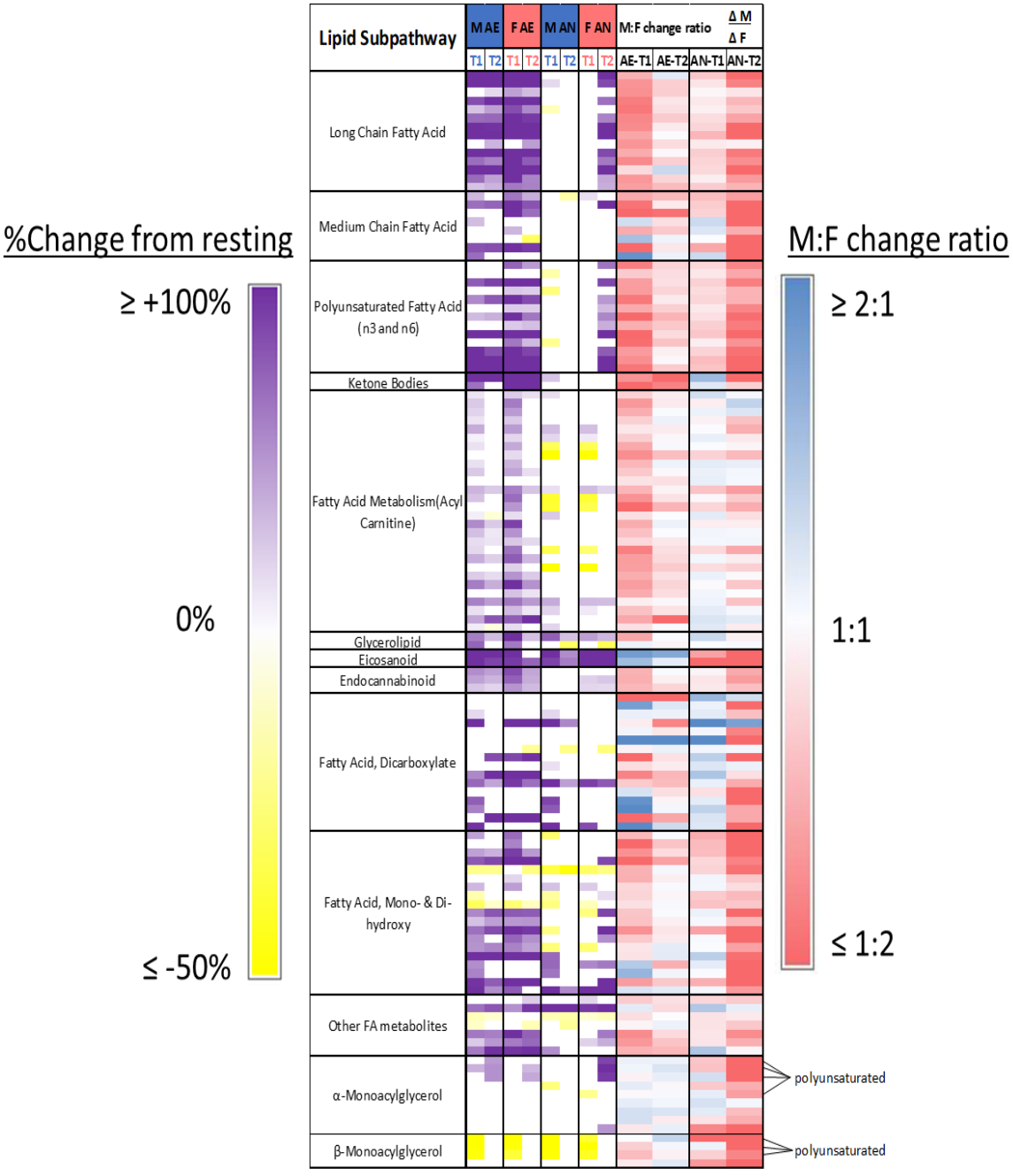
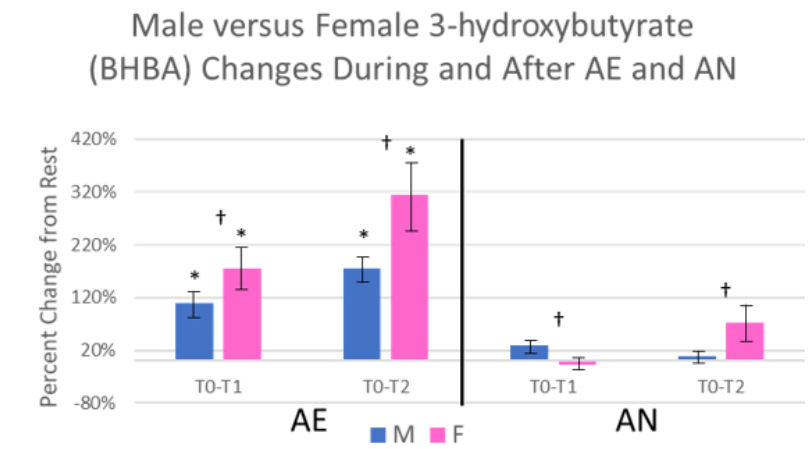


Figure 6. A) Compound heat map of lipid fatty acid metabolites. First 8 columns: shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest (T0) for the given group-exercise-time, respectively. White = no significant change from T0. Latter 4 columns use shades of blue and red to represent greater changes in M and F, respectively. Value determined as the ratio for the absolute value of percentage change values from resting level, $|\Delta M| : |\Delta F|$.
M= male; F= female; AE = aerobic exercise; AN = anaerobic exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercises; FA = fatty acid.

Figure 6.B) Ketone Body Metabolism



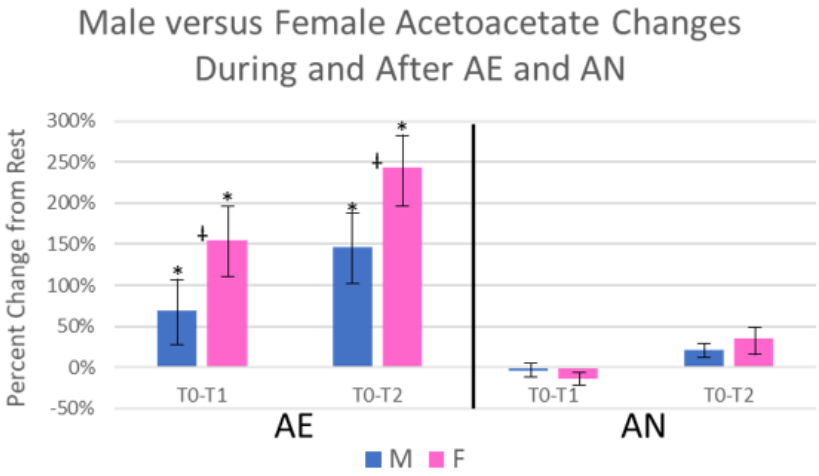
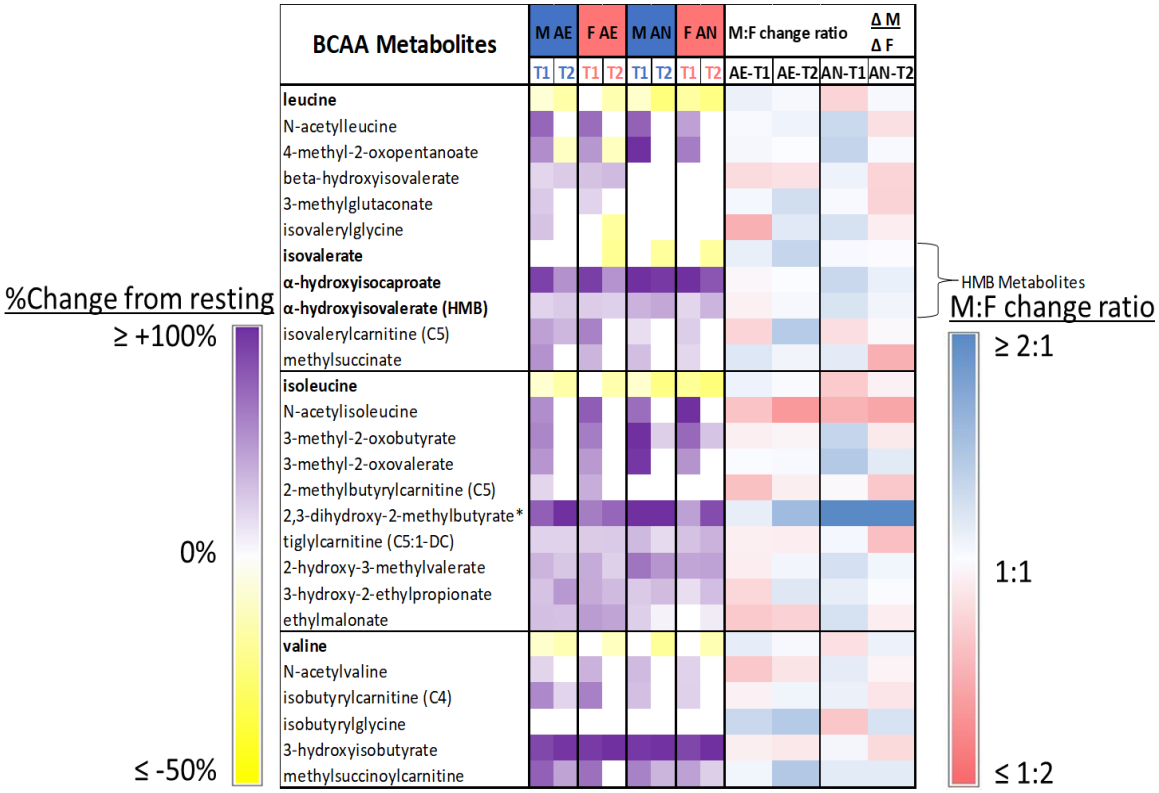


Figure 6.B). Changes in ketone bodies in response to the AE and AN. Values expressed as percentage change from baseline.
M = male; F = female; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise
* Significant difference from resting level
† Significant difference between sexes for relative % change

Figure 7. BCAA Metabolism

A



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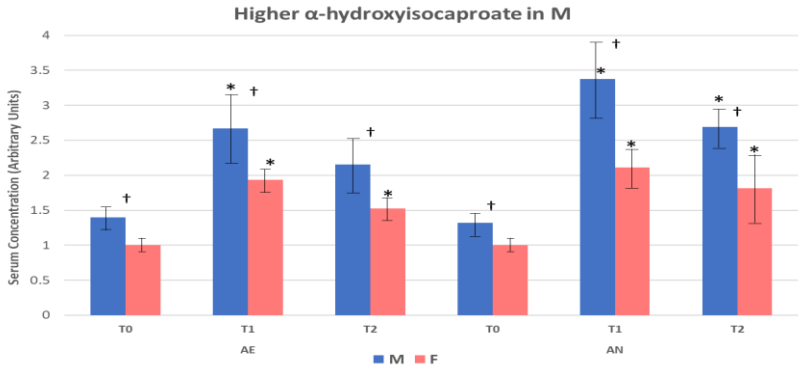


Figure 7. A) Compound heat map for BCAA metabolites. First 8 columns: shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest for the given group-exercise-time, respectively. White = no significant change from T0. Latter 4 columns use shades of blue and red to represent greater changes in M and F, respectively. Value determined as the ratio for the absolute value of percentage change values from resting level, |ΔM| : |ΔF|. Actual BCAA's and metabolites of HMB production are in **bold**.

* 2, 3-dihydroxy-2-methylbutyrate involved in all 3 BCAA catabolism pathways

Figure 7. B) Relative concentrations of α-hydroxyisocaproate in M & F for AE & AN at all times. M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise; BCAA = branched chain amino acid; HMB = hydroxy-methyl butyrate.

* Significant difference from rest

† Significant difference between sexes

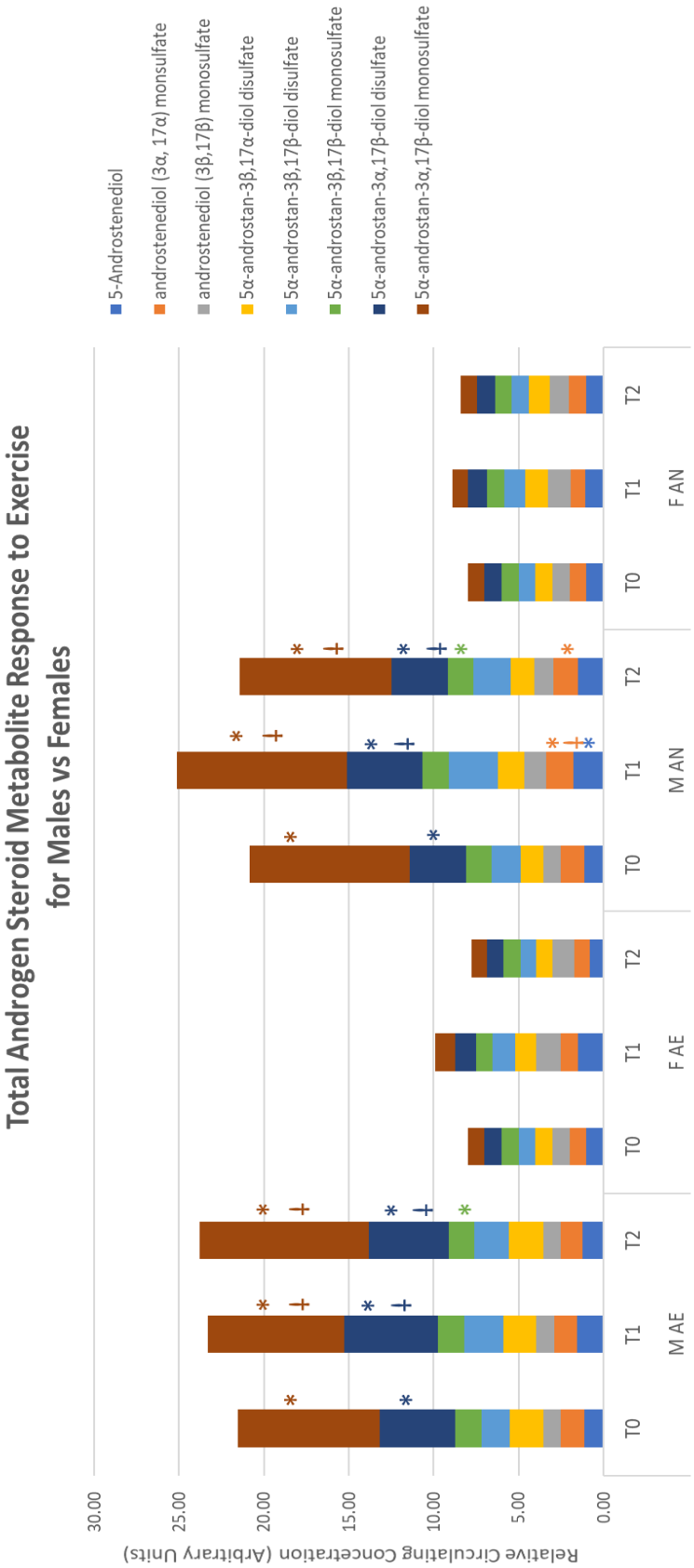
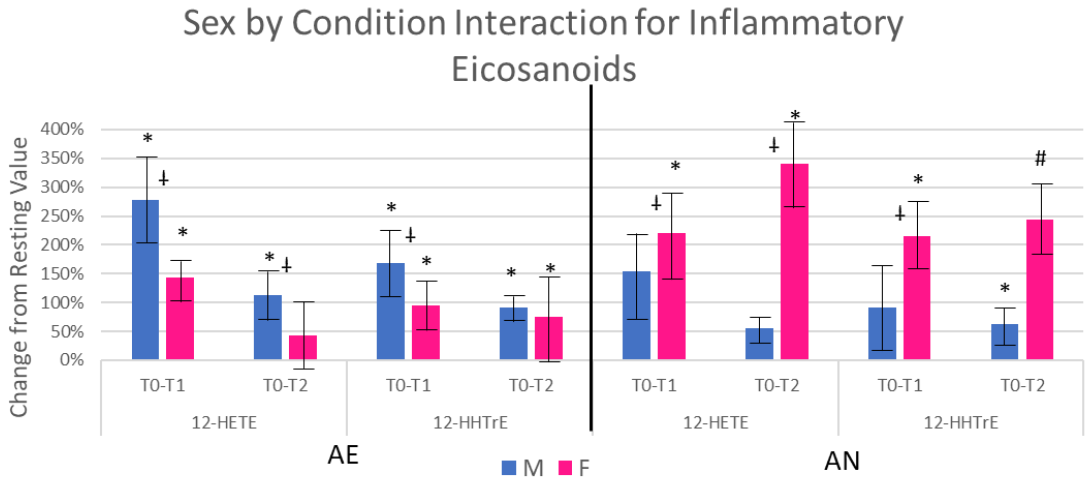


Figure 8. Androgen biosynthesis markers in response to exercise in M and F.
M = male cohort; F = female cohort; AE = aerobic exercise; AN = anaerobic exercise;
T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.
* Significantly difference between sexes for relative concentration
† Significantly difference between sexes for relative change from baseline

Figure 9, Inflammation

1. **Lipid Mediators**



2. **Cortisol**

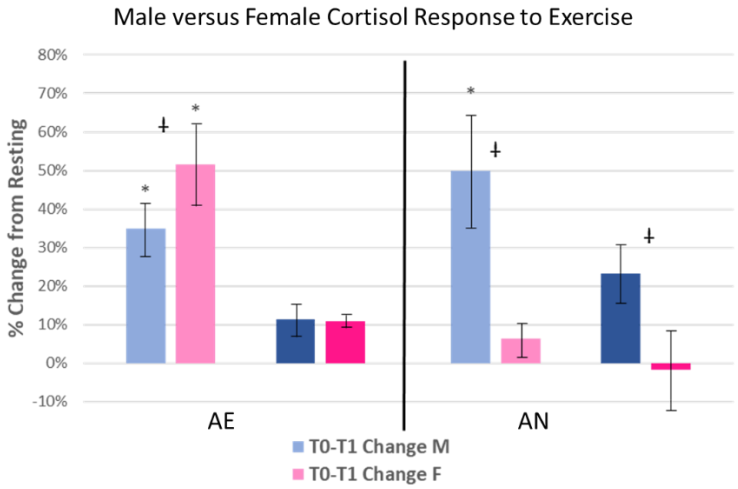
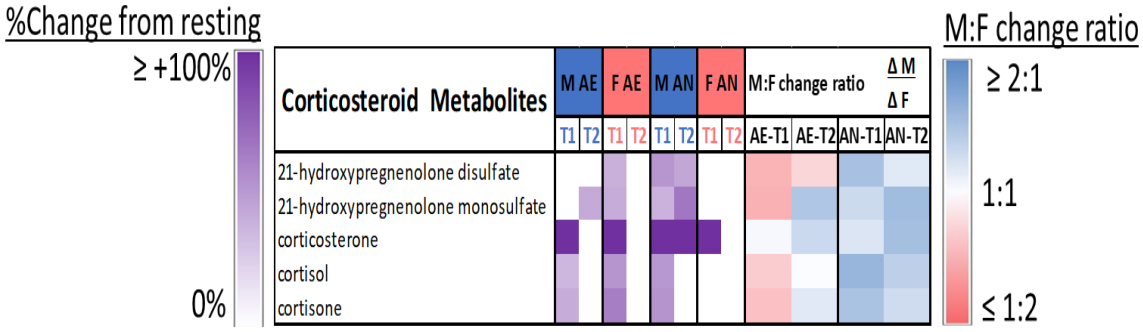


Figure 9. A) Sex differences for the changes in the inflammatory lipid mediators.
* Significant difference from resting value
† Significant difference between sexes for relative % change
# F AN 12-HHTrE near significantly elevated: T0-T2 value: p = 0.03, q = 0.11, and change for F near significantly > M change: p = 0.04, q = 0.13

Figure 9. B) Sex differences for the changes in cortisol.
M = male; F = female; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise.
* Significant difference from resting value
† Significant difference between sexes for relative % change

Figure 9.C) Glucocorticoids Heat Map



D

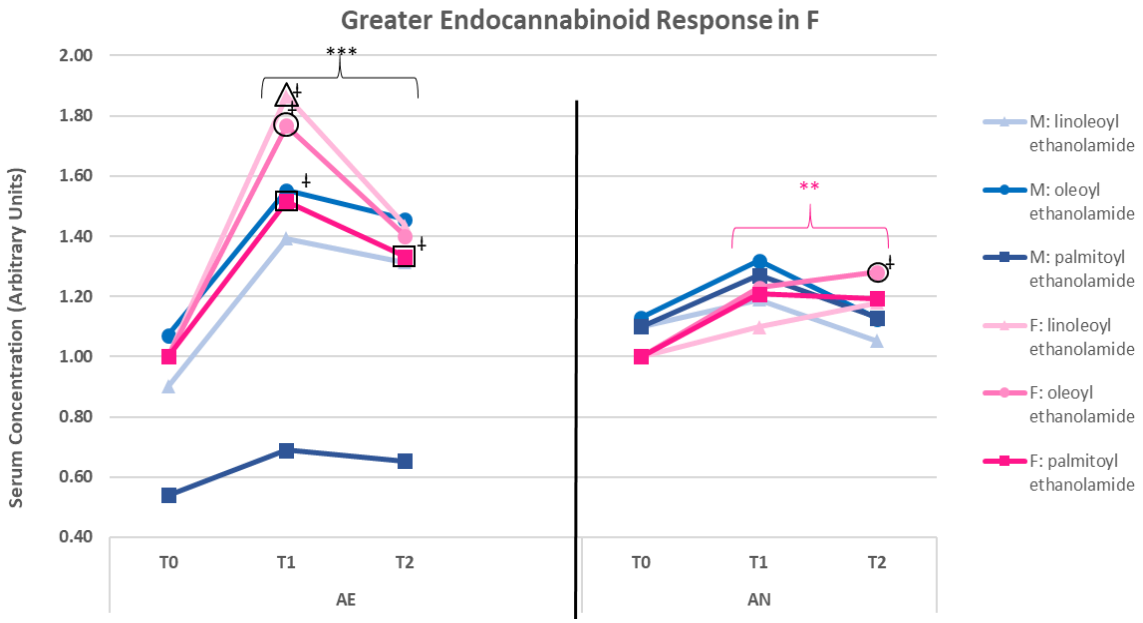


Figure 9. C) Compound heat map for corticosteroid metabolites. First 8 columns: shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest (T0) for the given group-exercise-time, respectively. White = no significant change from T0. Latter 4 columns use shades of blue and red to represent greater changes in M and F, respectively. Value determined as the ratio for the absolute value of percentage change values from resting level, |ΔM| : |ΔF|.

Figure 9.D) Endocannabinoid response to exercise. Line graph of time course for the relative circulating endocannabinoid levels from pre-to-60-minutes post-exercise.
M = male; F = female; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise; PEA = palmitoyl ethanolamide.
*** Significant increase for all 3 endocannabinoids at AE-T1 and AE-T2 for both M and F cohorts
** Significant increase above resting value in F cohort for linoleoyl ethanolamide and palmitoyl ethanolamide at AN-T1 and AN-T2
minutes post-exercise.
† Significant different between sexes for specified metabolite (all 3 endocannabinoids for F at AE-T1 and oleyl ethanolamide for F at AN-T2

Figure 10

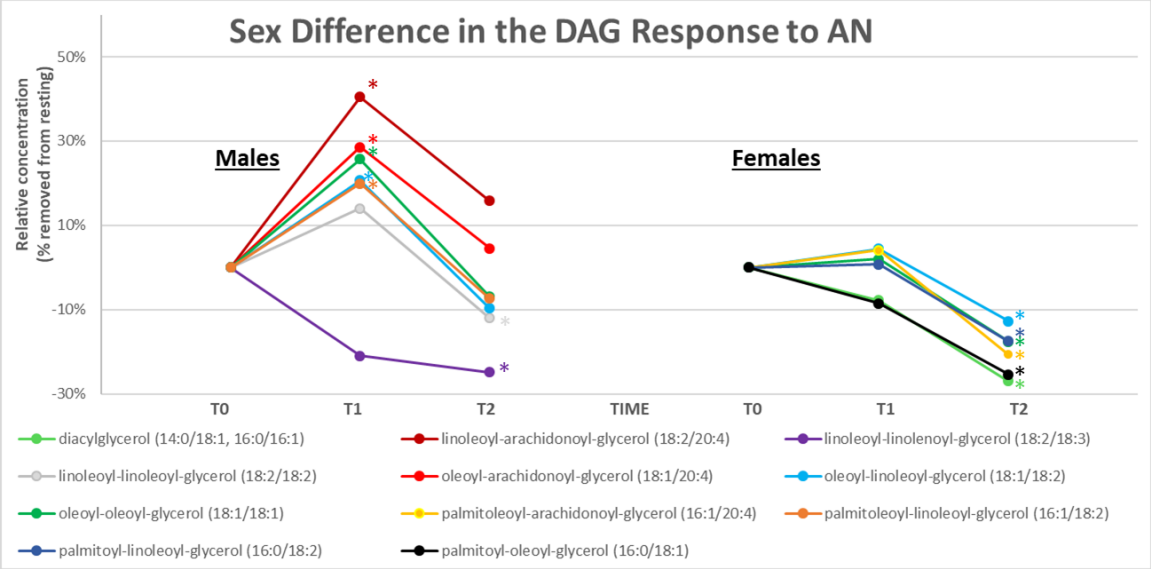


Figure 10. DAG response to AN. Line graph of time course of the relative circulating DAG levels from pre-to-60-minutes post-AN for the significantly altered DAG's.
M = male; F = female; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise; DAG = diacylglycerols.
* Significant difference from resting value

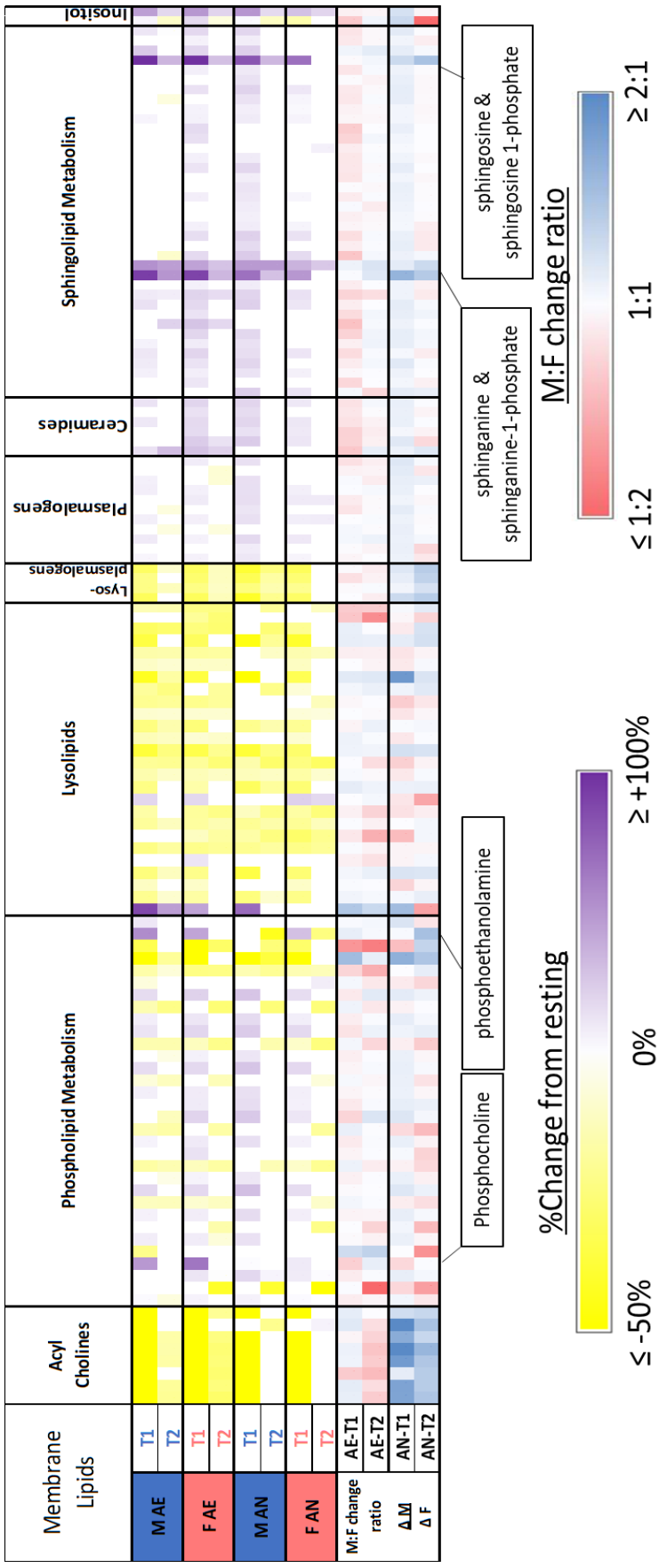
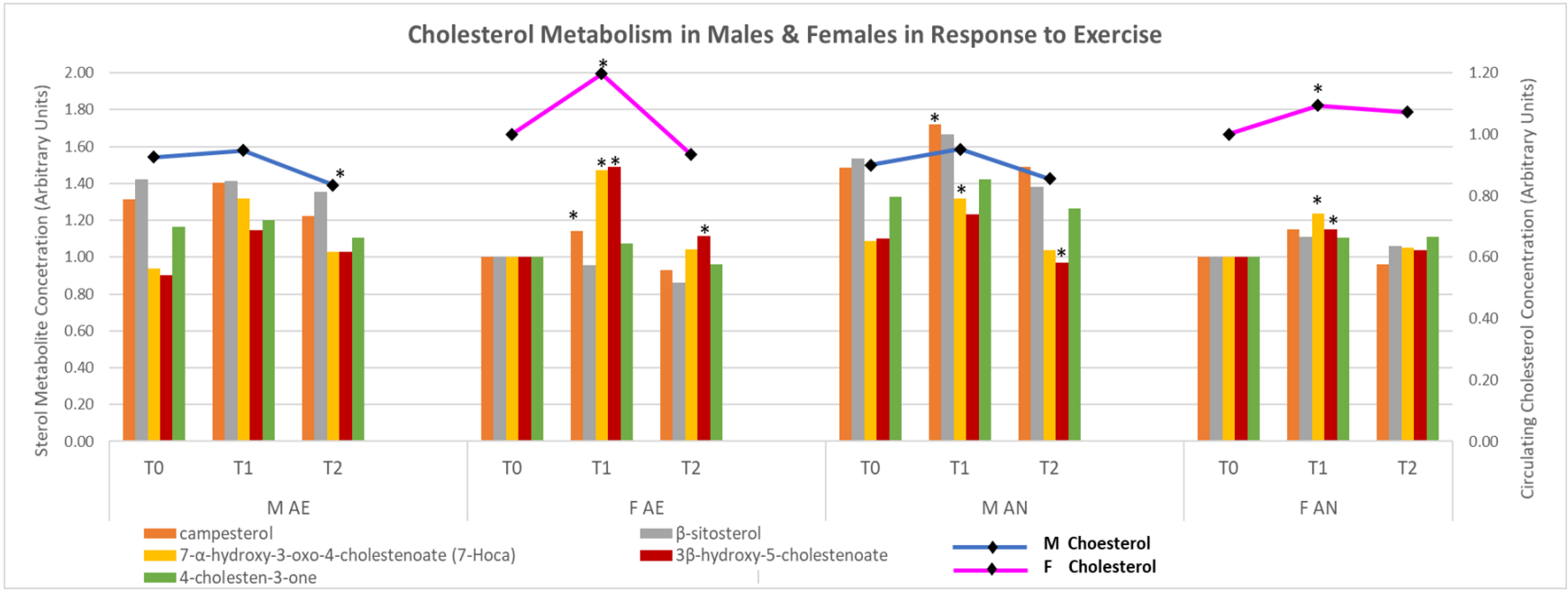


Figure 11.A) Compound heat map for the membrane lipid metabolites. First 8 columns: shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest (T0) for the given group-exercise-time, respectively. White = no significant change from T0. Latter 4 columns use shades of blue and red to represent greater changes in M and F, respectively. Value determined as the ratio for the absolute value of percentage change values from resting level, $|\Delta M| : |\Delta F|$. M= male; F= female; AE = aerobic exercise; AN = anaerobic exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercises; BCAA = branched chain amino acid; HMB = hydroxy-methyl butyrate.

Figure 11.B) Count of Lipid Membrane Metabolites to Differ by Sex

Mebrane Lipid Sex Differences		
Condition-Time	**M Only**	**F Only**
AE-T1	6 phospholipids 1 lysolipid 1 plasmalogen **8 TOTAL**	2 ceramides 3 lysolipid 5 phospholipids 2 plasmalogens 16 sphingolipids **28 TOTAL**
AE-T2	4 phospholipids 4 lysolipids 1 plasmalogen 1 sphingolipid **10 TOTAL**	1 acylcholine 5 phospholipids 2 lysolipids 2 lysoplasmalogens 1 plasmalogen 1 sphingolipid **12 TOTAL**
AN-T1	1 ceramide 1 lysolipid 4 phospholipids 20 sphingolipids **26 TOTAL**	8 lysolipids 3 phospholipids **11 TOTAL**
AN-T2	5 lysolipids 4 lysoplasmalogens 3 sphingolipids **12 TOTAL**	4 lysolipids 6 phospholipids 3 plasmalogens 1 sphingolipid **14 TOTAL**

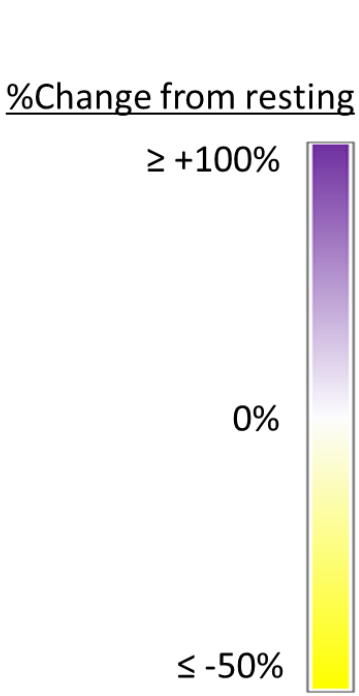
Figure 11.B) Table of significantly altered lipid membrane metabolite count organized by Kegg sub-pathway. Metabolites were counted when they were significantly changed from rest ($p < 0.05$, $q < 0.1$) in one sex only at each condition-time pairing. Total metabolomic perturbations were greater in F at AE-T1 and in M at AN-T1. There were 121 total metabolomic perturbations unique to 1 sex at AN- and AE-T1 and –T2 combined.
M= male; F= female; AE = aerobic exercise; AN = anaerobic exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercises.



Supplemental Figure 1. Cholesterol and related sterol metabolites in response to exercise. Cholesterol was increased immediately after both exercises in F only, but returned back to baseline in both instances. Cholesterol was significantly lower 60-mintues post-AE in M. The changes in cholesterol were never in excess of a 20% change from rest. Other sterol metabolites, including the phytosterol campesterol and bile acid synthesis metabolites 3β-hydroxy-5-cholestenoate and 7-Hoca, were altered with exercise as well, particularly for F in response to AE and M with AN.

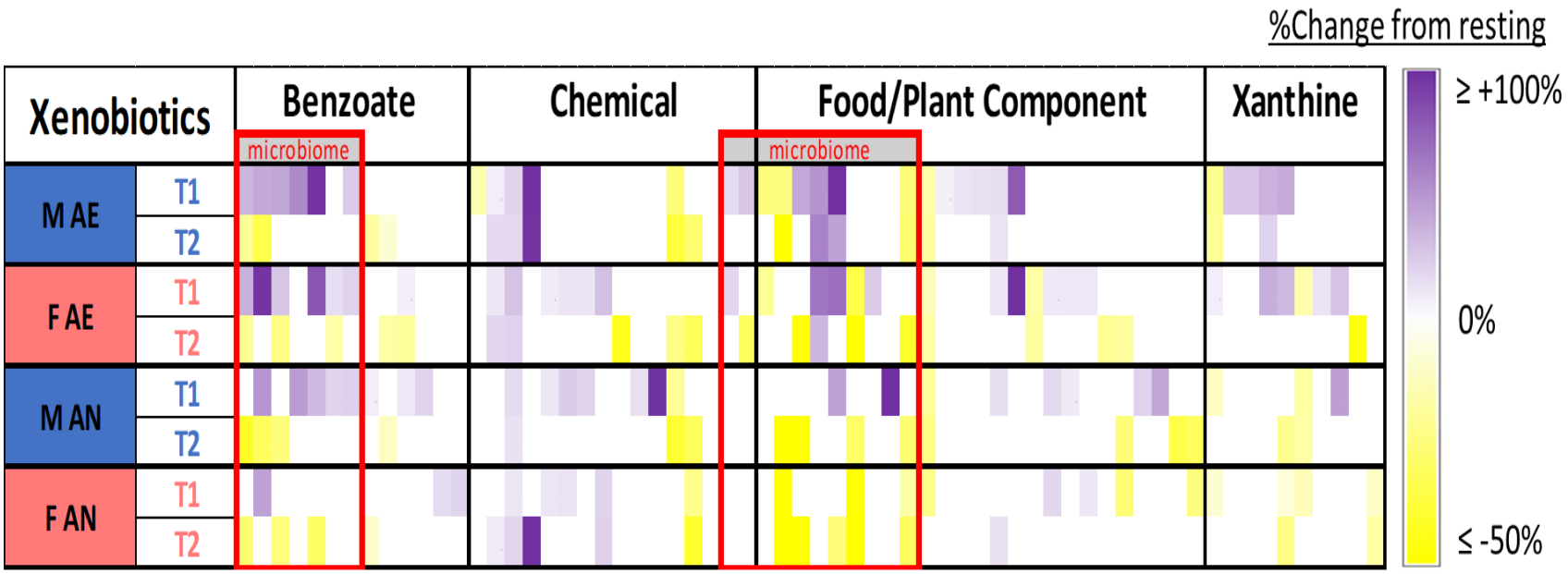
M = male; F = female; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise; 7-Hoca = 7-α-hydroxy-3-oxo-4-cholestenoate.

* Significant change from resting level



	Bile Acids	M AE		F AE		M AN		F AN		Difference in change from rest (MΔ - FΔ)			
		T1	T2	T1	T2	T1	T2	T1	T2	AE-T1	AE-T2	AN-T1	AN-T2
1°	chenodeoxycholate									10%	-7%	25%	14%
1°	cholate									6%	-17%	36%	-6%
1°	glycochenodeoxycholate									11%	-9%	16%	35%
1°	glycochenodeoxycholate glucuronide									10%	3%	-10%	13%
1°	glycochenodeoxycholate sulfate									10%	5%	-2%	10%
1°	glycocholate									14%	3%	9%	17%
1°	tauro-β-muricholate									-25%	-38%	-26%	-1%
1°	taurochenodeoxycholate									31%	-4%	7%	42%
1°	taurocholate									17%	8%	12%	34%
2°	3β-hydroxy-5-cholenoic acid									0%	-15%	3%	2%
2°	deoxycholate									1%	-5%	18%	9%
2°	glycocholenate sulfate									-33%	-11%	21%	11%
2°	glycodeoxycholate									6%	-11%	7%	10%
2°	glycodeoxycholate sulfate									2%	15%	-8%	2%
2°	glycohyocholate									9%	-9%	0%	17%
2°	glycolithocholate sulfate									5%	-2%	7%	16%
2°	glycoursodeoxycholate									8%	-15%	0%	26%
2°	hyocholate									22%	5%	16%	8%
2°	isoursodeoxycholate									17%	-19%	1%	-22%
2°	taurocholenate sulfate									-22%	-16%	48%	20%
2°	taurodeoxycholate									-8%	-6%	4%	18%
2°	taurolithocholate 3-sulfate									-5%	-8%	-25%	12%
2°	tauroursodeoxycholate									15%	-11%	-17%	35%
2°	ursodeoxycholate									-3%	-12%	11%	9%

Supplemental Figure 2. Compound heat map of the bile acids. First 8 columns: shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest (T0) for the given group-exercise-time, respectively. White = no significant change from T0. Latter 4 columns use shaded blue when M change was significantly larger than F change. Value determined as the difference between the absolute value of percentage change values from resting level, $|\Delta M| - |\Delta F|$. Bile acids tended to decrease in response to exercise with only 2 exceptions. They did not appear to differ by type (primary or secondary). There was a slight tendency for larger changes in M than F in response to AN scattered throughout the bile acids.
M = male; F = female; AE = aerobic exercise; AN = anaerobic exercise; T0 = pre-exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercise



Supplemental Figure 3. Compound heat map for Xenobiotic/Microbiome metabolites. Shades of purple (increase) and yellow (decrease) represent the fold change of each metabolite from rest (T0) for the given group-exercise-time, respectively. White = no significant change from T0. Metabolites known to link to the gut microbiome are outlined in red. The xenobiotics were largely unchanged with exercise, but changes were more common within those xenobiotics documented to change with the microbiome. M= male; F= female; AE = aerobic exercise; AN = anaerobic exercise; T1 = immediately post-exercise; T2 = 60-minutes post-exercises

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CONCLUSIONS

In the present study, exercise was explored using a comprehensive metabolomics panel. Specifically, serum metabolites were used to characterize the exercise response to two different acute bouts of exercise, one aerobic (AE), the other anaerobic (AN). Changes from resting values (T0) were calculated for the immediate (T1) and 60-minute post-exercise (T2) samplings. A targeted recruitment approach enabled comparisons between predominantly resistance-trained (RES) and endurance-trained (END) individuals to gain insight into the impact of training status on the metabolome pre- and post-exercise. Finally, male (M) and female (F) participants were included to allow for direct sex comparisons to be made. Many of the design parameters within this project are novel. This is the first explorative exercise metabolomics study to compare AE and AN, the first to consider training background in the exercise response, and the first to directly compare matched cohorts of M and F participants for their exercise response, let alone to interrelate all 3 of these. Findings reported expand on the current literature by offering a well-controlled (as evidenced by the low inter-day variability in resting metabolite levels) experimental design utilizing a comprehensive metabolomic panel (754 known metabolites) in a cohort of 40 healthy, active individuals.

FINDINGS SUMMARY

Initially, the primary aim of this investigation was to characterize the response to exercise, but the overwhelming similarity seen between groups (END and RES as well as M and F) at rest represented a major finding. Resting metabolomes between groups revealed differences in less than 4% of the measured metabolites by sex and less than 2% by training background. Notably, this was not due to a large heterogeneity within groups, but rather very similar resting values between groups. An extreme example of this could be seen when separated by training background within the F participants. F groups of END-F and RES-F did not differ for a single

metabolite at rest. It is thought that regular participation in physical activity by our participants led to a more homogeneous metabolome. This effect has been reported previously.[192] That the impact does not seem to vary based on exercise regimen, however, is novel. Importantly, these were free-training humans, meaning they all had entirely unique and disparate physical activity patterns, making this finding even more striking. Further differences were minimal across sexes as well. We believe the resting metabolome observed herein epitomizes the healthy condition and could potentially serve as a basis for comparison in future studies.

While the variance in training history may weaken the END/RES comparisons, it can also be interpreted to indicate a robustness in the effects of exercise across sex, especially in light of the similarity noted between M and F groups. It is human nature to gravitate towards differences rather than similarities when making a comparison, but the number of metabolites that were indistinguishable between groups in their exercise response was extremely low, especially when considering the volume or perturbations indicted by exercise. Both relative concentrations post-exercise and magnitudes of change from rest at T1 and T2 were also minimal. However, with regard to the similarities at rest, perhaps this is not surprising. Regardless, the observed exercise response likely exemplifies a normal response to both AE and AN for healthy, active young males and females.

Interestingly, and in a similar vein, the responses to AE and AN were also rather similar to each other. Both bouts increased energy dynamics, substrate metabolism, inflammation and antioxidant activity. Amongst these, purine salvage and TCAC metabolites included the biggest relative magnitudes of change for both exercises (several fold-change on average). Differences observed in substrate metabolism during exercise were mostly expected. FA metabolism was seen during AE, but not during AN, ketone metabolism was increased for AE only, glycolysis was higher with AN than AE, and there were greater BCAA perturbations with AN.

Though FA metabolism remained elevated post-AE, it climbed significantly post-AN, while metabolites of the other macronutrients steadily declined post-exercise. It seemed that regardless of the preceding exercise mode, once the stress of exercise is removed, most energy is derived through β-oxidation of FAs. Overall, pathways displaying the most potent responses were in agreement with what has been previously published in the literature.[181] However, the panel was able to identify novel metabolites and/or unanticipated changes to known metabolites within these pathways. Trans-urconate and sphingosine-1-phosphate may indicate changes in immune trafficking, and endocannabinoid changes following AE were unexpected given the relatively short duration of exercise. Still, outside of substrate metabolism, rather little differed between conditions.

The time-course of changes matched previous reports.[181] T1 saw the largest quantity and magnitude of change. Partial reversion towards the resting state was seen at T2. This is a general observation, and specific metabolites revealed unique and unexpected response patterns. For example, the methylated nucleosides, and 3-methyl-cytidine, in particular were elevated at T1 and T2 following AE and at T2 following AN. This was unforeseen, as these markers are indicative of increased tRNA breakdown, and epigenetic changes resulting in up-regulated translation typically take longer than this to occur, possibly indicating some other physiological phenomenon.

The sheer volume of metabolites significantly altered by exercise using $p < 0.05$ and FDR $q < 0.1$ stringency criteria was a remarkable 580 and 485 perturbed metabolites at AE-T1 and AN-T1, respectively. Intriguingly, applying a magnitude filter of just 2-fold reduced these numbers by nearly 90%. Both exercise protocols employed here were thought to be adequately rigorous, as duration and intensity prescriptions for AE and AN were on par or above what has been used elsewhere. Yet, many other studies have reported higher fold-changes in response to acute

exercise than were seen here. An exception to this was the purine metabolites, hypoxanthine and xanthosine, which indicate pressure on energy turnover. It is posited that due to the arduous nature of our acute exercise stress and the ability of trained individuals to push themselves, large responses in these sensitive metabolites[225] were seen. The 10- to 20-fold changes in our cohort are amongst the highest ever reported. Using trained individuals could be thought to reduce the impact of exercise, but this was not the case observed here or in pervious metabolomics reports.[188,189,192] Indeed, the literature indicates that sedentary and diseased individuals have blunted responses when compared to active or healthy individuals. Further, given our implementation of a matched relative workload, the individuals' training status allowed them to work at a concomitant absolute intensity, giving higher total workloads for "better-trained" individuals for each work bout. In turn, we observed bigger responses in the more aptly trained groups (END with AE and RES with AN).

Perhaps related to this, RES did appear to recover from AN better than AE, and END from AE better than AN, indicating familiarity with an exercise stressor better equips the individual to recover from that bout. When analyzed between groups, END displayed a more complete recovery of the metabolome by T2 than did RES for both exercises. This synchs with our observations of elevated FA metabolism post-exercise across groups and comparatively prolonged energy disruption (distressed mitochondrial activity and continued anaerobiosis) in RES. END are by definition better aerobically-trained individuals than RES, and so may be better set up to engage in aerobic metabolism to recover from exercise, particularly in terms of restoring energy balance.

In addition, and related to recovery rate, specific training background-dependent differences included larger ketone and FA metabolism in END during, but not after exercise and higher anabolic status for RES, particularly following AN, as well as greater glycolysis, purine

metabolism and inflammation in RES. Interestingly when differences were found between training groups, RES almost always had larger perturbations than END. Perhaps muscle mass is a driving force (higher in RES), as the same can be said of the sex comparison-when observed, changes were usually bigger in M than F (FFM: M > F). However, due to a relatively small sample and fairly homogenous values, with all participants being fit, correlations run between FFM and metabolite levels/changes did not provide any insight to this relationship. Overall, groups were more alike than different, and dissimilarities observed may have stemmed from higher absolute workloads in the more aptly trained group for each bout (END with AE, RES with AN).

On that note, it is teleologically impossible to match both absolute and relative intensity in individuals of different fitness. Matching by relative intensity was preferred because, individuals with a training bias (END or RES) made matching by absolute intensity impractical. Doing so would have resulted in either inordinately short AE or extremely long AN sessions, which are in contrast to the nature of AE and AN and would have confounded blood collection times. Moreover, if matched by workload, the AE and AN bouts would have been very different in perceived difficulty, much easier AE and more arduous for AN, biasing results. The workout routines chosen were done so with translation of findings in mind. Forty-five minutes is a reasonable estimate of a typical workout duration for AE or AN, and the lifting protocol and steady-state ride used were similar to those an avid exerciser might do on any given day. Regardless, using unmatched total workload and absolute intensity between groups, within the same session may have acted to influence results.

Finally, sex differences in the exercise response were largely in or derived from substrate selection. F relied more heavily on FA as a fuel source, and this impacted observed metabolism of essential FAs and downstream lipid mediators. Interestingly there was a sex by mode by time interaction, as PUFAs and lipid mediators were higher for F than M following AN, particularly at

AN-T2 but higher for M than F following AE, suggesting the possibility of differential regulation of PUFA metabolism according to sex by mode interactions. Inflammatory status followed a similar pattern, with greater pro-inflammatory status in M post-AE and in F post-AN. Perhaps as a cause or result of the lower FA use in M, there were further elevations in metabolites of glycolysis, BCAA metabolism, purine salvage and span-2 TCAC intermediates plus argininosuccinate in M than F. Downstream of the BCAAs, greater anti-catabolic leucine metabolites indicated heightened anabolic signaling in M compared to F, particularly post-AN. Elevated androgenic steroid biosynthesis markers in M likely impact this. Both the leucine and steroid markers were higher in M at all times, indicating a robust sex differences at rest and in the context of the exercise response.

Exercise presented a pervasive disruptor to the resting metabolome, as has been documented previously.[200,204] Resting metabolomes revealed near identical status across training background and sex, implying perhaps a universal “healthy metabolome” for participants in this study. Responses to exercise were fairly consistent across groups too, though pathway activation and specific metabolites were seen to differ post-exercise. Similarly, AE and AN were mostly alike by direction of changes wrought. Comparisons revealed differences in magnitude of change for a subset of metabolites, but never revealed an increase in response to one exercise or group and a decrease from the other exercise or in the other group. Prior to exercise, resting metabolomes looked identical between days and extraordinarily similar between groups. Dichotomously, both exercise modes brought about massive changes to the metabolome, but, they largely did not differ from one another. Perhaps due to the far-reaching effects each exercise had, there was a large degree of overlap by default. Similarly, the stress of exercise was required to reveal differences in training background, or sex, but the differences were largely overshadowed by the potent and pervasive stress of the exercise bout itself.

LIMITATIONJS AND DESIGN CONSIDERATION

Having overlapping groups for the END-RES and M-F comparisons meant there was some element of redundancy in data generation and statistical analysis. When this redundancy was eliminated by examining 4 different "sub-groups" of 10 individuals performing 2 different conditions each, metabolomic signatures and shifts brought on by exercise appeared representative of the more inclusive groups used for analysis. Moreover, training- and sex-dependent observations were largely maintained. Although power analyses were not possible to conduct for this design,[226,227] reduction in group size to n = 10 likely reduces power substantially.

Use of serum as a medium has both advantages and disadvantages. Blood flows extensively through all tissues and therefore represents an ideal medium for capturing a systemic response. Unfortunately, this same feature also makes the source of any observed metabolite changes difficult or impossible to detect. Because the majority of blood flow during exercise is to the active muscle, serum markers are often taken to reflect metabolic activity of this tissue during exercise. However, metabolomics has been used to demonstrate significant hepatic uptake and release of various metabolites into the circulation during exercise.[228] Ideally verification by simultaneous sampling of suspected tissue or venous and arterial collection around that tissue could be carried out to better ascertain specific sites of the identified metabolic activity.

A cohort of 40 is relatively large for a cross-over design using metabolomics, however; any findings and conjectures presented here must be tempered by the knowledge that this is orders of magnitude below that needed to generalize findings to a population.

Scaling of raw data was implemented here to report fold-change values. The common practice of scaling data makes inter-metabolite contrasts easier in terms of the scalar component, but more difficult in interpreting meaning. Actual metabolites concentrations and

absolute magnitudes of change were unknown. Thus, values reported may or may not have fallen within a clinically relevant range.

FUTURE DIRECTIONS

General. Further validation of the healthy metabolome purported herein is required. Ideally this would be done using actual, rather than relative metabolite levels. Additionally, use of multiple sampling mediums could provide insight into tissue specific metabolism (muscle). Though metabolomics is already widely used in clinical settings to characterize disease states, findings herein provide a basis for comparison with a healthy cohort and their response to different exercises. Long-term impacts of exercise are rarely studied, and attempts to track training responses over time should be made.

Not surprisingly, differences in exercise mode were largely recapitulated by those between training background. Also as expected, exercise modality was most impactful in determining the acute response. Furthermore, it appeared that while M and F differed more at rest than did END and RES, training background trumped sex as a driver of the exercise response. This highlights the power of exercise both as an acute stressor and in the capacity for long-term training to modify metabolic response systems.

Both F and RES and/or AN are largely understudied in the exercise metabolomics literature. Efforts to include F, RES and AN need to be made. Moreover, comparisons of M and F metabolomes in response to exercise are lacking. There is difficulty in cross-evaluation of results from different metabolomics studies. Examination of F along with M, RES with END, or AN with AE, as was done here, provides a direct basis of comparison for quantification. This context gives a frame of reference to findings for these otherwise under-characterized entities. Rather than study them in isolation, future studies should aim to include similar aspects to their designs to help contextualize findings.

Specific. This project revealed AE and AN to extensively activate nearly every biochemical (Kegg) pathway represented on our profiling panel. Roughly 100 sub-pathways contained significant perturbations to multiple metabolites in response to exercise. Though an extensive response to exercise was anticipated this was more prolific than predicted. Follow-up investigations on any of these myriad changes is justified, but should primarily focus on pathways that were identified to differ between groups or conditions and those that displayed the largest magnitudes of change and/or most pervasive disruptions. The largest magnitudes of change were in purine salvage markers indicative of ATP-turnover (xanthosine, hypoxanthine), lactate, the acyl cholines and TCAC intermediates. Future studies could follow a discovery science model. The acyl cholines present an opportunity for expansion, as no studies have reported on acute effects of exercise on acyl choline levels in the serum. In contrast, study of established pathways is warranted. For example, findings revealed intricacies in anaplerotic/catplerotic activity of the TCAC to differentiate between exercise condition and training background. This requires further investigation, preferably with muscle tissue sampling. The most pervasive disruptions were seen throughout substrate metabolism, inflammation and oxidative stress, but less-established pathways demonstrated widespread and or large perturbations in response to exercise as well (bile acid metabolism and microbiome metabolites). Further characterization and mechanistic studies are both needed in such areas.

Linking of metabolite response patterns across pathways also serves as a potential driver for future direction. As an example, parallel observations were made for specific androgenic steroid markers and leucine metabolites with anabolic signaling potential. These same biosynthesis steroids were also able to differentiate between M and F. Investigations into these relationships are needed to elucidate underlying mechanisms as well as impact of and interactions between

sex and exercise. Due to the extent of metabolic perturbations elicited by exercise, expansion on findings from this platform are virtually limitless.

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