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The Effects of Quercetin and Citrulline on Nitric Oxide Metabolite Production and Cycling Performance

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ACCEPTANCE

This dissertation, THE EFFECT OF QUERCETIN AND CITRULLINE ON NITRIC OXIDE METABOLITE PRODUCTION AND CYCLING PERFORMANCE, by JENNIFER A. KURTZ, was prepared under the direction of the candidate's Dissertation Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree, Doctor of Philosophy, in the College of Education & Human Development, Georgia State University.

The Dissertation Advisory Committee and the student's Department Chairperson, as representatives of the faculty, certify that this dissertation has met all standards of excellence and scholarship as determined by the faculty.

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THE EFFECTS OF QUERCETIN AND CITRULLINE ON NITRIC OXIDE METABOLITE PRODUCTION AND CYCLING PERFORMANCE

by

JENNIFER A. KURTZ

Under the direction of Jeffrey S. Otis, Ph.D.

ABSTRACT

There is a growing interest in the use of nutrition and dietary supplements to optimize training and time-trial (TT) cycling performance. Separately, quercetin (Q) and citrulline (CIT) have been used as ergogenic aids to improve oxygen (VO_2) kinetics, perceived effort, and cycling TT performance. However, it is currently unknown whether the combination of Q and CIT can provide additive benefits and further enhance cycling performance. We examined 28-days of Q+CIT supplementation on nitric oxide metabolite production (NO) and several physiological measures relevant to time trial (TT) cycling performance. Forty-eight highly trained cyclists were assigned to one of four supplementation groups: (1) Q + CIT (Q: 500 mg, CIT: 3.0 g), (2) Q (500 mg), (3) CIT (3.0 g), or (4) placebo (3.5 g of a zero-calorie flavored crystal light package). Supplements were dissolved in 16 oz. of water and consumed two times per day for 28 consecutive days. Participants performed a 20-km cycling TT race, pre- and post-supplementation to determine the impact of the combined effects of Q + CIT. There were no potential benefits of Q+CIT supplementation on TT performance, NO metabolite production, and several measures of physiological performance. Q+CIT does not seem to be beneficial for 20-km TT performance; further exploration with a focus on an increase in cycling duration or Q+CIT combined with additional polyphenols may amplify any perceived bioactive or metabolic effects on cycling performance. Until such studies are completed, the efficacy of Q+CIT supplementation to improve cycling performance remains ambiguous.

INDEX WORDS: quercetin, citrulline, cycling, nitric oxide metabolite production, oxygen uptake

THE EFFECTS OF QUERCETIN AND CITRULLINE ON NITRIC OXIDE METABOLITE PRODUCTION AND CYCLING PERFORMANCE

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JENNIFER A. KURTZ

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DEDICATION

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ABBREVIATIONS

ADP	Adenosine diphosphate
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	5’ adenosine monophosphate-activated protein kinase
AP1	Activator protein 1
AT	Adipose Tissue
ATP	Adenosine triphosphate
AS160	Akt substrate of 160 kDa
BCAA	Branched chain amino acids
Ca^{2+}	Calcium
CaMK	Calmodulin-dependent protein kinase
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
CIT or L-Citrulline	Citrulline
COX	Cyclooxygenase
Cr	Creatine
CrK	Creatine kinase
CrP	Creatine phosphate
CRP	C-reactive protein
DHA	Docosahexaenoic Acid
DSHEA	Dietary Supplement Health and Education Act
EAA	Essential amino acids
EGCG	Epigallocatechin 3-gallate
EIF4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ELK-1	ETS Transcription Factor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
ERRα	Estrogen-related receptor
FAD	Flavin adenine dinucleotide
FADH	Flavin adenine dinucleotide + hydrogren
FDA	U.S. Food and Drug Administration
FMD	Flow Mediated Dilation
fTRYP	Plasma BCAA: free tryptophan
GAP	Rab-GTPase-activating proteins
GI distress	Gastrointestinal distress
GLUT4	Glucose transporter 4
G_6P	Glucose-6-phosphate
GPx	Glutathione peroxidase
GSH	Glutathione
GSS	Glutathione synthetase
HDL	High-density lipoproteins
HR	Heart rate
ICAM	Intercellular adhesion molecule
ICC	Intraclass correlation coefficient

IKK	I kappa B Kinase
IKK-β	Inhibitor of nuclear factor kappa B kinase subunit beta
IL-	Interleukin
IMP	Inosine phosphate
iNOS	Inducible nitric oxide synthase
JNK1/2	C-Jun N-terminal Kinase
Keap1	Kelch-like ECH-associated protein 1
LDL	low-density lipoproteins
LDLR	LDL receptor
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MEF	Myocyte enhancer factor
MHC	Myosin heavy chain
MPS	Muscle protein synthesis
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NH_4^+	Ammonia
NFkB	Nuclear factor-kappa B
nNOS	Neuronal NOS
NO	Nitric Oxide
NOX	Plasma nitrite/nitrate
NO_2	Nitrite
NO_3	Nitrate
NRF1/2	Nuclear respiratory factors 1& 2
p-CREB	cyclic-AMP response element-binding protein
PGC1α	Proliferator-activated receptor-gamma coactivator 1-alpha protein
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PL	Placebo
PMA	Phorbol 12-myristate 13-acetate
P/O	Oxidative phosphorylation ratio
PPAR	Peroxisome proliferator-activated receptor
P38/MAPK	P38 mitogen-activated protein kinase
P70SK6k	Ribosomal protein S6 kinase beta-1
RE1B	NKkB, subclass
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
RyR	Ryanodine receptor
SAPK	Stress-activated protein kinase
SIRT1	Silent information Regulator
SOD	Superoxide dismutase

SOD-1	Superoxide dismutase-1
SR	Sarcoplasmic reticulum
STAT1	Signal transducer and activator of transcription 1
TBC1D4	TBC1 domain family member 4
TFAM	Mitochondrial transcription factor A
TGF-α	Tumor growth factor-alpha
TID	Training intensity distribution
TNF	Tumor necrosis factor
TRAF3	TNF Receptor Associated Factor 3
Q	Quercetin
QQ	Quercetin–quinone
UCP1	Uncoupling protein 1
VO_2	Oxygen consumption
VO_{2max}	Maximal oxygen consumption
WHO	World Health Organization

CHAPTER ONE
THE EFFECTS OF QUERCETIN AND CITRULLINE ON NITRIC OXIDE METABOLITE PRODUCTION AND CYCLING PERFORMANCE

Introduction

Time trials (TT) are gaining interest in competitions such as the summer Olympics and require a combination of technical, tactical, and power output demands. During TTs, the cyclist most often races individually and attempts to achieve the shortest possible time to cover a fixed distance [313]. TTs consist of varying power output, sprints, hills, and tight and technical corners that result in multiple up and down hill segments [273] (Figure 1), often close to and above maximal anaerobic and aerobic power. Power output is a standard performance measure of cycling performance for the purpose of aerobic capacity and training prescription [219, 264, 397]. Power output fluctuation can depend on multiple factors, such as the type of terrain, environmental conditions, or physiological and anthropometrical characteristics of the cyclists [259, 313]. The ability to achieve a high power output is indicative of an increased capacity for the activation of motor units and fast-oxidative energy-producing efficiency leading to an increased EMG, indicative of higher skeletal muscle activation and force-producing capabilities [391]. Additionally, TTs are implemented to predict cycling performance [313]. Cycling TT events range from 2 min to >6 hours depending on the discipline [30]. The minimum distance for a trial is generally 10 miles but most races are fixed at distances (10, 25, 50, 100 miles) or a fixed time (12 and 24 hours) [389]. To avoid fatigue, cyclists often pace themselves by choosing their own power output, speed, or energy expenditure across an event. However, power output is shown to progressively decrease from the start to the end of the trial [391]. For example, in an hour TT, professional cyclists ride on average at approximately 5.4 watts (W) per kilogram (kg) and during Iron man

competitions, cyclists ride at an average of 3.8 W/kg [250]. There are few little reports on the actual intensity of road or mountain bike time TTs [313]. However, TTs are often raced at 90-95% of maximum heart rate (HR_{max}) [313].

In cycling, the aerobic-power-producing capacity is associated with quantifying elements of cycling-specific performance and training adaptations. Typically, the signature of the cyclists' physical ability is based on a hyperbolic relationship between the record of power output over different durations (1 s to 4 hours) (Figure 2) and is characteristically used to compare data between different classes of riders, levels, establish training loads and zones and track fatigue [243, 356]. The aerobic-power sustainability profile of riders who specialize in TTs can be matched with data obtained through the power analysis of different kinds of races, such as TT races, grand tour stages, or even cycling sportive events [356]. Yet, predicting cycling performance from power profiling still requires scientific validations before any potential findings can be applied to training settings.

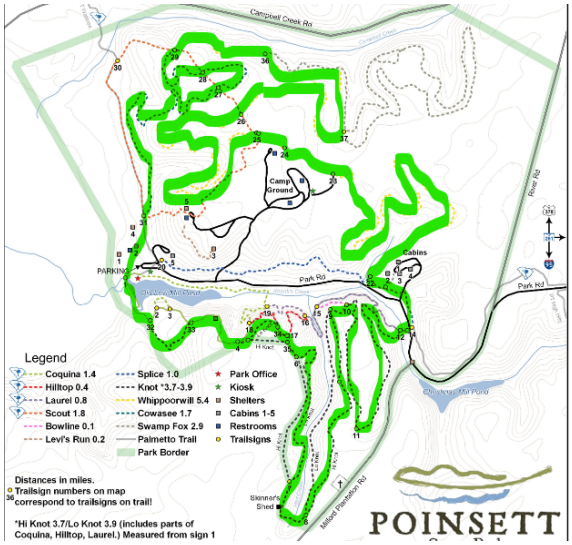

Figure 1. Example of a time-trial road map.

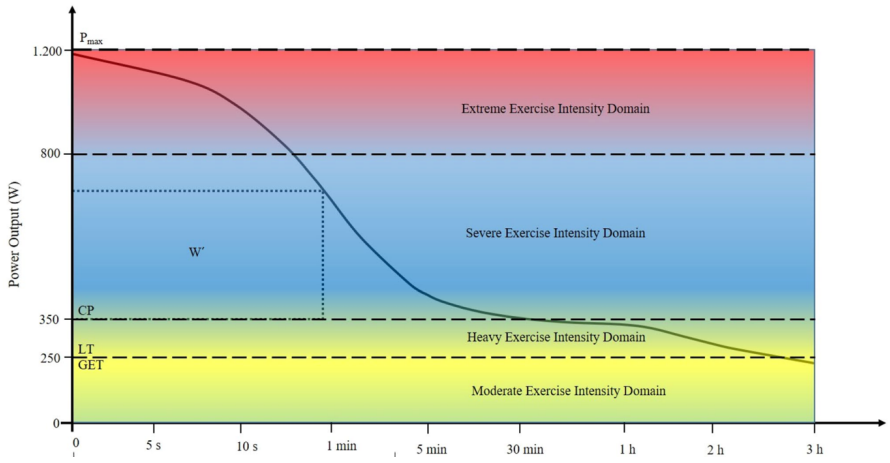

Figure 2. An illustration of the spectrum of physiological responses across the power-duration relationship using arbitrary power output values in cycling durations.

TT performance more accurately depicts athletes' high variable power output during cycling races on the ability to generate short-term maximal sprints that increase physiological demands [123]. However, traditional road cycling events may limit the application of research findings to actual sporting performances. Because of the relatively short nature and increasing popularity in these races, research to date, does not suggest proper fueling recommendations. However, multiple accelerations and decelerations without adequate nutrition can lead to muscle fatigue [33] and a lower repeated sprint ability [144]. Repeated sprint ability is the ability of an athlete to recover and maintain maximal effort during accelerations and sprints [392]. These races are maximal, including repetitive short power bursts and technical sections, which would require adequate nutrition (e.g., glucose and hydration) for optimal performance.

Nutrition for Cycling Performance

Muscle glycogen and blood glucose levels are the most important substrates for high-intensity TTs [338]. TTs and track cyclists spend considerable time near and above the individual lactate threshold and mainly utilize carbohydrates as their chief energy substrate [333]. During prolonged and high-intensity cycling bouts, fatigue is often associated with reduced blood glucose concentrations and muscle glycogen depletion [206]. Therefore, high pre-exercise muscle and liver glycogen concentrations are essential for optimal cycling performance. Such requirements can vary from 5 to 10g·kg−1·day−1 [51] depending on the individual and the race demands. Although some argue that athletes can meet caloric needs simply by consuming a well-balanced diet, it is often challenging for more prominent athletes and athletes engaged in high volume/intense training to consume enough food to meet and maintain caloric needs [37, 59-61, 231, 232]. For Extended (> 60 min) bouts of high intensity (> 70% VO_{2max}) exercise challenge

fuel supply and fluid regulation, carbohydrate should be consumed at a rate of ~30–60 g of carbohydrate/h in a 6–8% carbohydrate- electrolyte solution (6–12 fluid oz.) every 10–15 min throughout the entire exercise bout, particularly in those exercise bouts that span beyond 70 min [221]. When carbohydrate delivery is inadequate, adding protein may help increase performance, ameliorate muscle damage, promote euglycemia and facilitate glycogen re-synthesis. However, optimizing performance and recovery strategies is essential to maintain the variable power output demands during these races, maintain energy expenditure, decrease the onset of illness, decrease overtraining, increase oxygen delivery, and reduce high levels of oxidative stress.

Supplements for Cycling Performance

More recently, there has been more focus on the contribution of nutrition and dietary supplements to optimize training and cycling performance. Nutritional ergogenic supplements may enhance energy production via ATP synthesis, provide athletes with a competitive advantage, decrease fatigue, improve cycling efficiency, improve recovery, or assist in injury prevention during intense training [178]. An ergogenic aid may involve a training technique, mechanical device, nutritional practice, pharmacological method, or physiological process to improve exercise capacity and enhance training adaptations [232, 234]. Due to the competitive nature of TTs races, ergogenic aids have become more popular. Research shows that 62% of cyclists took supplements to improve their performance; 44% consumed energy drinks, while caffeine, carbohydrates and proteins, and vitamins were used by 24%, 30%, 30%, and 22%, respectively [314]. To meet the metabolic demands of TT style races, dietary supplements may enhance energy production and thus performance.

Molecular Pathways Associated with Energy Production

Cycling and the Phosphagen System

High-intensity exercise can result in up to a 1,000-fold increase in the rate of ATP demand compared resting conditions. To sustain high muscle contraction in TT style races, ATP needs to be regenerated to match the ATP demand of the cycling exercise. Three energy systems function to replenish ATP in the skeletal muscle: 1) phosphagen, 2) glycolytic, and 3) mitochondrial respiration. The three systems differ in substrates used, products, maximal rate of ATP regeneration, and their associated contributions to fatigue. In cycling performance, fatigue is best defined as a percentage of power production during muscle contraction despite the increased effort [20]. The replenishment of ATP during intense cycling exercise is the result of a coordinated metabolic response in which all three energy systems contribute to different degrees based on an interaction between the intensity and duration of the cycling exercise bout and, consequently the proportional contribution of the other skeletal muscle motor units [20]. Power output during high-intensity cycling bouts is dependent on the breakdown of ATP by ATPase [153]**.** The total quantity of ATP stored within the body cells is minimal (approximately eight mmol/kg wet weight of muscle) [20]. Thus, cells rely on other energy systems to supply ATP to support cell work. ATP production can include storing energy in more complex molecules such as glycogen and triacylglycerols when exercise requires more energy. More importantly, a sensitive control system rapidly increases metabolism during energy ATP demand. Depending on the demands placed upon the muscle tissue, it can vary its metabolic rate to a greater extent than any other tissue [153].

During TTs races, cells can detect, rapidly respond to, and successfully meet sudden increases in ATP by utilizing all three energy systems. Energy systems explain how cells

regenerate ATP—on three critical reactions that drive ATP production**.** TT races require short-term or a limited number of repeated, maximal, intense muscle contractions. During the initial 10–15 seconds of a cycling session, creatine phosphate (CrP) is solely responsible for ATP regeneration [20]. The creatine kinase and adenylate kinase reactions produce ATP in high-demanding cycling exercises. Yet, the creatine kinase (Cr) reaction has a greater capacity for ATP regeneration since the resting concentration of creatine phosphate is approximately 26 mmol/kg wet wt. Further, phosphocreatine hydrolysis does not depend on oxygen availability or necessitate the completion of several metabolic reactions to fuel ATP regeneration. The attainment of very high power output during cycle sprinting is derived from the anaerobic sources of phosphocreatine (CrP) degradation and glycogenolysis ending in lactate production [126] During exhaustive and maximal TT races, the energy yield from the phosphagen system may continue until the stores of CrP are depleted [43, 410]. This can occur within 10 s of the onset of maximal cycling sprints due to the rapid degradation of the CrP [20]. Research suggests that CrP resynthesis relies on oxidative metabolism, and the energetic capacity of this system depends on the concentration of creatine phosphate. The ability of cycling athletes to repeatedly recover their CrP stores and therefore produce high power outputs can significantly affect their performance.

During the creatine kinase reaction, the proton (H+) consumption accounts for the slight alkalinization of power at the onset of a cycling bout. High-intensity exercise such as TT races can result in intracellular protons flux and increases lactate concentrations [252]. However, metabolic acidosis can occur with the increased flux of H+. Metabolic acidosis activates AMP deaminase and results in the production of AMP and eventually ammonia (NH_3^+). However, the combination with the pre-existence of acidosis makes the H^+ consumption of this reaction of little consequence in the skeletal muscle. The phosphagen system, primarily used during TT cycling,

predominately the adenylate kinase reaction, is a potent allosteric activator of two enzymes important to glycolysis. First, AMP activates phosphorylase, which increases glycogenolysis and the rate of glucose-6-phosphate (G_6P) production, providing immediate fuel for glycolysis. Second, AMP activates phosphofructokinase within the energy investment phase of glycolysis (phase 1), allowing for the increased flux of G_6P through glycolysis. This increases rates of ATP through phase 2, the energy-producing phase. However, the third reaction, the AMP deaminase reaction, does not regenerate ATP.

During cycling, the phosphagen system demonstrates a theoretical understanding of bioenergetics, converting AMP to IMP, aiding in the phosphate transfer potential within the muscle. This system activates carbohydrate oxidation after the onset of the cycling bout [172] is caused by the production of AMP. AMP is increased by the release of intramuscular free calcium and inorganic phosphate. The AMP deaminase reaction produces NH_4^+, which is toxic to cells and subsequently removed into the blood for circulation to the liver and subsequent conversion to urea; this process is known as the urea cycle. This cycle is not the only source of ammonia during intense cycling bouts. Some ammonia is also produced from protein oxidation; it accounts for most ammonia production, which can substantially contribute to fatigue during sustained and intense TTs [20]. TT races are highly dependent on the phosphagen system.

Cycling and Glycolysis

When cycling continues longer than a few seconds, the energy to regenerate ATP is increasingly derived from blood glucose and muscle glycogen stores, called glycolysis [172]. The rate of the phosphorylase reaction results from calcium acting as an activator of phosphorylating, and inorganic phosphate is a substrate, and the immediate, increase in blood glucose uptake into

muscle is caused by muscle contraction. Glycolysis involves more reactions than the phosphagen system, slightly decreasing the maximal rate of ATP regeneration as cycling exercise demands. However, glycolysis remains a fast way to regenerate ATP during TT style races, compared with mitochondrial respiration [20]. Glycolysis contains two phases, phase 1 and phase 2. Phase 1 involves six-carbon phosphorylated carbohydrate intermediates called hexose phosphates, requiring four ATP molecules to operate. ATP provides the terminal phosphate in each of the hexokinase and phosphofructokinase reactions. Phase 1 prepares for phase 2, where phase 2 is the ATP regenerating phase of glycolysis. This phase occurs at a higher capacity than the cost of phase 1, resulting in a net glycolytic ATP yield. Each reaction of phase 2 is repeated twice for a given rate of substrate flux through phase 1, as phase 2 involves three-carbon phosphorylated intermediates or triose phosphates. The splitting of fructose-1,6-bisphosphate causes a doubling of reactions into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Triosephosphate isomerase catalyzes the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate. Consequently, two molecules of glyceraldehyde-3-phosphate (G3P) are now available for phase 2 of glycolysis. The two molecules of G3P allow for the doubling of each subsequent reaction when accounting for substrate flux and total carbons. The role of inorganic phosphate as a substrate in the glyceraldehyde-3-phosphate dehydrogenase reaction allows for free inorganic phosphate to bind to glyceraldehyde-3-phosphate, forming 1,3-bisphosphoglycerate. This process is a substrate level phosphorylation and effectively provides glycolysis to regenerate ATP. It provides the necessary phosphate to support additional phosphate transfer to ADP to form ATP in subsequent reactions. The two reactions that regenerate ATP in glycolysis are the phosphoglycerate kinase and pyruvate kinase reactions, resulting in four ATP from phase 2. For example, during 30 s of cycling at maximal effort, ATP resynthesis from glycolysis occurs almost immediately at

the onset of exercise [20]. However, ATP production from glycolysis does not reach its maximal regeneration rate until about 10 to 15 seconds of exercise and is maintained at a high rate for several more seconds. Throughout a 30-second cycling exercise, glycolysis's contribution (50-55%) [126] to ATP turnover is nearly double that of CrP [20, 35]. However, resynthesis of ATP to ~80–100% of resting value requires 2–4 minutes of recovery [126]. For competitive TT cyclists, resynthesizing ATP is optimal to finish the races faster.

In cycling, much of the performance reserve is conserved, involving anaerobic energy contribution, which can be utilized given the belief that the cycling bout will be sustainable [126, 369]. A recent laboratory-based protocol [123] with a range of power variations showed substantially greater physiological demands during variable power cycling than a sustained effort matched for mean power output training for a cycling race. Further, the implications of conserving repeated sprint ability and minimizing fatigue before the final sprint is a predictor of overall cycling performance [319]. The high power profile of TT cycling is best associated with the cyclist's ability to generate increased power efforts less than 2 minutes long, emphasizing the importance of the glycolytic energy system and the generation of short-term power for these racing events [18]. Additionally, ten trained cyclists completed four, 4000 m cycling TTs. During trials three and four, participants raced against a pacer which was set, in a randomized order, at a mean power output equal to 2% (+2% TT) or 5% (+5% TT) higher than their baseline performance [369]. This cycling performance improvement is likely attributable to a greater glycolytic contribution to total power output (Figure 3). The increased anaerobic usage was associated with an increase in blood lactate concentration. However, the increased lactate concentration would ordinarily be associated with a decrease in cycling intensity to maintain an appropriate surplus of anaerobic energetic supplies. However, this increase in lactate provided the subjects the ability to

retrieve latent anaerobic resources to convert to glucose (e.g., gluconeogenesis) to meet the metabolic demands of cycling while preserving fatigue. The anaerobic contributions to cycling performance enables the individual to produce greater total power output (Figure 3). Relatively brief but maximal sprint training can enhance glycolytic and oxidative enzyme activity [126], enabling the cyclists to utilize glucose more efficiently, increasing ATP production.

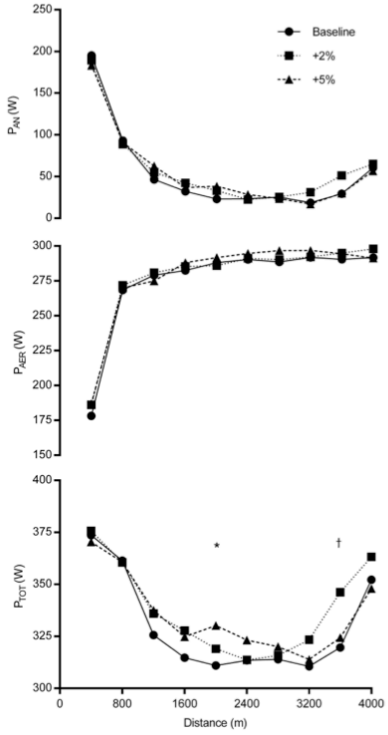

Figure 3. Serial power patterns of contribution to total power output (TOT), anaerobic contribution to total power output (TAN), and aerobic contributions to total power output (AER) during each 400 m section during baseline [369].

However, carbohydrate is the only nutrient whose stored energy can be used to generate ATP via glycolysis during high-intensity activities. When carbohydrate in the form of glucose or glycogen is catabolized during the high-intensity performance, only a partial breakdown or oxidation occurs, compared to the complete oxidation when reliant on mitochondrial respiration [20]. This is because pyruvate production occurs at rates exceeding the mitochondria's capacity uptake pyruvate. Pyruvate must be removed from the cytosol to prevent the product inhibition of glycolysis and a reduction in the rate of glycolytic ATP regeneration. However, some pyruvate is transported out of contracting muscle fibers, but most are converted to lactate via lactate dehydrogenase. The conversion of pyruvate to lactate not only oxidizes the reduced form of nicotinamide adenine dinucleotide (NADH) but also contributes to the recycling and lowering of the protons (H+) released from glycolysis [126]. The protons released from ATP hydrolysis require removal from the cell or cytosol, buffering to prevent the development of metabolic acidosis [20]. Lactate production is advantageous to cycling performance by maintaining cytosolic reduction-oxidation (redox), creating new glucose, and consuming and transporting H+ from the cell [126]. High levels of lactate are not detrimental to cycling performance, and of utmost importance, lactate assists with minimizing the effects of muscle fatigue by consuming more oxygen. The glycolytic system is arguably the most important for maximal sprints seen in TT races.

Cycling and Oxidative Phosphorylation

In more extended duration cycling events, the resynthesis of ATP by mitochondrial respiration occurs in mitochondria and involves fuel combustion in the presence of sufficient oxygen. The fuel can be obtained from sources within the muscle (free fatty acids and glycogen), outside muscle (blood-free fatty acids [from adipose tissue], and blood glucose [from dietary ingestion or

the liver]). The connection between the mitochondria and glycolysis is complete when pyruvate, the electrons, and protons from the glycolytic reduction of NAD^+ to NADH are transferred into the mitochondria as substrates for mitochondrial respiration. However, coenzyme A must be added to activate free fatty acids in the cytosol of skeletal muscle before transport into mitochondria. The inner mitochondrial membrane is impermeable to long-chain fatty acids; therefore, the fatty acyl CoA molecules are transported into the mitochondria via the carnitine shuttle [20]. Once inside the mitochondria, saturated fatty acids are degraded two carbons at a time in the four reaction β-oxidation pathway, releasing acetyl CoA, 1 NADH, and 1 FADH per cycle**.** However, the Krebs cycle is regulated by the availability of the NAD+ and FAD substrates, while high concentrations of NADH inhibit it [7]. The acetyl CoA is produced from β oxidation and then enters the Krebs cycle like that for the oxidation of acetyl CoA derived from pyruvate oxidation. The Krebs cycle comprises eight enzymes within the mitochondrial matrix except for the outlier succinate dehydrogenase, related to the respiratory chain on the inner mitochondrial membrane [7]. The cycle is a gateway for aerobic metabolism for molecules that can convert to an acetyl group or dicarboxylic acid. As mentioned above, organic molecules such as carbohydrates, lipids, and proteins are split. They transform into acetyl-CoA before entering the Krebs cycle, a molecule formed by an acetyl group and by an acyl transporter called coenzyme A. The acetyl group is then oxidized, and the energy obtained is used to synthesize ATP in cooperation with oxidative phosphorylation.

In the presence of oxygen and cycling lasting several minutes to hours, the oxidative metabolism of carbohydrates and fat provides almost all the ATP for contracting the skeletal muscle [172]. The primary function of mitochondria is the synthesis of ATP through oxidative phosphorylation. Carbon substrates are oxidized with electrons flowing through the respiratory chain

complexes to generate a proton gradient across the inner mitochondrial membrane [69]. This is accomplished by delivering electrons to the electron transport system from reducing equivalents produced in the metabolic pathways. The concomitant transport of electrons through complexes I, III, and IV leads to the pumping of protons from the mitochondrial matrix to the intermembrane space creating a proton gradient, i.e., a difference in electrochemical potential across the inner mitochondrial membrane, which energizes the ATP synthase to drive ATP synthesis [167]. This proton gradient provides the energy necessary to produce ATP. Besides, the proton gradient is the driving force for the mitochondrial uptake of positively charged ions and the transport of ADP from the cytosol to the mitochondria. High-intensity TT cycling can enhance oxidative enzymes [126], enhancing the ability to use oxygen and replenish sufficient ATP to meet cycling demands. During cycling TTs, all three energy systems play a role and contribute to ATP production to help meet cycling demands.

Cycling Effects on Oxidative Stress

Individuals produce free radicals as part of normal metabolic processes and exercise. Free radicals (e.g., superoxide, hydroxyl radical, hydrogen peroxide, oxygen singlet, etc.) are reactive molecular species with unpaired electrons that oxidize and cause damage to other substances (e.g., proteins, lipids, DNA, carbohydrates, etc.) [36]. Although these free radicals positively affect metabolic reactions such as mitochondrial biogenesis and hypertrophy, they also cause negative effects. Such as if the oxidative damage exists above a specific adaptation threshold for a chronic time, and is associated with an increase in the inflammatory response [119, 150, 194, 302], impaired exercise performance (e.g., force production), and induced muscle damage [220, 280, 302, 327, 375, 404], and accelerate fatigue [136, 220, 255, 302, 355, 365]. Finding the

optimal balance between negative-positive effects between physiological reactive oxygen species (ROS) and increasing adaptations is difficult [24]. Short high-intensity cycling, such as 10 km TT races, can lead to an increased in oxidative stress [107, 290]. However, optimal ROS concentrations can drive an increase in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) driving mitochondrial biogenesis, metabolism, and improve mitochondrial function (Figure 4). ROS production in cycling is proportional to the maximal aerobic power and is inversely related to the consumption of antioxidants [199]. For example, during an incremental cycling bout, thiobarbituric acid reactive substances were increased post-exercise in untrained subjects [199]. Though, they tended to decrease within 30 minutes of recovery. However, endurance training (e.g., TT racing), can increase antioxidant enzymes (.g., Krebs cycle succinate dehydrogenase, oxidative phosphorylation cytochrome c oxidase) levels in the skeletal muscle [46, 220]. In conditions that produce high amounts of ROS (e.g., stress, substances such as antioxidants help protect the cell from the harmful effects of free radicals.

Antioxidants are substances that may protect cells and provide oxidation-reduction balance from the damage caused by unstable molecules. In addition, they help give a consistent physiological level of ROS to elicit long-term adaptations (e.g., mitochondrial biogenesis, hypertrophy) [24]. Flavonoids are phenolic compounds which are antioxidant substances found in fruits and vegetables. The ingestion of flavonoids can reduce the risk of cardiovascular diseases (e.g., type II diabetes), liver damage, metabolic disorders, and gene mutation [100, 416]. These effects are likely due to the physiological activity of flavonoids in reducing scavenging free radicals, inhibiting low-density lipoproteins oxidation and platelet aggregation, and acting as vasodilators in blood vessels [36, 100]. Free radicals are constantly generated from exercise or disease. High levels can result in extensive damage to tissues leading to various disease conditions such

as cancer, Alzheimer's, renal diseases, cardiac abnormalities, etc., [100]. Antioxidants such as quercetin, may improve cycling performance.

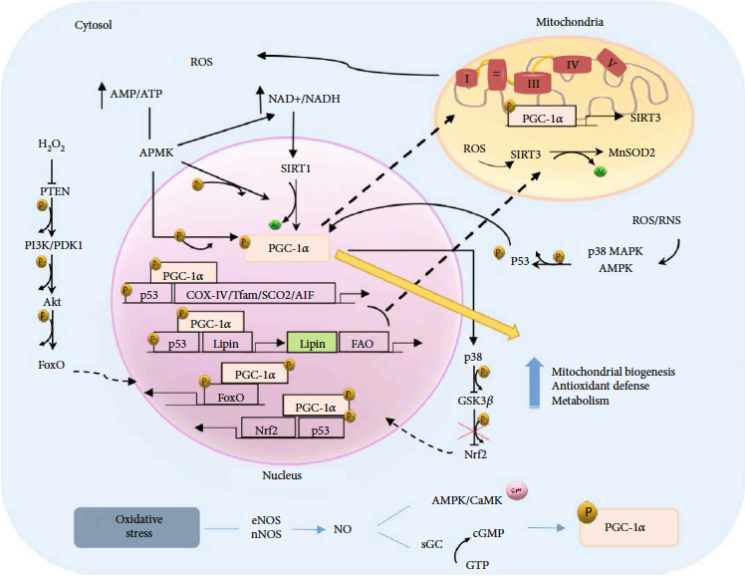

Figure 4. PGC-1α signaling pathway in response to ROS [334].

Cycling and Mitochondrial Adaptations

High intensity, volume, duration, seen in TT cycling can contribute to mitochondrial biogenesis by upregulation of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha protein (PGC1α) [321]. An increase in PGC-1α expression can lead to the expression of mitochondrial enzymes in the electron transport chain (ATP synthetase, cytochrome c oxidase subunits). Endurance exercise is shown to increase transcriptional protein PGC1α through post-translational modification [46], enhancing mitochondrial biogenesis through three main

mechanisms: 1) AMP concentration will be increased, and ATP concentration will be decreased. AMPK increases the phosphorylation of the AMP-activated protein kinase (AMPK) [213]. This protein is activated upon alterations in the cellular ROS concentrations and AMP/ATP ratio. Once activated, AMPK quickly regulates metabolic enzymes through direct phosphorylation and stress-activated protein kinase (SAPK) gene expression from increased ROS from exercise. SAPK proteins are p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal Kinase (JNK1/2), and extracellular signal-regulated kinases (ERK), which can play a role in increasing PGC-1α expression and glucose metabolism [316]. 2) PGC-1α expression responds to calcium signaling by activating calmodulin-dependent protein kinase (CaMK) and calcineurin (a calmodulin-dependent serine/threonine phosphatase). Calcineurin activates the myogenic transcription factor, the myocyte enhancer factor (MEF), which drives the PGC-1α transcription [237]. AMPK also causes the redox of NAD+ and NADH, which operates the silent information regulator (SIRT1) [339]. SIRT1 activates PGC-1α through deacetylation, but this activity depends on the prior phosphorylation of PGC-1α by AMPK and the cell's redox state (Figure 4-5). Since the cellular redox balance of NAD^+ and NADH is highly related to catabolic fluxes, it has been postulated that SIRT1 (NAD+ dependent deacetylase) could act as a redox sensor that directly connects metabolic flux changes with increasing transcriptional outputs, such as PGC-1α [72]. Furthermore, the increased flux of ROS (i.e., seen during cycling exercise) can increase PGC-1α [107, 290]. PGC-1α enhances mitochondrial biogenesis and oxidative capacity via upregulation of nuclear respiratory factors 1& 2 (NRF1/NRF2) and estrogen-related receptor (ERRα), which in turn regulates mitochondrial transcription factor A (TFAM). TFAM plays a role in mitochondrial replication and expression of mitochondrial DNA-encoded proteins [217] (Figure 4-5). Another critical transcription factor involved in regulating the expression of mitochondrial proteins

is the peroxisome proliferator-activated receptor coactivator, which controls the expression of the mitochondrial fatty acid oxidative enzymes. The upregulation of these mitochondrial proteins is mainly associated with the physiological adaptation in mitochondrial density and oxidative enzyme activity. The mitochondrial enzymes in fatty acid oxidation, Krebs cycle, and oxidative phosphorylation (e.g., cytochrome c oxidase) increased with a cycling stimulus, elicited improved mitochondrial efficiency [73, 263, 366]. The pronounced mitochondrial adaptation increases the size and number of mitochondria via PGC-1α upregulation.

High-intensity efforts can stimulate PGC-1-α [254], mitochondrial biogenesis, can enhance whole-body oxidative capacity and maximal oxygen consumption (VO_{2max}) [66, 149], and elicit high levels of oxidative stress [362]. To date, limited research exists investigates the effects of TT cycling on PGC-1α [4]. More recently, studies have shown that an acute bout of high-intensity interval cycling can increase the nuclear abundance of PGC-1α and activates mitochondrial biogenesis. For example, six weeks of hyperoxia (increasing oxygen-carrying capacity) supplemented with cycling high-intensity interval training led to an improvement in cycling performance (oxygen consumption), hemoglobin mass, mitochondrial oxidative phosphorylation capacity (e.g., citrate synthase, electron-transferring flavoprotein complex, state four respiration, complex I, complex I+ II, uncoupling transport system capacity, and cycling efficiency [73]. With 10 days of high-intensity cycle training (6×5 min; 90–100% VO_{2peak}) and six prolonged moderate-intensity sessions (45–90 min; 75% VO_{2peak} in healthy untrained individuals, PGC1-α protein expression and cytochrome oxidase complex IV increased [366]. In a randomized crossover fashion, trained cyclists completed six weeks of a polarized (80%, 0%, and 20% of training time in low-, moderate-, and high-intensity zones) and a threshold cycling model (57%, 43%, and 0% training-intensity distribution) [295]. Endurance performance, peak power output, lactate

threshold, monocarboxylate transporter 4, and high-intensity exercise capacity all increased over both training periods. However, no changes in mitochondrial enzyme activities or monocarboxylate transporter 1 were observed following training, and markers of muscle metabolic adaptation are essentially unchanged [295]. In a counterweighted single-leg cycling study [265], ten young men performed unilateral graded-exercise tests to measure single-leg $\dot{V}O2$, peak, and peak power (W_{peak}). Each leg was randomly assigned to complete six sessions of high-intensity interval training (HIIT) [4 × (5 min at 65% W_{peak} and 2.5 min at 20% W_{peak})] or moderate-intensity continuous training (MICT) (30 min at 50% W_{peak}), which were performed 10 min apart on each day, in alternating order. Post-training, citrate synthase maximal activity and mass-specific oxidative phosphorylation capacities (complex I, complexes I, and II) were more significant in HIIT than MICT; however, mitochondrial function was measured under various conditions unaffected by training [265]. Single-leg cycling performed in an interval compared to a continuous manner elicited superior mitochondrial adaptations in human skeletal muscle despite equal total work. Metabolomics markers suggest different cellular metabolic exercise stresses to increase muscle metabolic adaptations. However, future research needs to investigate the impact of TT cycling on PGC-1α and oxygen delivery. Understanding the mechanisms of ATP production and mitochondrial biogenesis in skeletal muscle in response to TT cycling is critically essential.

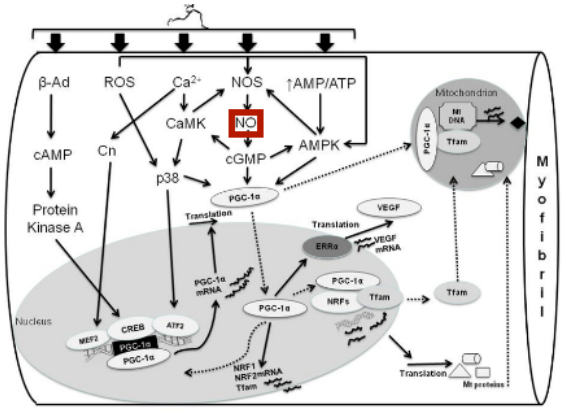

Figure 5. Upregulation of PGC-1α by exercise and the subsequent effect on regulating mitochondrial and nuclear-encoded genes [195].

Endothelial Nitric Oxide in Skeletal Muscle

The metabolic demands increase during high-intensity aerobic and anaerobic [46]. Along with the adaptation to muscle fibers (e.g., mitochondrial biogenesis and fiber transitions), cardiovascular adaptations can also improve vascular and endothelial function [312]. Exercise training exerts systemic effects on the arterial endothelium and vascular changes in the heart and blood vessels [29]. Endurance exercise (e.g., cycling) increases the vessel radius and endothelial vasodilation to the working muscles [312]. The mechanism of how this occurs is an increase in nitric oxide (NO). NO is an endogenous vasodilator continuously synthesized from the amino acid L-arginine (arginine) in endothelial cells by the calcium-calmodulin-dependent enzyme endothelium NO synthase (eNOS) that metabolizes one of the nitrogen's of arginine to NO and its metabolites, nitrite, and nitrate [15, 386]. Nitric oxide synthases (NOSs) includes endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and induced nitric oxide synthase (iNOS) [129]. Flavin mononucleotide and flavin adenine dinucleotide are cofactors, while

reduced nicotinamide adenine dinucleotide phosphate (NADPH) molecular oxygen is a necessary co-substrate for optimal NOS activity [15]. Additionally, 5,6,7,8-Tetrahydrobiopterin (BH4) is an essential cofactor for multiple enzymes, including NOS [129]. BH4 homeostasis determines the role of NOS, affecting the production of NO and oxygen free radicals [129]. Under conditions of oxidative stress (e.g., cycling), BH4 is diminished due to its oxidation which leads to NOS uncoupling and the generation of highly oxidative free radicals [129].

NO has a wide range of biological properties that maintain vascular homeostasis, modulating vascular dilator tone, increasing mitochondrial biogenesis (PGC-1α) (Figure 4-5, 11), enhancing oxygen delivery, and protecting the vessel from injurious consequences of platelets and cells circulating in the blood, playing in this way a crucial role in the standard endothelial function [15, 312, 386]. Changes in eNOS gene expression increase following endurance exercise [86] and plasma arginine levels enhancing NO [373, 374], keeping blood vessels dilated and increasing blood flow and oxygen delivery. An increase in blood flow to the skeletal muscle is required to provide an additional supply of oxygen and nutrients [415]. This is especially important for high-intensity TT races on PGC-1α. races that can cause endothelium shear stress, eliciting an increased metabolic demand by activating gene expression [308]. This gene expression can lead to an increase in capillary growth and maintain homeostasis during high-volume activities. One hypothesis theorizes that eNOS and AMPK cooperatively regulate PGC-1α and induce effects in the skeletal muscle [253]. Given that NO enhances blood flow and oxygen delivery, a higher NO concentration may enhance power output in cyclists.

Time-Trial Endurance Capacity Adaptations

Time-trial performance and endurance capacity influence metabolic adaptations and high-performance capacity. For example, a 20-minute TT can predict a cyclist's functional threshold power (FTP) [387]. FTP is the highest amount of power a cyclist can maintain in a quasi-state for approximately 60-minutes without the onset of fatigue [52]. FTP is recognized as a critical measurement in cycling performance and adaptation, determining an cyclist's VO_{2max} and aerobic capacity [110], lactate threshold [110], maximal metabolic steady state and ventilatory threshold two [52, 54, 219, 226, 243, 303]. Time-trial exercise intensities above FTP deplete the selected energy reserves due to an insufficient oxygen supply for aerobic metabolism. Assessing and tracking the intensities above FTP can provide knowledge to modify cycling training programs to elicit and improve adaptations. In TTs, FTP is shown as an adaptation showing the measure of a maximum steady-state of aerobic metabolism [110] and to determine the severe and heavy intensity domains in cycling training which is correlated to muscle capillarity, inorganic phosphates, and hydrogen ions [81]. High-intensity cycling seen in TTs can increase the fixed work performed within the severe intensity domain and endurance capacity. Further, physiological markers are found to be predictive of cycling performance, including power output at the lactate threshold (LT2); peak power output (Wpeak), indicating a power/weight ratio of ≥5.5 W/kg; the percentage of type I fibers in the vastus lateralis; maximal lactate steady-state, representing the highest exercise intensity at which blood lactate concentration remains stable; Wpeak at LT2; and Wpeak during a maximal cycling test [126].

The gene expression of skeletal muscle fibers is based on their specific contractile function of speed (slow or fast) based on the myosin heavy chain (MHC) isoform or density of mitochondria (low or high) [46]. Type I muscle fibers are termed slow oxidative and have the least

force output. In parallel, Type I fibers are highly oxidative, have a high mitochondria density, and increased endurance capacity [46]. They contain the MHC protein isoform with a slow contractile speed and use less ATP per unit of work because of its lower myosin ATPase activity [46]. Lower ATPase activity allows the fibers to be more metabolically efficient, while the high mitochondrial density allows an oxidative supply of ATP for contractile work and more excellent resistance to fatigue [10]. Studies show that endurance sports such as cycling have the highest concentrations of mitochondrial enzymes and a higher percentage of type I fibers [46]. However, type II fibers are more prone to fatigue and have poor endurance capacity than type I fibers [46]. Type II fibers have two standard classifications, fast oxidative (IIa) and fast glycolytic (type IIx) [46]. Type IIx is known for its quick contractile time and considerable power output, large motor unit, faster innervation of muscle fibers, high ATPase activity, and higher concentrations of MHC(3) [46]. Type IIa is intermediate fibers with moderately fast contractile time, medium motor units, reasonably high resistance to fatigue, moderate power produced, high mitochondrial density, high oxidative capacity, high glycolytic capacity, and intermediate MHC(2) isoform, and medium capillary density [46].

In the wake of a high-intensity cycling bout, sprint training regimen, [118, 200] adaptations can result in a shift from slow-to-fast MHC expression as demonstrated by an increase in MHCIIa, and a decrease in MHCI/β mRNA. High-intensity sprint training can lead to a slow-to-fast transformation and improve glycolytic capacity, repeated sprint ability, and cycling performance. Further, endurance activity can also increase calcium-calmodulin dependent serine/threonine protein phosphatase calcineurin [415]. Activation of calcineurin promotes the expression of slow-twitch muscle genes. This activation of calcineurin in skeletal muscle can increase the number of slow-twitch fibers and elevate the expression of slow-twitch troponin I, myoglobin,

glucose transporter 4 (GLUT4) pyruvate dehydrogenase kinase, mitochondrial enzymes, and PGC-1α [415].

Moreover, this can lead to an increase in lipid oxidation and a decrease in carbohydrate oxidation. Further, these cellular and performance adaptations can reduce the appearance of glycolytic products, increase resting glycogen content in the working muscle and increase muscle maximal oxygen delivery and uptake [325]. These acute exercise transient changes and phosphorylation of PGC-1α is an essential regulator of exercise-induced adaptations in skeletal muscle and induced in response to cycling exercise.

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Nutritional Interventions & Performance

Ergogenic Aids

Athletes often feel less at a given exercise every day and use ergogenic aids to enhance training or competition performance. So many individuals try to go beyond the assigned exercises and techniques using substances, often referred to as ergogenic, to gain an advantage. An ergogenic aid is any training technique, nutritional practice, pharmacological practice, or psychological technique that can improve performance capacity (e.g., run faster, lift more, work during a given task, etc.), efficiency of exercise, tolerate heavy training, improve recovery or enhance training [232]. Data shows at least 50% of the general population reported using supplements, while about 75% of college athletes, 100% of bodybuilders[5], and nearly half of all athletes reported using supplements [326]. The 2021 Supplement Business Report detailed a record-breaking year in 2020, with a 14.5% increase in sales to $55.75 billion from $48.67 billion in 2019. Before2020, the supplement market has grown between $2 billion and $2.5 billion each year since 2015 [296]. The industry's growth was led by vitamin sales, which spiked to 22.3% growth in 2020 amid the pandemic. Vitamins added $3.24 billion to total supplement sales, accounting for nearly half of the real dollars added in 2020 [296].

The International Society of Sports Nutrition defines a dietary supplement as ergogenic, effectively promoting further muscle hypertrophy or performance increases with exercise training in human studies. Still, a supplement cannot be classified as ergogenic if data is supported by cell culture or rodent studies field [232]. However, the Food and Drug Administration regulated dietary supplements before 1994 until Congress passed the Dietary Supplement Health and Education Act (DSHEA) 1994, which placed dietary supplements in a special category of "foods." [232]. DSHEA defined a dietary supplement as a product ingested by the mouth that contains a

"dietary ingredient," which may include vitamins, minerals, herbs, or other botanicals, amino acids, or other substances (e.g., enzymes, organ tissues, glandular, or metabolites) [232]. Dietary supplements can be sold in tablet, capsule, powder, soft gel, liquid, or bar form. However, they must be clearly labeled as dietary supplements. According to the DSHEA Act of 1994, dietary supplement manufacturers are not required to submit to the FDA the evidence it relies upon to substantiate safety or effectiveness before or after it markets the ingredients [232]. However, DSHEA grants FDA greater control over supplements containing new dietary ingredients [232] since indirect detection methods exist. However, when individuals decide to use an ergogenic aid, whether a dietary supplement, method, or other substance, they must consider the product's efficacy, safety, and legality before using it [326].

Plant-based Foods as Ergogenic Aids

Alternative medicine, often using plant extracts, plant leaves, or concentrates, is widely used in the management treatment of ailments or ergogenic substances to enhance performance. Dietary supplements that contain plants have become one of the most popular ergogenic aids on the market. The World Health Organization (WHO) estimates that 80% of people rely primarily on traditional remedies, such as herbs, for medicinal purposes [424]. The therapeutic value of certain plants is due to the presence of phytochemicals, polyphenols, elemental composition terpenoids, flavonoids, and alkaloids which have several physiological effects on the human body [34, 349, 424]. Polyphenols which constitute the active substances found in many medicinal plants, modulate the activity of a wide range of enzymes and cell receptors [269].

Furthermore, they exert a powerful antioxidant effect. Polyphenols inhibit lipid oxidation by acting as chain-breaking peroxyl-radical scavengers and can protect lipoproteins from

oxidation [360]. Plants are also widely sought for their biological properties: anti-allergic, anti-atherogenic, anti-inflammatory, hepato-protective, antimicrobial, antiviral, antibacterial, anticarcinogenic, antithrombotic, cardioprotective, antioxidant, and vasodilatory properties [34, 92, 236, 360, 371]. Yet, few studies have conclusively shown the exact mechanisms of antioxidant, anti-inflammatory, performance improvements, and physiological adaptations from specific plants used as ergogenic aids. These antioxidant characteristics and redox properties play an essential role in oxidative damage stabilization by free radical neutralization, oxygen scavenging, or decomposition of peroxides [349]. This information has led to studies focusing on the role of herbal supplements in reducing exercise-induced oxidative stress to enhance muscle recovery and energy maintenance during intensive exercises [349]. However, outcomes have varied in previous studies due to factors like the type of the plant, the geographic location from which was gathered, and the method of extraction used [349].

Further, most studies do not mention possible threats to health factors by interferences with medications [121] or risks in athletes [349]. There is limited evidence investigating the use of ergogenic herbals plant supplements. Still, the interest in herbals or plants remains as common ergogenic aids in sports performance.

Herbal supplements like capsaicin, ginseng, ginkgo Biloba, ephedrine, caffeine, and moringa oleifera have been used to study their ergogenic effects. Ginseng roots contain approximately 13 glycosylated steroidal saponins (ginsenosides), the probable active agents to stimulate the central nervous system [80]. Ginseng is thought to be a stimulant that can improve vitality, health, and longevity. In addition, ginseng possesses antioxidant properties whereby it scavenges hydroxyl radicals and inhibits lipid peroxidation [80]. Ginseng also has a stimulant effect by

improving alertness and decreasing fatigue and stress [11]. Other potential ergogenic effects of ginseng include favorable metabolic, hematologic, and cardiovascular improvements [408].

Ginseng ingestion's possible mechanisms and effects contribute to enhancing human sports performance by acting as a physical performance enhancer [80, 349]. However, a review of the available data on the effects of ginseng on human exercise performance reveals equivocal results relating to its dose-response and duration effects [80]. Capsaicin, ephedrine, and caffeine are similar in that they are stimulants of the central nervous system, which increases catecholamine secretions and enhances lipid oxidation and carbohydrate usage [408]. Moringa oleifera is used to improve glucose uptake and utilization, the expression of uncoupling protein (UCP1), SIRT1, and PGC-1α, which is involved in mitochondrial function, metabolic energy production, and modulating lipid metabolism [357]. There is an increasing number of herbal supplements marketed as ergogenic aids. Still, research is limited in evaluating herbal supplements' effects and metabolic pathways on human performance and recovery [408]. Much research fails to account for the plant's health risks, content and purity, and accurate dosages. However, quercetin is a promising, safe, protective, therapeutic, and performance-enhancing plant-derived supplement which assist with TT performance.

Quercetin

Plant Background

Quercetin (Q), a plant pigment, is an antioxidant, polyphenol, and a flavanol belonging to the flavonoid group [36, 343]. Q can be extracted from various plants, including *Moringa oleifera and Sophora Japonica* L. [394]. It can also be found in food sources such as apples, elderberries, citrus fruits, red wine, red onions, hot peppers, berries, kale, and a large amount can be

found in buckwheat tea and capers [36, 201, 416]. Literature suggests Q possesses antioxidant, anti-inflammatory, and cardioprotective effects, which may help reduce chronic inflammation and oxidative stress. The activity of Q and its protective effects against cardiovascular disorders, cancer, inflammatory, oxidation, and anti-viral activities is extensively documented in animal models [143, 164, 247, 267, 294, 417, 424]. Q is an effective and potent free radical scavenger in the flavonoid family, and it is 6.24 times higher than Trolox, which is used as an antioxidant reference [306, 341, 416]. However, quercetin combined with metal ions (i.e., vanadium, copper, magnesium, iron, ruthenium, cobalt and cadmium, calcium, and rare earth elements) elicits a higher antioxidant activity [416]. Quercetin becomes oxidized when employed as an antioxidant to generate quercetin–quinone (QQ). QQ is toxic because of its ability to arylate protein thiols [341]. Protection against QQ may arise from binding with glutathione (GSH), the most abundant thiol (prevents uncontrolled oxidative reactions). With low concentrations of GSH QQ, the QQs may become free to react with other thiol groups (e.g., protein sulfhydryl) and may cause ROS [44, 341]. The potentially toxic effects of QQ species have not yet been studied in humans. Further studies are needed to elucidate these exact mechanisms. The properties and structure of Q as a promising agent inhibit oxidative stress in damaging bouts of exercise. Q may increase aerobic performance to a greater extent than anaerobic performance. There is an interest in its impact on exercise, especially regarding recovery from damaging bouts of exercise [346].

Structure, Pharmacokinetics, and Bioavailability of Quercetin

The structure of Q is glycosylated (sugar group at the 3-position). Glycosides consist of simple or several sugar groups, which is the main compound that contributes to the potential beneficial effects of Q [158] (Figure 6). Therefore, these glycosylated structures are most

common when assessing the antioxidant properties of Q. The structural composition of Q contains B ring *o*-dihydroxyl groups, a 4-oxo group in conjugation with the 2,3-alkene, and 3- and 5-hydroxyl groups. Because of Q's structure, it can act as an antioxidant by donating electrons to stabilize reactive oxygen species [36]. Q has served as a more powerful antioxidant than vitamins C and E [343].

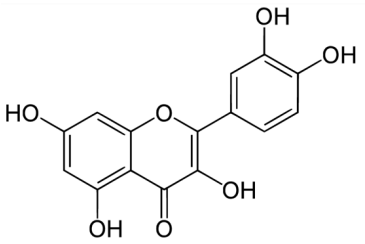

Figure 6. Chemical structure of quercetin.

Q shows relatively higher bioavailability than other phytochemicals, such as vitamins (carotenoids), and food polyphenols, such as flavonoids, phytoalexins, phenolic acids, indoles, and sulfur-rich compounds [97, 341, 343]. Bioavailability is the extent to which absorption occurs. The average daily intake of Q in the diet has been estimated as 5–40 mg/day [341] with plasma concentrations between 0.06 and 7.6 μM [36]. Although these levels can increase up to 200–500 mg/day in individuals who consume high quantities of fruits and vegetables rich in Q (e.g., apples, onions, tomatoes) [341]. However, Q in foods is not present with sugar groups, aglycon, but it is otherwise glycosylated. Q's bioavailability depends on the type of glycosides present in different food sources and food handling. Onion-derived Q (containing Q glucoside)

revealed a higher bioavailability than apple-derived Q (containing Q rhamnoside and Q galactoside) [341, 343].

Further, boiling can cause a significant decrease in the Q bioavailability [36]. Furthermore, storage effects (e.g., shelf life) can also affect Q content in digestion and absorption. High levels of ultraviolet-B rays (location grown) can also affect the flavanol content of Q. Thus, Q content varies with aglycons, geography, type, storage, boiling, and freezing [36, 122, 318]. The biochemical explanation for the higher bioavailability of Q glycosides (e.g., onion-derived than apple-derived Q) likely resides in the deglycosylation processes at the intestinal level and/or the carrier-mediated transport [158, 341]. The biological activity of Q found in food is diminished during small-intestinal and hepatic metabolism [235], resulting in decreased potency after absorption into the blood compartment. Q is primarily absorbed in the small intestine. However, Q may be difficult to digest; Q may undergo further digestion by the intestinal microflora to produce bioavailable sugar compounds that can be easily absorbed in conjugates such as Q-methyl, Q-sulfate, or Q-glucuronides groups [394]. Q-glucuronides can be further glucuronidated, sulfated, or methylated in the liver [304]. However, Q's metabolism and bioavailability are believed to lower its bioactivity in vivo [167] substantially. Q glucosides can pass through the epithelial cell layer. Still, they have a lower efficiency than the Q-aglycone (the absorbed unit of quercetin which is very reactive and insoluble in an aqueous solution) [36, 394]. The hydrolysis of the glycoside to the aglycone can accelerate the absorption of Q in the enterocyte. The attached sugar molecules or other chemical substances on Q must be removed in the enterocyte, usually by brush border enzymes (e.g., lactase phlorizin hydrolase), which eliminates the sugar groups from flavonoids [394]. The enzymes on the brush border are more glucose-specific,

so the absorbability of quercetin glucosides is rapid compared to other forms of glycosides such as rutin (quercetin-3-*O*-rutinoside).

However, rutin must undergo deglycosylation from the aglycone via the actions of enzymes from the gut microflora to increase the absorption [394]. When quercetin is absorbed into the enterocytes, it is glucuronidated, sulfated, or methylated by UDP-glucuronosyl transferases, sulfotransferases, and catechol-*O*-methyl transferase. However, Q absorption can be improved in the gut or hepatic cells when combined with alcohol, nondigestible oligosaccharides, or a high-fat diet [394]. Thus, Q absorption depends on the variety and position of the sugar groups attached. However, 5 to 10% of Q undergoes complete absorption in the small intestine, whereas about 90 to 95% of quercetin is absorbed in the colon [394]. After absorption, Q is metabolized in different organs, such as the small intestines, colon, liver, and kidney. Then, the molecule is conjugated to methyl and sulfate groups and glucuronic acid to generate its primary conjugates in humans: 30-O-methyl Q (isorhamnetin), Q -3-O-glucuronide, 30-O-methyl Q -3-O-glucuronide, and Q -30-O-sulfate [174]. Generally, quercetin glucuronides are in a more stable state for transportation into the bloodstream. However, neither glycosides of Q nor free aglycone are present in the plasma [381]. The bioavailability of Q may be increased by incorporating conjugates, which further enhance its activity (i.e., antioxidants).

However, it has been proposed that Q does not necessarily need to be absorbed to exert an effect [36]. The beneficial effects of Q in humans are primarily dependent on its bioavailability after administration. Q's dietary intake with other compounds' ingestion is further examined, but little research exists on how this affects Q's bioavailability and the mechanisms involved. For example, trained cyclists consumed Q for two weeks combined with 30 mg of epigallocatechin 3-gallate from green tea extract, 100 mg of isoquercetin, and 100 mg of N_3- poly-

unsaturated fatty acids (55 mg of EPA and 45 mg DHA from fish oil). The bioavailability of Q was improved, and its bioactive effects were prolonged [299]. In addition, the absorption of Q is influenced by gut microflora, which, in rats, converts more than 95% of the Q -40-glucoside to phenolic acids [341]. As a result of its absorption and metabolism, total Q derived from the diet is present in plasma at the nanomolar range (<100 nM) but can be increased to micromolar concentrations after supplementation (1 g, 28 days) [91, 341]. These variations are explained by evoking the different bioavailability of Q glycosides and the polymorphism of intestinal enzymes in humans compared to animals [341]. The detection of Q can occur in the plasma within 15–30 min of ingestion of a 250 or 500 mg Q chew, reaching a peak concentration at approximately 120–180 min and returning to baseline levels at 24 h [32]. Further, Q has a peak bioavailability time of 12 to 19 h [266]. Q's bioavailability mainly depends on co-ingestion with other nutrients, gut microbiota, and glycosides.

Q's half-life provides useful information serving as an antioxidant and anti-inflammatory in exercise. The half-lives of the molecule and its metabolites range between 11 and 28 hr, which suggests the possibility of significantly increased plasma concentrations upon continuous supplementation [31, 32, 341]. The plasma half-life of Q is 6–12 hr., and peak concentration occurs in 1–3 hours [116]. However, there does not appear to be a toxicity threshold and absorption rates from high dosages (>3.5 g/day) of Q. With limited evidence existing in humans, Q and Q metabolites are widely distributed in rat tissues with the highest concentrations in the lung (3.98 and15.3 nmol/g tissue for 0.1% and 1% Q diet, respectively). Further studies are needed to elucidate the effects of Q degree and rate of absorption with varying dosages and forms.

Mechanisms of Action: Quercetin in Exercise

Quercetin and Oxidative Stress

Several investigations have used Q supplementation to decrease health-related concerns, such as reducing high levels of oxidative stress and inflammation, increasing lipid metabolism via transcription proteins, and improving sport and exercise. Q can act as an antioxidant by inducing copper and ferrous iron through catechol in its chemical composition [416]. In an animal study with rats, Q inhibited ferrous iron lipid peroxidation by binding ferrous and inhibiting iron overload in alcoholic liver disease [36, 416]. Ferrous in compounds containing dihydroquercetin is inactive, unable to catalyze the decomposition of hydrogen peroxide, and thus unable to trigger further generation of free radicals [416].

Further, Q could inhibit oxidative damage by inhibiting lipid peroxidation (i.e., low-density lipoproteins (LDL)) by increasing the expression of LDL-R and reducing the secretion of proprotein convertase subtilisin/Kexin type 9 serine protease, which plays a role in cholesterol metabolism [36, 416]. Further, Q combined with liposomes and glycerol nanoparticles could scavenge free radicals and protect human keratinocytes from free radicals (e.g., hydrogen peroxide) in vitro [270]. A high antioxidant capacity of Q *in vivo* largely depends on a high concentration and gradient dependence. Q manifests itself in the glutathione pathway to enhance antioxidant capacity [412]. Superoxide dismutase-2 captures the oxide molecule and converts it into hydrogen peroxide when reactive oxygen species are present. Plasma glutathione peroxidase catalyzes the degradation of hydrogen peroxide to water molecules, which requires glutathione to provide a reducing hydrogen [412]. Thus, Q can assist with the glutathione pathway by regulating the levels and increasing glutathione synthesis [412]. Increasing the glutathione pathway via Q may help in antioxidant defense, nutrient metabolism, and regulation of cellular events (e.g.,

gene expression and protein synthesis). Although only performed in animal and cell studies, Q shows a promising supplement to minimize high levels of ROS through the glutathione pathway and enzyme activities. Q can help restore the normal redox status and promote intestinal calcium absorption via glutathione and the glutathione-dependent enzymes system [394].

Q can increase the expression of antioxidant enzymes, such as acetylcholinesterase and butyryl-cholinesterase, by binding the -OH groups on the side phenyl ring of Q to critical amino acid residues at the active site of the two enzymes [412]. The pretreatment with Q significantly enhanced the expression levels of endogenous antioxidant enzymes such as copper/ zinc superoxide dismutase, manganese superoxide dismutase (Mn-SOD), catalase (CAT), and glutathione (GSH) peroxidase in the hippocampal CA1 pyramidal neurons of animals suffering from ischemic injury [412]. This suggests that Q may be a potential neuroprotective agent for transient ischemia in animals. Further, Q can increase the expression of some antioxidant enzymes, such as glutathione transferase and Aldo-ketoreductase, in the rat liver [305]. Q treatment in rats increased the levels of SOD and CAT and reduced the level of malondialdehyde after lipopolysaccharide-induced endotoxemia., suggesting that Q enhanced the antioxidant defense system [6].

The Q molecule (Figure 6) shows a protective effect by upregulating the expression of oxidative stress-related genes: superoxide dismutase-1 (SOD-1), CAT and glutathione synthetase (GSS) in menopausal rat ovaries in vivo and in vitro [406]. Further, Q activates or inhibits the p38MAPK/iNOS pathway, upregulates antioxidants (e.g., SOD, TRAF3, GSH, CAT, Nrf12), or downregulates the oxidative stress MDA pathway and many molecules in the antioxidant signaling pathway and pro-inflammatory molecules (e.g., NF-κB including IKK and RelB) [416]. Quercetin can induce antioxidant effects by activating the nuclear respiratory factor proteins (e.g., Nrf2/NRF1) transcription pathway. This pathway upregulates the expression of

peroxiredoxins, an antioxidant family responsible for catalyzing hydrogen peroxide reduction [394]. Nrf2 transcription affects the downstream targets such as CAT, SOD 1, glutathione peroxidase (GPx) 2, heme oxygenase 1, and thioredoxin genes, ameliorating oxidative stress.

Q inhibits oxidative stress by regulating the antioxidant defense systems by balancing oxidant and antioxidant effects. By influencing the signaling transduction pathways, Q may modulate the enzymes or antioxidant substances that enhance antioxidant capacity, preventing oxidative stress in high volumes of TT events. Due to Q's effect, stimulating genes responsible for antioxidative effects may be beneficial during sport and practice as it may assist in anti-inflammatory processes.

Quercetin and Inflammation

Q is confirmed to be a long-acting anti-inflammatory agent in animal and human cells by scavenging free radicals [36, 416]. Free radicals can activate transcription factors to upregulate pro-inflammatory cytokines [36]. For example, Q can treat respiratory and food allergies by inhibiting IL-8 and IL-6 [416]. Further, Q exerts anti-inflammatory effects on endothelial and monocyte/macrophage systems in vitro [258]. In animal models, Q inhibited the production of TNF-α induced by LPS in macrophages [148] and lung A549 cells LPS-induced IL-8 production [147]. Furthermore, in glia cells, Q can suppress LPS-induced mRNA levels of TNF-α and interleukin1α: neuronal cell death is also reduced [65]. Q can inhibit the enzymes that produce inflammation cyclooxygenase (COX) and lipoxygenase (LOX) [299]. The COX-2 and iNOS were inhibited by Q by suppressing AP-1, NF-κB, and STAT-1 signaling in cytokine- or LPS-induced HUVECs and macrophages [170, 351]. The expression of pro-inflammatory cytokines in calcium ionophore-and PMA-induced mast cells was attenuated by Q. Moreover, the TNF-α-

stimulated NF-κB recruitment to pro-inflammatory gene promoters was also suppressed by Q in murine intestinal epithelial cells [315, 340]. The TNF-α- or PMA-induced expression of intercellular adhesion molecule (ICAM-1) in human endothelial cells was decreased by Q [227]. The LPS-stimulated NF-κB and nitrite oxide production was also inhibited by Q in mice. The properties of Q in the inflammation process may assist in repair and recovery after exercise.

Quercetin's Role in Glucose and Lipid Metabolism

Glucose Metabolism

The research on improving lipid metabolism with supplements is surging, especially with plant polyphenols. Q improves lipid metabolism in animal models by modulating the AMPK/PPARs signaling pathways via upstream (PI3K) and downstream enzymes (acetyl-CoA carboxylase). AMPK plays a crucial role in regulating lipid and glucose metabolism (Figure 7). AMPK signaling pathway coordinates glucose metabolism by regulating glycolysis and gluconeogenesis; additionally, AMPK controls lipid metabolism by acting on fatty acid synthesis and fatty acid oxidation [407]. Insulin-mediated GLUT4 translocation is a critical signaling pathway promoting glucose uptake in adipocytes and muscle cells. Insulin promotes glucose uptake in muscle and adipose tissue (AT) by facilitating glucose transporter 4 (GLUT4) translocation, which plays a vital role in whole-body glucose homeostasis. Insulin-signaling pathway leading to GLUT4 translocation is mediated through the activation of phosphatidylinositol 3-kinase (PI3K) [413], which subsequently triggers the activation of Akt. The downstream of Akt, two closely related Rab-GTPase-activating proteins (GAPs), Akt substrate of 160 kDa (AS160, also known as TBC1D4) and TBC1D1, have been demonstrated to associate with GLUT4 traffic in response to the insulin [216, 336, 342]. AS160 and TBC1D1 are negative regulators of basal GLUT4

exocytosis, but when phosphorylated, these Rab-GAPs release the inhibition on GLUT4 vesicles and then allow GLUT4 translocation to the plasma membrane to facilitate glucose uptake [342]. In addition to insulin, adenosine 5′-monophosphate-activated protein kinase (AMPK) contributes to glucose homeostasis by regulating GLUT4 [186, 187, 281]. Both exercise-associated and agent-induced AMPK activation in muscle induces phosphorylation of AS160 [111, 383, 388], indicating that AS160 is not only an Akt substrate but also a substrate for AMPK in the regulation of GLUT4 translocation.

Quercetin is the most important dietary source for flavone intake, with beneficial effects on glucose and lipid homeostasis [205, 224]. Some studies also reported that quercetin ameliorated insulin resistance by inhibiting inflammation in adipocytes and muscle cells [11, 83]. However, some studies show Q causes adverse effects on insulin-mediated glucose uptake in adipocytes [166, 209], giving rise to a somewhat paradoxical situation, evaluating the impact of quercetin on insulin action in adipocytes. Quercetin is a known AMPK activator [117], and several reports show that quercetin promoted glucose consumption in muscle cells and ameliorated endothelial dysfunction through regulation of the AMPK activity [117, 352, 413] (Figure 7). Evidence indicates that quercetin negatively affects insulin-regulated glucose homeostasis in inflamed cells under normal conditions through the AMPK-mediated mechanism [413].

Quercetin and Lipid Metabolism

AMPK suppresses the expression of NF-κB by increasing the expression of SIRT1, thereby minimizing the inflammatory response [422]. It has been well documented that the Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta (IKK-β) can regulate the expression of inflammatory molecules, including TNF and IL-6, through subsequent NF-B activation [413].

IKK/NF-B signaling activation links inflammation to insulin resistance by evoking the expression of a wide range of pro-inflammatory cytokines, inducing serine phosphorylation of IRS-1. This vital pathway blocks the functional interaction between IRS-1 tyrosine phosphorylation and downstream PI3K signaling in response to insulin [68]. Quercetin inhibited macrophage derived-induced IKK-β phosphorylation and showed beneficial regulation of serine/tyrosine phosphorylation of IRS-1, demonstrating its anti-inflammatory activity implicated in the regulation of IRS-1 function [413]. The practical regulation of IRS-1 function should be responsible for improving insulin signaling, as Q treatment inhibited the phosphorylation of tyrosine, Akt, and AS160. As a result, quercetin successfully restored GLUT4 translocation when cells were exposed to inflammatory challenges (Figure 7). However, quercetin may act as a competitive antagonist to insulin under normal conditions but confirms its opposite regulation of AS160 in adipocytes [413]. However, the law of AMPK activity is involved in both actions of quercetin.

Further, Q supplementation increased the phosphorylation of AMPK in cultured smooth muscle cells and aortic arteries, exhibiting increased levels of acetyl CoA carboxylase, a downstream protein of AMPK. Q supplementation increases the upstream proteins of AMPK, namely PI3K and PKB (enhancing glucose uptake), fatty acid synthesis enzymes (e.g., acetyl-CoA carboxylase), and beta-oxidation enzymes (e.g., carnitine palmitoyltransferase), and peroxisome proliferator-activated receptors (PPARs) [407]. Q phosphorylating the upstream proteins demonstrates its role in numerous signaling pathways. In rats, Q supplementation improved the expression of SIRT1 (improves cellular ability to remove ROS and increase mitochondrial biogenesis) [422]. Although most research is performed in animal models, Q increase may improve lipid metabolism and oxidation via the expression of AMPK via stimulating PI3K-PKB/AKT kinase activity [407]. Current literature states that Q may serve as a lipid metabolism signaling modulator

by accelerating lipolysis, fatty acid oxidation, and inhibiting lipid synthesis. Q may have promising effects on inhibiting inflammation and oxidative stress by modulating the AMPK/SIRT1 pathway.

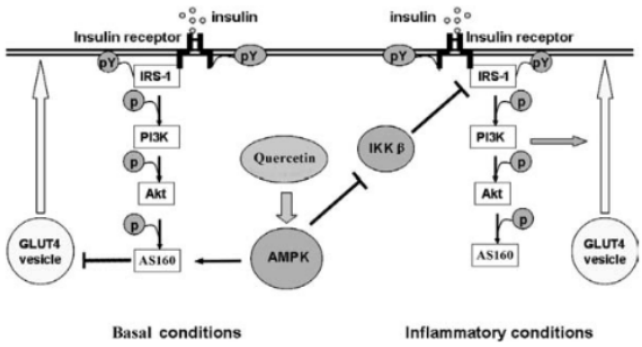

Figure 7. Proposed action pathway of quercetin in the regulation of GLUT4 translocation in adipocytes [413].

The benefit of Q is ascribed to its role as an antioxidant and anti-inflammatory molecule, which may help increase exercise performance. Q shows more of a health benefit (decreasing blood pressure, inflammation, and oxidative stress) in clinical populations [47, 201, 323, 416]. Still, future research is warranted to investigate its oxidative, anti-inflammatory, and recovery effects in exercise. Due to Q's ability to modulate antioxidative systems and inhibit the enzymes that produce inflammation, minimal information exists on how Q impacts sports performance and muscle-damaging bouts.

Quercetin Toxicity and Safety

The International Agency for Research on Cancer stated that Q is not classifiable as carcinogenic to humans [341]. Human studies do not show any adverse effects associated with the

oral administration of Q in a single dose of up to 4 g [341] or after one month of 500 mg twice daily for 4-8 weeks [202, 311, 354]. Clinical trials of Q currently recommend a dose of 1400 mg/m2, which corresponds to about 2.5 g for a 70 kg individual, administered via intravenous infusion at 3-week or weekly intervals [420]. At higher doses, healthy individuals consumed up to 50 mg/kg (about 3.5 g/70 kg), but renal toxicity was detected without signs of nephritis or obstructive neuropathy [420]. Additionally, the pharmacy compounding Advisory Committee Meeting approved quercetin dihydrate, part of inclusion on 503A bulk drug substance evaluation and dietary supplements for use in compounding (health symptoms, asthma, cancer treatment, and hypertension) [138]. FDA committee members stated the number of these products containing quercetin dihydrate for these conditions is already proven to be safe and efficacious. The consumption of Q in high doses (200-500mg, often found in diets rich in fruits and vegetables) is likely safe. However, no longer-term studies to date have been performed in humans. Previously mentioned, there does not appear to be a toxicity threshold from high Q dosages (>3.5 g/day).

Amino Acids as Ergogenic Aids

Along with plants, protein and protein breakdown constituents are among the most popular dietary supplements and ergogenic aid marketed to athletes. Protein supplements are recommended to athletes to enhance nitrogen retention, increase muscle mass, prevent protein catabolism during prolonged exercise, enhance muscle protein synthesis (MPS), increase muscle glycogen resynthesis following exercise, and prevent sports anemia by promoting an increased synthesis of hemoglobin, myoglobin, oxidative enzymes, and mitochondria during aerobic training [70, 409]. However, there is debate on how much protein athletes need, concluding that very active individuals require higher protein, 1.4-2.0 g/kg [70]. It is suggested that resistance athletes need

1.6–1.7 g protein/kg body weight while endurance athletes need approximately 1.2–1.4 g protein/kg, values that are about 150–200 percent more than the current United States Recommended Dietary Allowances [70, 409]. However, there is novel evidence that suggests higher protein intakes (>3.0 g/kg/d) may have positive effects on body composition in resistance-trained individuals and may promote loss of fat mass [70]. It is recommended that acute protein doses should strive to contain 700–3000 mg of leucine and a higher relative leucine content, in addition to all the essential amino acids (EAAs), and should be ingested evenly every 3–4 h across the day to most effectively stimulate MPS [70]. A great deal of literature supports pre-exercise protein ingestion, and even intra-exercise protein ingestion can help higher levels of MPS [70, 385]. For instance, whey protein ingested close to resistance exercise promotes a higher phosphorylation of mammalian target of rapamycin (mTOR) (an essential signaling protein found in myocytes that are linked to the synthesis of muscle proteins) and its downstream mRNA translational signaling proteins (i.e., Ribosomal protein S6 kinase beta-1 (P70S6K) and Eukaryotic translation initiation factor 4E-binding protein 1 (eIF4BP)) suggesting timed ingestion of protein may favorably promote heightened muscle protein synthesis (hypertrophy) [70]. However, the timing of protein near (± two h) aerobic and anaerobic exercise training appears to provide greater activation of the molecular signaling pathways that regulate myofibrillar and mitochondrial protein synthesis and glycogen synthesis [70]. Further, evidence suggests post-protein consumption sooner rather than later after exercise since post-workout MPS rates peak within three hours and remain elevated for an additional 24–72 h [64, 70].

Further, there are many different types of protein with varying branched-chain amino acid (BCAA) compositions. For example, plant-based or animal-based protein. Mostly consuming high-quality, animal-based products (meat, milk (e.g., whey and casein), eggs, and cheese),

an individual will achieve optimal growth as compared to ingesting only plant proteins [70]. Further, digesting ability of the protein affects MPS. For example, casein and fatty cuts of meat are slower digesting proteins than fast-digesting proteins (i.e., whey). [70] The literature concludes that athletes should seek fast-digesting protein sources, essential amino acids, and high leucine (increases rates of MPS) content to maximally stimulate rates of MPS (98% greater with whey) while decreasing muscle protein degradation and possibly aiding in recovery from exercise [70]. Further, a combination of proteins is likely to appear in many ergogenic supplements to elicit additional benefits. For example, a variety such as whey and casein yielded the most significant increase in fat-free mass [222] and muscle fiber area [173], and a positive and prolonged amino acid balance when compared to the ingestion of whey protein alone.

The optimal protein intake per serving for athletes to maximize MPS is dependent upon age, intensity, volume, progression of exercise, length of the training program, training status, energy intake, quality and quantity of protein intake, recent resistance exercise stimuli, and co-ingestion of additional dietary ingredients that may impact strength (e.g., creatine) [70]. It is recommended that whole-food sources that contain high-quality (e.g., complete) sources of protein should be consumed before dietary protein supplements; however, supplemental amino acids and protein are a safe and convenient method of ingesting high-quality nutritional protein to enhance performance, increase the secretion of hormones, prevent overtraining, and prevent mental fatigue. For example, some research states that increased serotonin levels may cause fatigue [297]. During prolonged aerobic endurance exercise, muscle glycogen may become depleted. The muscle may increase its reliance on BCAA for fuel, decreasing the plasma BCAA: free tryptophan (fTRYP) ratio (forms serotonin). Since BCAA competes with fTRYP for entry into the brain, a low BCAA:fTRYP balance would facilitate the access of fTRYP to the brain and result in the

formation of serotonin [409]. Hypothetically, BCAA supplementation may delay central nervous system fatigue and enhance performance in prolonged aerobic endurance events by increasing the BCAA:fTRYP ratio, mitigating the formation of serotonin, and delaying mental fatigue during performance. Further, arginine supplementation may be theorized to be ergogenic because it is a substrate for NO synthesis by increasing L-citrulline concentration. This potent endogenous vasodilator may benefit blood flow and endurance capacity [409]. Research regarding the ergogenic effect of protein and amino acids is promising and safe but may interfere with protein metabolism if consumed in excess. However, a non-essential amino acid, citrulline may assist with energy production during cycling via eNOS.

Citrulline

Background

Citrulline (CIT) (Figure 8) (commonly found as L–citrulline) is a non-essential amino acid, a component of the urea cycle in the liver and kidneys, and a Krebs cycle intermediate. It demonstrates unique metabolic properties, emerging as a promising pharmaconutrient and ergogenic aid [8, 127, 394]. It is rarely found in food but often found in high concentrations of watermelon [89, 135, 394]. The concentration of l-citrulline in the United States grown and fresh watermelon can range from 1.6 to 3.5 g/kg [8, 101, 376]. The consumption of approximately 1–1.5 kg/day (2.2–3.3 lbs/day) of fresh watermelon would be needed to achieve the minimum effective dose of l-citrulline (3 g/day) and 3.3–5.0 kg/day (7.3–16.5 lbs/day) would be needed to achieve the maximum effective dose of l-citrulline (10 g/day) [8, 376].

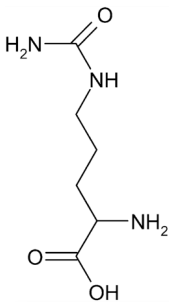

Figure 8. Chemical structure of citrulline.

Citrulline is implicated in several regulatory roles, including gut modulation [394], assisting as an antioxidative [17, 103] and anti-inflammatory [1, 8], protein synthesis and recovery [156, 157], upregulating endothelial NO synthase (eNOS) (Figure 9-10) expression, improving endothelial function in animal models [177, 411], NO production via arginine production [17, 179, 347], cardiac function [8, 22, 103], skeletal muscle function (e.g., mitochondrial biogenesis) [400], oxygen uptake, high-intensity exercise performance [19], vascular health, lipid, and energy metabolism [203], protein synthesis [284], and thermoregulation [8, 378]. Further, CIT's role accounts for 90% in the urea cycle [394] and assists in converting toxic ammonia, originating from protein catabolism, into urea and ultimately excreted in the urine [26]. In the skeletal muscle and peripheral tissues, glutamate accepts this free ammonia which results in the formation of glutamine. Glutamine is then exported from the skeletal muscle and tissue and utilized by the liver. Glutaminase breaks down glutamine into glutamate and ammonia. This free ammonia is incorporated into hepatocyte mitochondria and results in the formation of urea [26]. Further, CIT can bypass hepatic catabolism, allowing CIT to effectively increase the peripheral levels and tissue contents for l-arginine and NO.

Due to its role in L-arginine (arginine) production and NO, citrulline increased cycling TT performance [364, 374] and oxygen uptake in males [19]. However, much research focuses on the combination of citrulline and malate in the performance [94, 151]. CIT is often supplemented with malate (1:1 ratio), or as watermelon extract, as with many other NO-boosting supplements. It has received much interest for its potential cardiovascular and anti-hypertensive capabilities [8]. However, the role of malate in combination with citrulline is largely undetermined. Since malate is an important tricarboxylic acid cycle intermediate, this could account for improvements in the muscle function [405]. Therefore, it is unclear whether these benefits can be solely attributed to citrulline and what role it may play in aerobic and anaerobic performance.

Bioavailability and Metabolism of Citrulline

Citrulline is directly formed from arginine via the activity of NOS enzymes (e.g., eNOS, inducible NOS (iNOS), and neuronal NOS nNOS). Arginine supplementation is largely ineffective at increasing NO synthesis, causing gastrointestinal distress symptoms [8]. Since citrulline is found in high concentrations in watermelon [89, 135] and is a neutral amino acid (formed by enzymes in the mitochondria), it serves as a substrate for recycling arginine [8]. Unlike arginine, citrulline is not extracted from the gastrointestinal tract (i.e., enterocytes) or liver; therefore, it is more effective at increasing arginine levels and NO synthesis [8]. Further, CIT metabolism in the liver is somewhat compartmentalized to the urea cycle, and exogenous CIT typically bypasses hepatic metabolism [8, 394]. CIT is synthesized from glutamine in the enterocytes within the small intestine; citrulline is released into blood circulation for metabolism and conversion into arginine by the kidneys [394]. The small intestine is established as the primary site for citrulline production due to the abundance of citrulline synthesizing enzyme (pyrroline-5-

carboxylate synthase) and the lower activity of citrulline catabolizing enzymes, such as argino-succinate synthase and argininosuccinate lyase [394]. Pyrroline-5-carboxylate synthase is almost solely located in the intestinal mucosa. The synthesis of CIT from l-glutamine occurs through a transaminase reaction in the enterocytes [394]. The citrulline is produced from the enterocytes, released from the gut, and absorbed by proximal tubular cells in the kidney. The enzymatic actions of arginosuccinate synthase are converted into arginosuccinate, which is then converted into arginine by arginosuccinate lyase [163, 394] (Figure 9-10).

CIT serves as the direct precursor of arginine. The synthesis of arginine from CIT is essential for downregulating urea formation in the liver during periods of low protein intake to increase nitrogen retention [8]. This amino acid is involved in several physiological roles: protein synthesis, urea cycle function, creatine and polyamines synthesis, ammonia detoxification, and NO synthesis [394]. This partial urea cycle meets the demand of the body's arginine requirement, NO responses (Figure 9), and helps maintain protein homeostasis. However, arginine is required for NO formation via eNOS, iNOS, and nNOS. This is particularly useful in endothelial cells via eNOS and activated macrophages via iNOS to sustain CIT as a precursor to l-arginine to produce NO [8]. However, physiologically high concentrations of CIT are necessary to maximally stimulate iNOS activity in cultured smooth muscle cells [8]. This is because aortic smooth muscle cells take up CIT at a relatively slower rate than arginine due to a low infinity transporter [8]. For conditions associated with impaired arginine metabolism, arginine deficiencies, and NO metabolism disorders, CIT supplementation offers a potential therapeutic strategy.

Following oral l-citrulline ingestion, circulating l-arginine concentrations peak after ~1–2 h [284]. As shown for both l-arginine and l-ornithine, circulating concentrations of l-citrulline return to baseline within eight hours [284]. There is higher activity bioavailability of CIT

than arginine due to the absorption in the small intestine's enterocytes [8]. For example, 0.75 grams of CIT ingested twice daily (1.5 g total) increased the l-arginine concentration similarly to consuming 1.6 g of l-arginine twice daily (3.2 g total) [8]. CIT does not cause gastrointestinal distress compared to arginine. This may be because of utilizing a different transport system. Arginine is mainly transported across the intestinal membrane through Na+-independent cationic amino acid transporters [85]. In contrast, a CIT transporter has not been identified; however, the B^0-transporters have been suggested to play a role in the Na^+ dependent transport of l-citrulline across macrophages, glial cells, aortic smooth muscle, with the highest enterocytes reported in the enterocytes [8]. However, to date, only a few studies have investigated the pharmacokinetics of the CIT supplementation [8, 284].

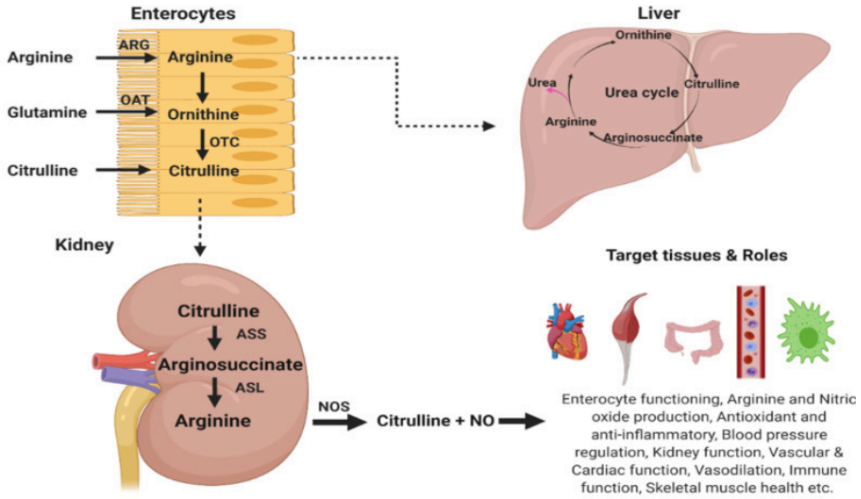

Figure 9. Citrulline metabolism and related amino acids.

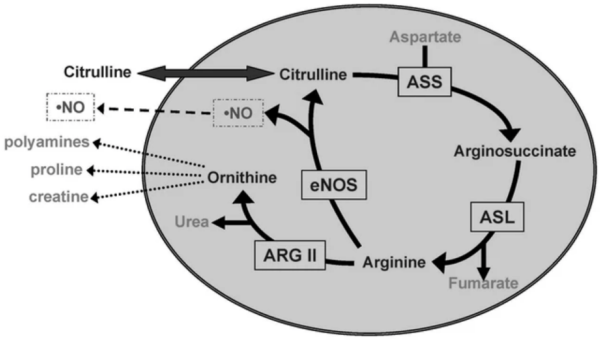

Figure 10. Citrulline-Arginine-NO pathway in endothelial cells.

Mechanisms of Action: Citrulline's Role in Exercise

Citrulline's Role in Nitric Oxide Production

In endothelial cells, NO is synthesized from l-arginine (precursor) by eNOS, generating NO and l-citrulline (end-products) (Figure 9-10) [8, 129, 395]. NOS activity and NO production increase during contraction of muscle cells [188]. NO released from the endothelium as a gas initiates a signaling cascade involving the activation of soluble guanylate cyclase (sGC) to increase cyclic guanosine monophosphate (cGMP) synthesis [8] (Figure 12). Increased cGMP levels act as a second messenger to increase NO and vasodilation by relaxing the smooth muscle cells and resistance arteries [13]. NO production increases endogenous production of nitrate/nitrite limb blood flow and improves oxygen consumption during exercise via nitrates and nitrites, reducing back to NO [209]. It is suggested that this alternative pathway may complement the l-arginine–NOS–NO path by enabling NO production in conditions of low oxygen availability in which NOS activity (which is oxygen dependent) may be reduced [261] (Figure 10). In addition to its generation using the NOS system, body stores of nitrate and nitrite may also be increased exogenously through the diet, mainly through the consumption of green leafy vegetables such as lettuce, spinach, rocket, celery, cress, and beetroot, which typically contain over 250 mg (>4 mmol)

nitrate per 100 g fresh weight [191, 261]. Evidence suggests that enhancing NO bioavailability by augmenting the nitrate–nitrite–NO pathway may influence muscle function and exercise performance [8]. However, the potential role of CIT in regulating skeletal muscle protein metabolism and cell size remains unknown.

Nitric Oxide Effects in Exercise

NO can modulate skeletal muscle function by regulating blood flow, contractility, glucose calcium homeostasis, mitochondrial respiration, and biogenesis [363], in vivo. However, in humans, the influence of NOS blockade is more controversial, but there are indications that NO is involved in regulating blood flow and VO_2 [57]. NO/cGMP increases mitochondrial biogenesis in vitro and in vivo, and this stimulation improved mitochondrial function, resulting in an enhanced formation of ATP [301]. Enhanced ATP production was accompanied by an increased expression of display an increase in mitochondrial DNA content, cytochrome *c* and COX IV protein expression levels [62], as well as PGC-1α, NRF-1, TFAM, satellite cell activation [168], mediated by NO/cGMP signaling pathway [301]. NO confirmed the crucial role in the cGMP pathway and plays a critical role in mitochondrial biogenesis, mitochondrial respiration, glucose uptake, and calcium handling (Figure 11). In addition, NO plays a role in overload-skeletal muscle hypertrophy and may serve as a protective mechanism against catabolic stimuli, modulating muscle protein metabolism and attenuating muscle wasting [168]. However, these increases do not necessarily imply that the resultant mitochondria are functional and apply to humans.

In the skeletal muscle, calcium (Ca^{2+}) is released from the sarcoplasmic reticulum (SR) via ryanodine receptor (RyR) channels. Previous studies indicate that NO increases the open probability of the RyR channel in the absence of a RyR agonist [183]. Few studies show NO's

effects on SR Ca^{2+} release in intact cells. In a recent study, NO increases Ca^{2+} concentration responses during submaximal activation in mouse single muscle fibers [10]. However, it was uncertain whether this resulted from increased RyR channel mediated Ca^{2+} release or impaired Ca^{2+} reuptake. NO can react with thiol groups via either *S*-nitrosylation or by influencing the disulfide formation [183]. Therefore, the RyR Ca^{2+} release channel is a possible target for NO. The hypothesis that NO may modulate SR Ca^{2+} release is promising under low-frequency electrical signals and fatigue by controlling thiol groups [183]. However, under conditions of high electrical signals and activation, NO may decrease the open probability of the RyR Ca^{2+} release channels and may alter the force generation [183]. NO also increases SR Ca^{2+} leak through RyR, changing the beta-adrenergic tone and the activation of Ca-calmodulin-dependent protein kinase (CaMKII) and the subsequent phosphorylation of the ryanodine receptor in wild type ventricular myocytes [96]. However, this effect was ablated by the inhibition of NOS. Evidence supports that NO regulates CaMKII and the release of Ca^{2+}. However, the degree of electrical stimulation that determines NO's effects on Ca2+ release and force generation is unknown. NO has a complex impact on Ca^{2+} handling and the extent of force generation (via inhibition of cytochrome c) [62]. This may affect exercise performance by NO inhibiting cytochrome c oxidase during oxidative phosphorylation, competing with oxygen and thus limiting ATP production. This can cause irreversible inhibition of the respiratory chain, uncoupling (proton leak), permeability transition, and cell death [62]. Thus, NO inhibition of cytochrome c oxidase may involve the physiological regulation of respiration rate having a greater affinity for oxygen during exercise.

NO is produced within the skeletal muscle and has various functions in the skeletal muscle. There is evidence that NO may be essential for glucose uptake during contraction. It is consistently stated that NO inhibition limits glucose uptake during contraction in animal models and

humans [188]. The NO-mediated increase in skeletal muscle glucose uptake during contraction/exercise is likely due to the modulation of intramuscular glucose signaling that ultimately increases glucose transporter 4 (GLUT4) translocation, independent of the blood flow [188]. This may occur through the phosphorylation of the primary downstream signaling mediators of NO, including cGMP, PKG, peroxynitrite, or via various post-translational protein modifications (e.g., *S*-nitrosylation or A-glutathionylation) [188]. Evidence for these signaling mechanisms in stimulating skeletal muscle glucose uptake during contraction/exercise is very limiting. Peroxynitrite causes tyrosine nitration and activates proteins associated with glucose uptake such as PI3K and AMPK [425], and protein kinase C [21], suggesting a possible role of peroxynitrite in muscle glucose uptake during contraction. Evidence indicates that NO plays a vital role in skeletal muscle glucose uptake during exercise, highlighting the cGMP target; however, the source of the NO and the downstream mechanisms involved are not fully elucidated.

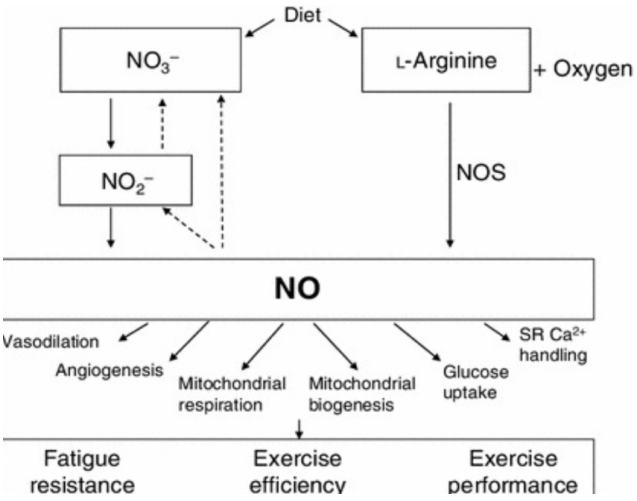

Figure 11. NO pathways and performance adaptations [353].

High NO levels also reversibly inhibit mitochondrial oxygen consumption by competitive binding to the heme a_3 site of cytochrome oxidase in competition with the oxygen [57]. Higher (mM) NO concentrations lead to peroxynitrite production and irreversibly inhibit complexes I and II of the electron transport chain. However, studies examining mechanisms of blood flow control have revealed conflicting findings on whether NO increases or decreases VO_2 during contraction [57, 209, 241]. In humans, NOS inhibition is found to have little or no effect on $\dot{V}o_2$ across the exercising leg or forearm measured by the Fick method [185, 241]. High nitrate levels can lower oxygen cost by increasing abundances of ATP, ADP, lactate, glycolytic intermediates (e.g., fructose 1,6- bisphosphate), tricarboxylic acid (TCA) cycle intermediates (e.g., succinate), and ketone bodies (e.g., β-hydroxybutyrate) by 1.8- to 3.8-fold, compared to nitrite in zebrafish [16]. Further, NO could reduce oxygen cost during physical exercise, accompanied by improved mitochondrial oxidative phosphorylation efficiency (P/O ratio) and a decrease in state four respiration measured ex vivo in isolated mitochondria [241]. However, the nitrate–nitrite–NO pathway may influence muscle function and exercise performance, but research is limited, especially in humans.

Reduced Nitric Oxide Effects on Disease and Exercise

Reduced eNOS expression and NO production/bioavailability have been reported in patients with essential hypertension, healthy older individuals, and heart failure patients [8, 223]. Further, reduced NO bioavailability has both direct and indirect effects on skeletal muscle metabolism, energy production, and exercise performance that likely contribute to the development of insulin resistance and type 2 diabetes and age-related muscle wasting [8, 384].

Elevations in ROS, especially superoxide, can reduce NO's bioavailability by generating peroxynitrite; this can promote reductions in NO synthesis and may cause endothelial dysfunction commonly associated with cardiometabolic diseases [140]. Thus, augmenting l-arginine levels in the circulation may represent a potential therapeutic mechanism to increase NO synthesis, bioavailability, vasodilation, and oxygen consumption during exercise. However, oral l-arginine supplementation is ineffective mainly due to gastrointestinal and hepatic extraction of l-arginine [76] and a dose-dependent presentation of gastrointestinal distress [161]. Alternatively, oral CIT supplementation consistently increases l-arginine and NO bioavailability and may assist in increasing vasodilation, muscle perfusion, lipolysis, mitochondrial biogenesis, and protein synthesis [8, 284, 347]. In gastrointestinal situations where arginine synthesis is compromised, CIT becomes a conditionally essential amino acid, thus justifying dietary supplementation of CIT [284]. CIT is a significant precursor of arginine (through renal conversion) (Figure 12) and suggests that CIT might be particularly useful for patients with impaired arginine metabolism, in short-bowel-syndrome patients, or malnourished patients [284].

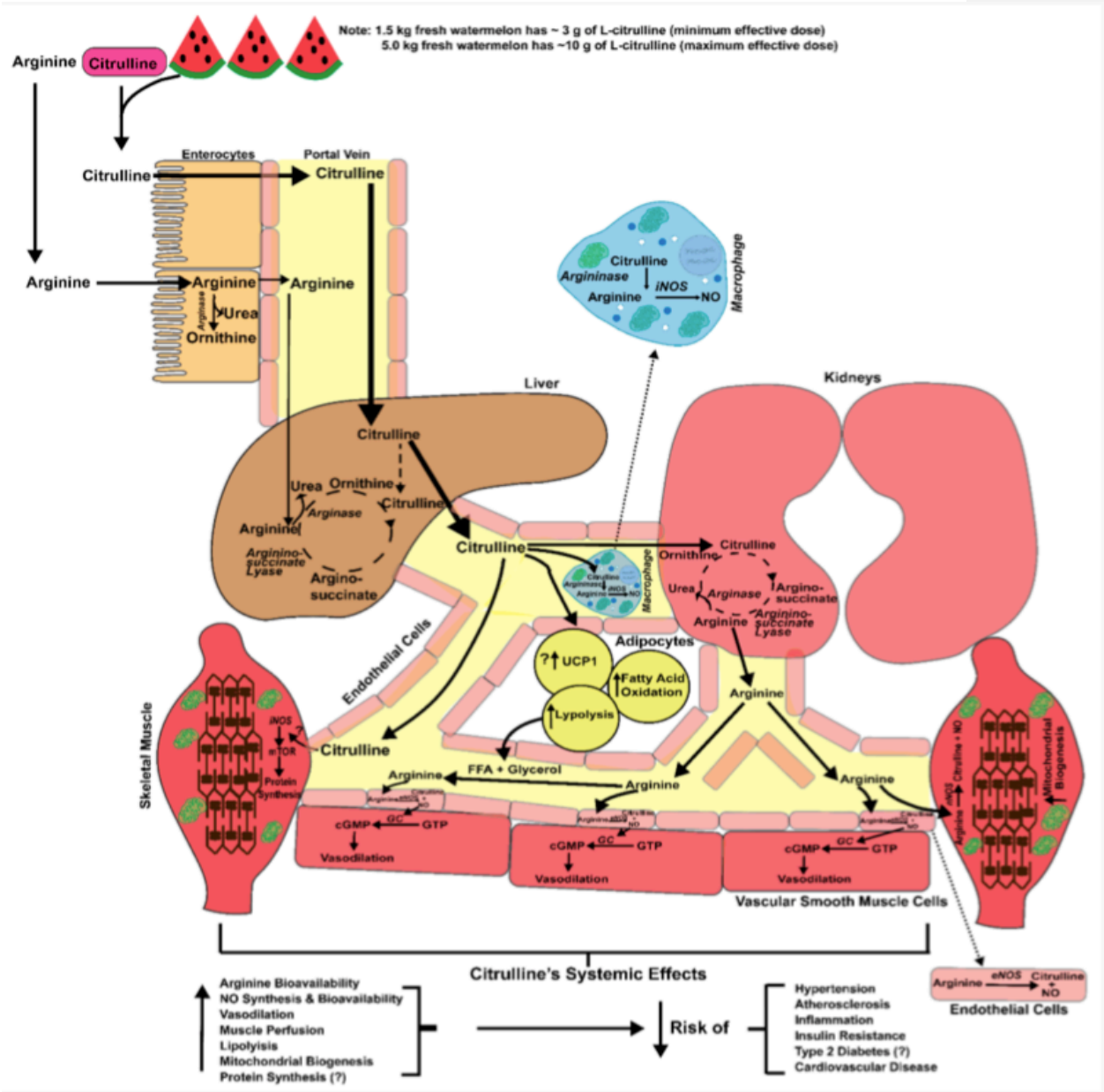

Figure 12. Comparison of L-Citrulline on mechanisms of action versus L-Arginine.

Citrulline's Role in Skeletal Muscle Health

Supplementation with essential amino acids, especially branched-chain amino acids (BCAAs), is extensively studied to improve skeletal muscle, MPS, and cardiometabolic health [8, 377]. In a human study, participants with anabolic resistance to increased protein intake did not experience elevated MPS following resistance training with the co-ingestion of CIT and whey protein but only improved arginine concentrations, contrasting to improvements in aged malnourished rats [8]. This is consistent with other studies that CIT alone may not sufficiently stimulate NO-mediated vasodilation to increase MPS [223, 347]. However, supplementation with CIT for three weeks did increase lean body mass in a subgroup of women. CIT may benefit older women due to reductions in age-related skeletal muscle vasodilatory capacity and blood flow by its vasodilatory effects on MPS [57]. As mentioned above, the generation of NO by NOS can increase mitochondrial biogenesis by activating PGC1α. One study shows that supplementing with CIT (250 mg/kg) for 15 days in mice resulted in elevations in PGC1-α mRNA and protein expression in hindlimb and forelimb muscles [400]. These elevations in PGC1α mRNA and protein expression were associated with improvements in exercise performance as measured by time to exhaustion during a standardized swim test [400]. CIT prevented inflammation and oxidative stress-induced muscle cell wasting [168]. Muscle cell wasting is the loss of skeletal muscle or atrophy from a chronic imbalance between rates of muscle protein synthesis and breakdown [168]. It is associated with a lack of muscle function, diseases, and conditions such as chronic heart failure, cancer and can reduce the quality of life. As such, the development of strategies to prevent muscle wasting especially post-exercise is of importance. The hypothesis of how CIT prevents muscle cell wasting is mediated through iNOS, a factor that contributes to skeletal muscle wasting. iNOS promotes the induction of antioxidant genes, specifically SOD, CAT, and

reduces the expression of specific muscle ubiquitin ligase atrogin-1, responsible for tagging the protein for removal [168]. Increased expression of ROS is responsible for muscle-wasting conditions; CIT improved antioxidant gene expression and indicates enhanced the antioxidant defense system and preserve the rates of protein synthesis [168] CIT supplementation increased L-arginine concentration and may represent an effective anabolic treatment on protein metabolism [168]. In rodents, L-citrulline effectively restores muscle L-arginine stores and reduces muscle wasting in L-arginine-deficient and low-protein intake conditions. Pre-clinical studies support the benefit of CIT supplementation on skeletal muscle bioenergetics. Still, future studies are warranted to determine the potential effects and mechanisms of CT in older adults and various populations.

Citrulline's Role in Vascular Function and Vasodilation

Chronic CIT supplementation increases NOS, decreases blood pressure, and may increase peripheral blood flow [135]. These changes improved skeletal muscle oxygenation and performance during endurance exercise. In adults with prehypertension or hypertension, citrulline/watermelon supplementation showed an antihypertensive effect, but not in the normotensive group [135]. However, CIT supplementation may attenuate the blood pressure response to exercise in normotensive men. The beneficial vascular effects of CIT/watermelon supplementation may result from improvements in the arginine/NO pathway. CIT/watermelon supplementation may show reductions in resting blood pressure with cardiovascular diseases and implications for individuals with prehypertension and hypertension [135].

Further, CIT may improve endothelial vasodilation function microcirculatory (gut villi) blood flow by reducing the synthesis of eNOS. Endothelial NOS plays a significant role in the

endothelial dysfunction associated with aging, menopause, and cardiometabolic disease [8]. The endothelial function may be enhanced by l-citrulline supplementation's capability to increase arginine levels. However, improvement in endothelial function, as measured by flow-mediated dilation (FMD), have not been reported with acute or short-term (~7 days) administration of CIT, despite significantly increased arginine bioavailability and increased urinary nitrate/nitrite (NOX) [8, 223, 328]. Further, older adults with heart failure acutely ingested l-citrulline (10 g) and l-arginine, and NO synthesis were increased after supplementation. However, CIT supplementation did not improve forearm blood flow during reactive hyperemia (measured by plethysmography) in these subjects' [223]. However, CIT supplementation at 800 mg/day for eight weeks elevated plasma arginine levels and improved FMD in humans with vasospastic angina [287]. Blood flow and vascular conductance during exercise were improved at higher workloads, following 14 days of CIT (6 g/day) in older men but not in the women's group [155]. Although blood flow enhancement is a proposed mechanism for the ergogenic potential of CIT, evidence supporting acute improvements in vasodilation, vascular conductance, and skeletal muscle tissue perfusion after supplementation is scarce and inconsistent. Several studies have reported that CIT supplementation can enhance exercise performance and recovery [156]. The research elucidates the potential synergistic effects of CIT with other dietary ingredients (e.g., arginine, antioxidants, nitrates, and branched-chain amino acids). However, future studies should continue to investigate the effects of both acute and chronic supplementation of CIT on blood flow markers, oxygenation, and exercise performance and explain the mechanisms underlying such effects [156]. However, the acute time course, length of treatment, and the health state of the participants may explain the lack of benefit of CIT supplementation.

Citrulline's Role in Cardiometabolic Health and Disease

Endothelial dysfunction, often associated with obesity-induced insulin resistance, is thought to be a significant factor in the development of cardiovascular disease [8]. Dietary factors, such as high fat/high cholesterol diet, adipocyte-derived factors, and aging [8], have also been implication in promoting low-grade inflammation and further exacerbated endothelial dysfunction, contributing to the development of cardiovascular disease. Subsequent CIT or arginine treatment increased high-density lipoprotein (HDL) levels and reduced serum alanine and aspartate aminotransferase (indicative of liver stress and damage) in diseased patients [8]. It confirmed modest structural changes in the endothelial structure of the thoracic aorta [8]. Also, endothelial dysfunction is associated with the inability to oxidize fatty acids (lipolysis) in adipose tissue and adipocytes [8, 207]. However, few studies have examined the impact of CIT on lipolysis, but most show CIT is ineffective by increasing mitochondrial uncoupling [8, 41]. CIT's role in the adipocyte has yet been explored.

Data supports the idea that CIT may protect against the liver damage and endothelial dysfunction induced by chronic exposure to a Western DIET (high fat/high cholesterol/high fructose) diet. Further, CIT can preserve endothelial function in animal models during exposure to a high cholesterol diet by decreasing the production of superoxide and associated oxygen-sensitive proteins (e.g., ELK-1 and p-CREB) [177]. The mechanisms of CIT to improve endothelial dysfunction, in conditions such as insulin resistance (i.e., type II diabetes), are most likely mediated via direct reduction of ROS (e.g., hydroxyl radicals) and oxidative stress, direct action on vascular smooth muscle, and indirect action of NOS [8]. Further, CIT supplementation is also likely to indirectly benefit vascular health by modulating chronic low-grade inflammation by reducing inflammatory cytokine concentrations, such as IL-6, tumor necrosis factor (TNF)-alpha, and C-

reactive protein in both aged animals and humans [8]. Although the exact mechanisms underlying the CIT-mediated improvements in systemic inflammation remain unknown, CIT may exert anti-inflammatory effects by dampening the macrophage cytokine production [58]. CIT may also improve immunity by reducing cytokine-induced low-grade inflammation. The amino acid CIT can directly increase immune cells' metabolism, function, and survival (e.g., CD4+ T-cell) [8]. Supplementation with CIT shows a promising intervention as a blood pressure-supplement (both resting and stress-induced) in adults with pre-/hypertension, and for atherogenic-endothelial protection, in animals [8]. In obese pre and hypertensive men, treatment with watermelon extract containing six g/day of l-citrulline/l-arginine for six weeks exhibited reduced ankle and brachial systolic and diastolic blood pressure [133].

For example, short-term treatment for 7–14 days with l-citrulline (5.6 g/day) reduced arterial stiffness in healthy and overweight middle-aged men [344] and six weeks of watermelon extract (6 g/day l-citrulline) showed evidence of reduced arterial stiffness in obese postmenopausal women [134]. CIT promotes favorable adaptations in blood vessel wall stiffness, improves blood flow responses (via eNOS), and reduces hypertensive and pre-hypertensive effects. CIT ingestion in patients with heart failure improved right ventricular function by increasing right ventricular ejection fraction (stroke volume/end-diastolic volume) [309] and left ventricular ejection fraction [22], attributing to decreases in systolic pulmonary artery pressure. CIT has likely clinical benefits in cardiometabolic risk populations, especially in decreased insulin secretion [27] and insulin resistance, hypertension, long-term blood pressure regulation, cardiac function, oxidative stress, and inflammation. However, the patient population, dose, and duration of treatment appear to impact the magnitude of these effects and warrant future research.

Citrulline Toxicity and Safety

Clinical dose-ranging and tolerability studies have also been conducted for CIT supplementation. A human study demonstrated a tolerance of up to 15 g CIT per day in the healthy volunteer's [284]. In comparison, high doses of l-arginine (~13 g) can induce significant gastrointestinal complications [8, 161]. However, at 15 g doses of CIT, a lower fractional absorption rate and plasma retention of l-citrulline were observed, potentially due to saturation of its transporters (e.g., $B^{0,+}$-amino acid transporter) or reduced renal conversion of CIT to arginine. A dose of 10 g o-f CIT is suggested for clinical use [284]. However, amounts of CIT as low as 3 g are adequate [8]. Thus, the minimum effective dose of CIT is ~3 g/day, where a sufficient amount may be as high as ten g/day, depending on the initial concentrations. However, much CIT research focuses on short-term ingestion (weeks). Further, human umbilical endothelial cells have shown that repeated exposure to arginine via CIT supplementation can promote oxidative stress by increased superoxide formation and reduced eNOS protein expression [8]. Prolonged exposure to l-arginine may promote cellular tolerance and maladaptation. Further, most CIT studies fail to report the plasma and serum concentrations. Additionally, a balancing of evaluation criteria weighs in favor of CIT for oral administration being added to the list of bulk drug substances that can be used in compounding under section 503A of the FDA Food and Drug Committee Act [208]. Further, the Adverse Event Reporting System (CAERS) reported CIT combined with productions, herbs, vitamins, and/or other amino acids showed 3 deaths out of 332 reports [208]. However, decades of CIT have not been associated with safety concerns. Additionally, chronic oral use of CIT is the standard of care. There are published dosing recommendations for CIT [8, 165], according to dosages of peroral drugs for long-term treatment of urea cycle disorders [165]. Additionally, Eight fasting healthy males underwent four separate oral loading tests (2, 5, 10 or 15 g

CIT) in random order for an 8 hr period [284]. Results showed that none of the subjects experienced side effects whatever the CIT dose. Even at high doses, urinary excretion of CIT remained low (< 5 %) [284]. Plasma insulin and growth hormone were not affected by CIT administration. Short-term CIT administration is safe and well-tolerated. CIT is a potent precursor of ARG. However, at the highest doses, CIT accumulated in plasma while plasma ARG levels increased less than expected. However, there have been no clinical trials to assess safety or efficacy; still, long-term studies (>6 months) require further investigation, especially in cardiovascular diseases.

The Combination of Quercetin and Citrulline

Citrulline is a unique amino acid that exerts effects on protein synthesis, lipid, energy metabolism, cardiovascular function, and metabolic and vascular health; however, CIT's exact mechanisms in sports performance require investigation. Several studies show the efficacy of CIT in improving NO, blood flow, and mitochondrial respiration. Quercetin decreases cardiovascular disorders, such as high levels of oxidative stress and inflammation. It increases lipid and glucose metabolism via phosphorylation of transcription proteins, which can improve sport and exercise. The benefits of Q are ascribed to its combination of antioxidant and anti-inflammatory activity with other ingredients (e.g., Vitamin C, copper, magnesium, iron, ruthenium, cobalt and cadmium, calcium, and rare earth elements). Co-congestion may improve each substance's bioavailability and elicit synergistic effects on exercise performance. A polyphenol combined with an amino acid has unique chemical properties that determine some specific actions in different cellular compartments. Although evidence is lacking, one study, [394], has looked at the co-ingestion of Q and citrulline on gut function. The gut microbiota's fundamental roles include: regulating energy metabolism, inflammation, nutrient harvesting, maintaining the gut-epithelial barrier

(via tight-junction proteins), and is associated with cardiovascular events such as regulating blood pressure, vasodilation, and producing short-chain fatty acids that upregulate PGC-1α, driving mitochondrial biogenesis [84, 291] and an improvement in performance [274]. Q + CIT can elicit potential synergistic roles to attenuate intestinal inflammation, promote gut health and increase energy metabolism and endurance performance capacity. High intensity and prolonged exercise are associated with exercise-induced muscle damage, oxidative stress, and inflammation and, in some cases, can cause gut dysbiosis [77, 131, 286]. The inflammatory process releases inflammatory cytokines such as TNF-α, IL-1β, and TGF-α, which can alter tight junction proteins on the endothelium. This can increase gut permeability, cause inflammation, lead to gut dysbiosis, decrease metabolism and energy expenditure, reducing exercise performance [262].

It is well known that quercetin interacts with other dietary components, such as selenium, polyunsaturated fatty acids, sulfur-containing amino acids, minerals, and other antioxidants to improve its bioavailability [123-125], gut function [394], and decrease inflammation and oxidative stress. Further, studies have shown that quercetin augments NO production and vasodilation via eNOS activation [257], eliminating endothelial dysfunction. Recently, quercetin induced vasodilation by enhancing NO synthesis and promoting intracellular calcium-activated potassium channels [422, 423]. Quercetin also restored the intravascular homeostasis and endothelial functions by attenuating excess NO production induced by ATP, decreased intracellular calcium flux, and eNOS activity in vascular endothelial cells [109]. Further, quercetin reversed the endothelial damage arising from excessive NO by attenuating nitrification stress and protecting the endothelial cells [394]. Since citrulline functions to increase cellular metabolism and is an efficient precursor for NO synthesis, blood flow, and endothelial functions in the gastrointestinal system [394], there is a promising link between the co-ingestion of quercetin and citrulline. The

combinational effects of each ingredient may increase endothelial function, cellular intestinal integrity and function, and combat oxidative stress (elevating antioxidant enzyme levels) and inflammation.

The Safety of Combining Quercetin and Citrulline

Q combined with other antioxidants, such as vitamins and minerals have been deemed safe to enhance bioavailability and there do not appear to be interactions between quercetin and foods or other herbs and supplements [343]. Additionally, Q is being used for preventive purposes, as well as in combination with multiple drugs to determine their abilities to potentiate or synergistically interact with these chemical agents, consequently reducing their side effects and related toxicity, at the same time increasing their overall efficacy and safety (as in the case of antitumor drugs) [343]. According to previous literature and FDA approval of these compounds, Q and Cit (combined with other ingredients or ingested alone), do not appear to cause any major side effects or health concerns. The combination of these two ingredients appears safe.
This suggests the beneficial role and interconnected role of NO in exercise performance.

Conclusions

Since Q plays several biological roles, just as CIT promotes gut health, decreasing inflammation, and oxidative stress, we propose that there would exist synergistic and beneficial interaction when these two bio-active substances are utilized together. Further, these molecules may also increase the gut microbiome diversity and richness, ultimately leading to an increase in energy metabolism and exercise performance. Studies investigating the interaction between citrulline, and quercetin are unavailable in exercise studies, specifically TT performance. Citrulline

and Q's roles in exercise performance are unknown and pose a considerable knowledge gap. Q and CIT have been shown to reduce inflammation, attenuate oxidative stress, energy metabolism, and improve cardiovascular health and muscular performance. Since citrulline and quercetin are natural bioactive compounds found in various foods such as vegetables and citrus, we suggest that the co-ingestion will elucidate various and synergistic biological properties, including anti-inflammatory and antioxidant, nitric oxide production, and gut-modulating effects which will likely improve exercise performance. Separately, in parallel, these supplements have been used as ergogenic aids to improve endurance performance. However, the co-supplementation effects of Q and CIT have not been explored and require further investigation.

Statement of Problem

To the best of our knowledge, no studies have examined the mutual effects of citrulline and quercetin on nitric oxide metabolite production and cycling performance. This is likely due to the lack of investigation of the possible and beneficial synergistic roles of Q+CIT targeting specific cellular mechanisms and processes. The beneficial role and interconnected role of Q and CIT targeting the NO pathway in exercise performance is promising. Endurance athletes undergoing repeated physiologic stress from training and competition may benefit from Q + CIT supplementation. **The combination of Q+C will likely serve as an antioxidant and anti-inflammatory, improve gut function, increase muscle function, and increase endothelial function (via NOS), enabling cyclists to perform at a higher capacity. The proposed research will investigate the effects of daily Q and CIT supplementation for 28 days on TT performance in healthy trained cyclists. This is a randomized, double-blind, placebo-controlled trial. Outcomes of interest include the impacts of Q + CIT or placebo on [1] TT cycling performance,**

[2] NO metabolite production, [3] cycling performance (mean power, VO_2, submaximal and maximal heart rate, respiratory exchange ratio (RER), and rate of perceived exertion (RPE) and 4) GI distress.

Specific Aims (SA) and Hypotheses:

Q suggests antioxidant [47, 48], anti-inflammatory [225, 245], glucose regulation [187, 281] cardioprotective (via NO signaling) [211] effects [131], which may help improve aerobic performance. Improved performance is operationally defined as (cyclists) producing a higher power output, increasing oxygen consumption, improving time to fatigue, and reducing TT performance. CIT serves as an antioxidant and cardiac modulator by its ability to improve aerobic capacity (e.g., oxygen consumption kinetics) [19] by increasing NO [396]. With only one study that exists combining Q and CIT to improve gut function, we can base our rationale on previous research stating that: an improvement in gut microbiota function, can lead to regulating and improving energy metabolism, inflammation, nutrient harvesting, maintaining the gut-epithelial barrier (via tight-junction proteins), regulating blood pressure, vasodilation, and producing short-chain fatty acids that upregulate p38, AMPK, and PGC-1α, driving mitochondrial biogenesis and energy production [84, 291], and a performance improvement [274]. When combined, Q and CIT with their slightly different mechanisms of action in cellular compartments, both are touted to impact gut function and mechanisms that influence their respective bioavailability and aerobic-power activity. However, it is unknown what cellular compartments Q and CIT target. To address these gaps in knowledge, the present study is purposed to determine the possible effects of chronic daily consumption (4 weeks) of the combination of Q + CIT, Q, and CIT on cycling performance (TT) variables and NO metabolite production.

Our primary outcome is time-trial completion time, and our secondary aims are: 1) blood plasma NO metabolites, 2) cycling performance outcomes (mean power, VO_2, submaximal and maximal heart rate, respiratory exchange ratio (RER), and rate of perceived exertion (RPE) and 3) GI distress. We hypothesize that Q+C will improve NO metabolite production, provide additional performance advantages compared to Q and CIT alone, and 3) explore gastrointestinal (GI) distress, providing evidence that the co-supplementation of these ergogenic aids will provide a safe and effective nutritional strategy to improve cycling VO_2 kinetics and performance.

SA #1.

Investigate the effect of Q (500 mg, 2x / day) + citrulline (CIT) (3 g, 2 x/ day), Q (500 mg, 2x / day), CIT (3 g, 2 x/ day), or placebo (PL) (3.5 g, 2 x/ day) powder dissolved in a zero-calorie orange flavored crystal light package and water (16 oz) each day after meals on 20-km TT performance. We hypothesize that Q+C will decrease 20-km time-to-completion after 28 days of supplementation.

SA #2.

Investigate the effect of Q (500 mg, 2x / day) + citrulline (CIT) (3 g, 2 x/ day), Q (500 mg, 2x / day), CIT (3 g, 2 x/ day), or placebo (PL) (3.5 g, 2 x/ day) powder dissolved in a zero-calorie orange flavored crystal light package and water (16 oz) each day after meals on NO metabolite production. We hypothesize that Q+C will improve NO metabolite production at rest and post-exercise.

SA #3.

Investigate the effect of Q+CIT, Q, and CIT, or PL on cycling performance measures (e.g., mean power, VO_2, submaximal and maximal heart rate, RER, and RPE). We used a Wahoo Kickr

Trainer and Ultima Series Metabolic Unit for gas collection. We assessed performance through a 20-km TT.

We hypothesize:

1. Q+CIT will outperform Q or CIT alone and increase mean power
2. Q+C outperform Q or CIT alone and increase oxygen consumption
    a. CIT will outperform Q and increase oxygen consumption
3. Q+CIT will outperform Q or CIT alone and decrease submaximal and maximal heart rate
    a. CIT will outperform Q and decrease submaximal and maximal heart rate
4. Q+CIT will outperform Q or CIT alone and improve RER by increasing fat oxidation
5. Q+CIT will outperform Q or CIT alone and decrease RPE,

in the four weeks after supplementation, as compared to the placebo. We hypothesize that RPE will be lower in the Q+CIT condition as compared to the placebo.

SA #4.

Investigate the effect of consuming three weeks of the co-ingestion of Q+C, Q, CIT, or PL on gastrointestinal distress. To achieve this aim, we used an online-version questionnaire to assess gastrointestinal distress. Participants will rate their perceived GI distress at all visits and during all phone call check-ins. We hypothesize that Q+CIT will not cause GID prior to visit 3 (pre-exercise and post-supplementation period), as compared to the placebo.

Collectively, these specific aims will examine whether the combination of Q and CIT are effective ergogenic aids and evaluate their use in the mitochondrial function ($\dot{V}O_2$ kinetics), cycling performance, and NO metabolite production in the skeletal muscle.

CHAPTER 2

THE EFFECT OF QUERCETIN AND CITRULLINE ON NITRIC OXIDE Metabolite PRODUCTION AND CYCLING PERFORMANCE

Introduction

Cycling time trials (TT) are gaining interest in competitions such as the Summer Olympics require a combination of technical, tactical, and power output demands. TTs consist of varying power output, sprints, hills, and tight and technical corners that result in multiple up and downhill segments [273] often close to and above maximal anaerobic and aerobic power. To avoid fatigue, cyclists require adequate nutrition to maintain optimal performances during an event. Because of the relative novelty of these races in elite competition, research to date has not suggested proper fueling recommendations. There is growing interest in optimizing training, adaptations, and time-trial (TT) performance using pharmaceutical or nutritional supplementation strategies. Aerobic adaptations include an increase in mitochondrial biogenesis (peroxisome proliferator-activated receptor-gamma coactivator α, mitochondrial density and capacity, oxygen consumption, glucose and fatty acid oxidation, glycogen storage, and insulin sensitivity) [128, 193]. These aerobic adaptations can be detected after four weeks of high-intensity training in trained individuals [93, 105, 193].

Due to the competitive nature of TTs and adaptation time, ergogenic aids are of particular interest. Nutritional ergogenic supplements may improve performance by increasing oxygen consumption and improving ATP production [130, 139], decreasing fatigue, improving cycling efficiency and power output, or assisting in recovery during intense training [178]. As the popularity and competitiveness of TTs increase, it is important to identify safe, effective ergogenic aids and nutritional strategies to improve cycling performance. While few studies have looked at the ergogenic effects of quercetin (Q) and citrulline (CIT) separately on cycling performance, to the best

of our knowledge; no studies have examined the ergogenic effects of co-supplementation. We seek to elucidate the potential beneficial role of Q and CIT via the nitric oxide (NO) pathway and cycling performance.

Background: In endothelial cells, NO is synthesized from l-arginine (precursor) by eNOS, generating NO and l-citrulline (end-products) [8, 129, 395]. NO production increases endogenous production of nitrate/nitrite limb blood flow and improves oxygen consumption during exercise via nitrates and nitrites, reducing back to NO [209]. It is suggested that this alternative pathway may complement the l-arginine–NOS–NO path by enabling NO production in conditions of low oxygen availability in which NOS activity (which is oxygen dependent) may be reduced [261]. Animal models support multiple pathways for the effects of NO on endothelial function that vary performance improvements [307]. Evidence suggests that enhancing NO bioavailability by augmenting the nitrate–nitrite–NO pathway may influence muscle function and exercise performance in humans [8]. However, the influence of Q and CIT on NO production or metabolites has not been sufficiently investigated.

Quercetin (Q) is a plant pigment, an antioxidant, a polyphenol, and a flavanol belonging to the flavonoid group [36, 343]. Quercetin is a powerful antioxidant and anti-inflammatory compound commonly found in apples, elderberries, citrus fruits, red wine, red onions, hot peppers, berries, kale, buckwheat tea, dark green leafy greens, and capers [36, 201, 416]. Improving endothelium-dependent vasodilation is believed to be one possible mechanism by which flavonoids function [256]. Q improves blood flow by stimulating the endothelium nitric oxide synthase (eNOS) activation [257], increasing nitric oxide (NO) production, and increasing vasodilation in rats. In parallel, Q restored intravascular homeostasis and endothelial functions by attenuating excess NO production induced by ATP, decreased intracellular calcium flux, and eNOS activity in

vascular endothelial cells [109]. Further, Q reversed the endothelial damage arising from excessive NO by attenuating nitrification stress and protecting the endothelial cells [394]. However, research does not exist investigating Q's effects on NO and its metabolites in humans.

Recently, Q has received attention due to its effects on improving oxygen consumption VO_2 kinetics [95, 266]. However, there is modest evidence to suggest Q improves cycling performance in trained individuals [82, 298, 299]. Previously Q (1000 mg) combined with epigallocatechin gallate (EGCG; 120 mg) resulted in an enhanced granulocyte oxidative burst activity and resisted inflammatory markers after 3 days of heavy exercise but resulted in no change in mRNA expression related to mitochondrial biogenesis [299]. Additionally, a 6-week supplementation of Q (600 mg) combined with vitamins and minerals improved 30-km TT performance in similarly trained male athletes [266]. These multifaceted metabolic effects of Q combined with other molecules make it a potential ergogenic aid to improve endurance performance. However, the effect of the combination of a polyphenol (Q) with an amino acid is unknown on endurance performance.

Citrulline (CIT) is a nonessential amino acid found in high concentrations in watermelon [89, 135, 394]. Emerging evidence suggests that CIT can be used as an ergogenic aid since it has strong antioxidant [17, 103] and anti-inflammatory properties [1, 8], promotes muscle protein synthesis [214, 414], skeletal muscle metabolism, oxygen uptake [14, 19, 401], improving post-exercise muscle function, RPE, and recovery effects [332]. CIT is formed from arginine, an amino acid involved in several physiological roles including the urea cycle, protein synthesis, creatine, and nitric oxide synthesis [394]. CIT can be formed through the activity of nitric oxide synthase enzymes (i.e., eNOS, inducible NOS, and neuronal NOS yielding NO [8, 17, 140, 141, 168, 179, 347, 400]. Further, CIT can serve as the direct precursor of arginine, through the oxidation of arginino-succinate.

Several lines of research suggest when provided separately, that both Q and CIT may improve components of cardiovascular health, metabolism, oxidative stress, and performance as an ergogenic aid by possible synergistic interactions. For example, a dose of 6 or 8 g of L-citrulline modestly improved endurance cycling performance in trained individuals [364, 374]. Additionally, 1000 mg/day of Q combined with other ingredients positively improved performance [82, 98, 102, 266, 277, 298, 299]. Although evidence is sparse, a review [394] summarizes the modulatory effects of the co-ingestion of Q and citrulline on gut function, intestinal integrity, and gut microbiota. However, to date, no studies exist investigating Q + CIT's potential synergistic roles to attenuate intestinal inflammation, promote gut health, and increase energy metabolism, endurance performance capacity, and VO_2 kinetics. Moreover, no research exists investigating the combined and potential synergistic effects of these two compounds, specifically in TT performance.

The interaction of Q+CIT may improve each substance's bioavailability and elicit synergistic effects by targeting specific cellular mechanisms and processes during exercise performance [394]. A polyphenol (Q) combined with an amino acid (CIT) has unique chemical properties that determine some specific actions in different cellular compartments which may result in an improvement in cycling performance. Citrulline and Q's roles in exercise performance are unknown and pose a considerable knowledge gap. The role of Q and CIT targeting the NO pathway in exercise performance is understated. Endurance athletes undergoing repeated physiologic stress from training and competition may benefit from Q + CIT supplementation. When combined there is a promising link that Q and CIT are touted to influence their respective bioavailability and aerobic activity. However, it is unknown what physiological mechanisms Q and CIT target to improve cycling performance. To address these gaps in knowledge, the present study tested the ergogenic effects of daily consumption of Q + CIT, Q, CIT, or placebo (PL) for 28 consecutive days on

cycling TT performance variables and NO metabolite production. We hypothesized that Q+C will improve NO metabolite production and provided additional performance advantages compared to Q and CIT alone. We aimed to assess Q+CIT as a novel, safe, and effective nutritional strategy and to investigate its effects on cycling metabolic and cardiovascular changes (i.e., NO metabolites and VO_2 kinetics) and aerobic performance in trained cyclists.

METHODOLOGY

Protocol

We employed a randomized, placebo-controlled study design with a quercetin + citrulline (Q + CIT), Q, CIT, or placebo conditions as the independent variable and TT cycling performance, NO metabolite production, cycling performance (mean power, VO_2, submaximal and maximal heart rate, respiratory exchange ratio (RER), rate of perceived exertion (RPE) and GI distress as the dependent variables. We recruited 60 participants to visit Georgia State University (GSU; Atlanta, Georgia) Applied Exercise Physiology laboratory on three separate occasions scheduled throughout the day (0700 – 1600) at the same time of day (+/- 2 hours), over a five-to-six-week period. Participants were randomly assigned to one of four supplementation groups: (1) Q + CIT, (2) Q, (3) CIT, or (4) placebo (PL). Supplement composition, dosages, and duration will be described below in *Supplementation Conditions*. Visits to GSU required participants to perform cycling performance tests to obtain time-trial (TT) completion, mean power, VO_2, submaximal and maximal heart rate, RER, and RPE) (for clarity, please see Figure 13). The study was approved by the Georgia State University Institutional Review Board IRB # H23189. Data were collected in accordance with the Declaration of Helsinki.

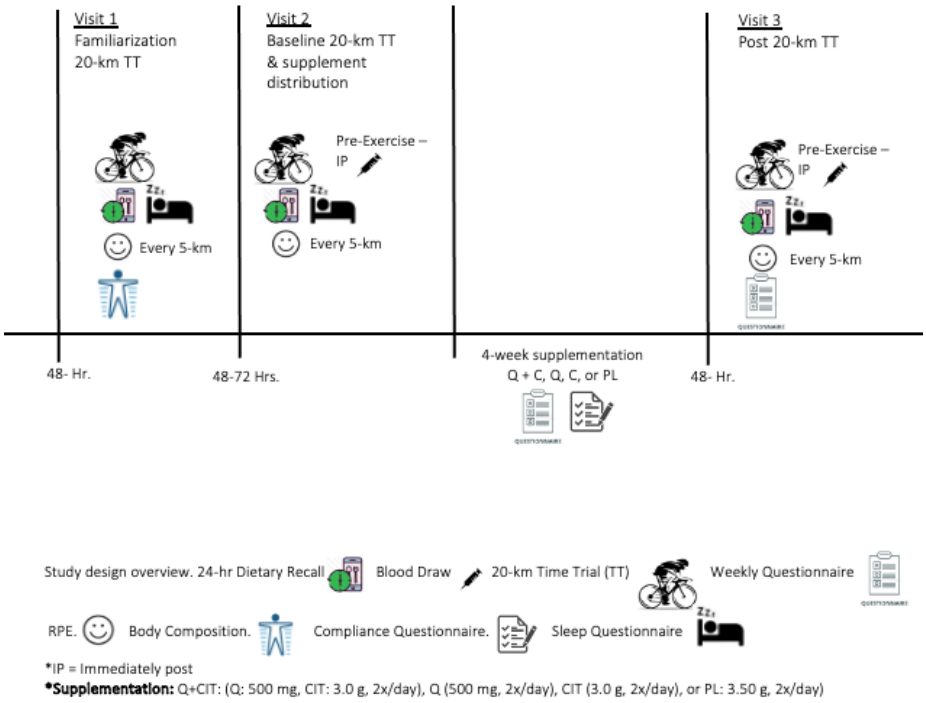

Figure 13. Study design overview.

Participant Inclusion Criteria

Participants were defined as Tier 2 of a 6-Tier framework developed previously to classify exercise/training and/or sports performance levels [279]. Tier 2 is defined as a trained, developed individual who identifies with cycling as their main sport [279] and provides a sport-specific metric of training volume (e.g., kilometers per week, rather than days/week). However, this Tier framework is still ambiguous. Additionally, we recruited cyclists who regularly train at least three times per week, currently training with a stationary bike/trainer either indoors or outdoors for at least three to five hours per week, over the past three years [226, 421], and who are training with

a purpose to compete [176, 279]. Due to the lack of uniformity of pre-experimental protocols and various performance indicators and criteria (i.e., Tier framework) to classify cyclists for cycling competitions and across studies, it is difficult to translate scientific results into practice and interpret and extrapolate research data [106]. Thus, the purpose of including these guidelines is to 1) provide standardization and classification of trained cyclists according to performance indicators and 2) observe how cyclists improve or do not improve relative to their performance level.

Female Inclusion Specifics

All females were tested during their follicular phase (approximately day 0 to day 16, assuming a 30-day cycle [63], assuming regular cycles), where female sex hormone concentrations are relatively stable and most similar to other women [75]. Endurance performance in some intermittent endurance tests is greater in the follicular phase [159, 215] due to the absence of increased thermoregulatory and cardiovascular strain seen during the luteal phase [104, 192, 272]. We documented if a female's cycle has not been regular but did not exclude them from participating in the study. Aerobic performance outcomes are likely to be enhanced, but strength performance is likely diminished in the early follicular phase [75]. Thus, we tested during the follicular phase because aerobic performance is higher compared to the luteal phase [75]. A regular cycle typically lasts for 24 to 35 days; however, the cycle length varies amongst women [160]. An irregular cycle was defined as shorter than 24 days, more than 38 days long, falls outside the female's "regular cycle length range," or if the length varies significantly from month to month; doctors refer to this as oligomenorrhea [181].

We determined the menstrual cycle and oral contraceptives (OCs) use by asking the female during the Pre-Screening Call, (see Pre-Screening Phone Call section); it is normal for females to

record their cycles. OCs are common among elite female athletes [67]. OCs reduce cycle-length variability and provide a consistent 28-day cycle by controlling concentrations of endogenous sex hormones. For instance, half of the women who competed at the Rio Olympic Games used some form of OC [239] and approximately 75.3% of elite Australian female athletes use an OC pill [240]. The type of OC administered (monophasic, biphasic, or triphasic), and the type and dose of estrogen and progestogen within, will have varying effects on exercise performance [67]. It seems that the effects of OCs on aerobic capacity are more pronounced in triphasic OC formulations than in monophasic OC formulations [67]. For example, trained female athletes who used triphasic OCs saw changes in endurance performance, hemoglobin levels, maximum heart rate, respiratory exchange ratio, ventilation [67], glucose flux and substrate oxidation [370], or fatty acid re-esterification [197], suggesting performance effects may be affected with the type of OC consumed [67]. However, there may be a decrease in peak oxygen uptake (volume of O_{2peak} per minute) using triphasic OCs, but no effect was reported on ventilation in active women [67].

Although the research in this area is sparse, well-controlled studies indicate that there are no significant effects of monophasic or biphasic OC formulations on cardiovascular responses at rest or during exercise [67]. As such, we included females with medically prescribed monophasic or biphasic OCs. However, if a female did not have a regular period and did not consume an OC, we still included them. If, for some reason, a female's cycle changed, we documented and reported this. Additionally, we included perimenopausal women; however, their strength and power-producing capabilities may be decreased [45].

Participant Exclusion Criteria

We excluded the following participants: performing greater than two days of resistance training per week which could cause delayed onset muscle soreness and fatigue; consuming triphasic oral contraceptives [67]; menopausal and post-menopausal women due to the large variations in hormones [120]; daily use of nonsteroidal anti-inflammatory drugs (naproxen and cyclooxygenase-2 inhibitors) and/or use of anti-hypertensive medications (e.g., angiotensin-converting enzyme inhibitors or beta-blockers); smoker (in the past six months); smoked or use THC or CBD products; failed to abstain from strenuous exercise in the past 48 hours prior to each visit [169] (i.e., cycling competition or race in the next two months, or eccentric resistance training; suffer from systemic and inflammatory diseases that change physical or laboratory tests (thyroid, arthritis, hepatic, respiratory, gout); pregnant women since this study requires the use of devices, agents, or procedures that pose safety concerns for the developing fetus, participating in a high-intensity exercise protocol; females who have not had a period in the past 6 months (i.e., amenorrhea) or use triphasic OCs; documented intolerance to iron; failed to abstain from caffeine consumption 24 hours before testing [169]; documented daily Q, CIT, creatine, B-alanine, antioxidant medications, tocopherols, or flavonoid supplementation in the past three months; have an orthopedic injury that may impact cycling performance testing.

Recruitment

A G*Power analysis revealed a repeated measures within-factor sample size of 48 with an effect size of 0.25 required with an alpha level of $p \leq 0.05$ to detect significant differences (power = 0.80). The medium effect size is based on time-trial completion time which is the primary outcome variable in this study [238] (for clarity, see Statistics section). We attempted to recruit 60

participants ages 18-55 to account for an anticipated 25% attrition rate. Our recruitment strategy included an active and passive effort. Our passive effort included emailing local cycling teams around Atlanta, GA, and posting flyers at local cycling shops and cycling trails. Our active effort was accomplished through presentations at cycling events and conversations on Atlanta cycling trails. Interested volunteers received a phone call prior to visit 1 to answer basic health and medical history questions to determine eligibility.

Participant Screening

All participants were screened and informed verbally about the aims and risks of the study and provided written informed consent prior to study enrollment and participation (visit 1). The screening process involved a pre-screening phone call prior to visit 1 (see Pre-Screening Phone Call section) to scan for basic eligibility and discuss cyclists' training volume, the purpose of the study, and supplement intervention. It did not require informed consent because no personal identifiable information about the individuals was collected.

Pre-Screening Phone Call

Interested volunteers received a telephone screening call from the co-PI asking basic pre-screening health and medical questions. The screening process included asking 20-28 (if female) basic health, physical activity, and medical questions to determine possible eligibility. The questions asked cyclists about their gender, age, current disease status (e.g., metabolic syndrome, systemic or inflammatory disease, cardiovascular disease), smoker status, personal health history (e.g., food intolerances, allergies, disease, syndromes), supplementation use, history of cycling and current training volume, and previous or current injuries. For women, we asked them about their

menstrual cycle when their last menstrual cycle was, if it was normal, and if they consumed OCs, and recorded it electronically. If participants met the basic inclusion criteria and were still interested in the study, we scheduled them for visit 1.

Participant Enrollment Participation

Enrolled participants were cyclists who were eligible for participation (i.e., met the inclusion criteria for the study), and gave written consent. Screened participants are individuals who gave informed consent and participated in screening procedures to determine eligibility. Screen failures are individuals who gave informed consent and participated only in screening procedures to determine eligibility but were determined to be ineligible to participate in the study. Screen-failure individuals were not enrolled in the study.

Visit Descriptions

Recruited cyclists arrived having completed the following: well-rested and well-nourished. Well-rested is having adequate sleep to function and focus optimally during waking hours. Well-nourished was defined as consuming adequate and optimal consumption of macronutrients and micronutrients for an endurance training bout [403]. We instructed them to follow their normal physical activity but avoid strenuous exercise for at least 48 hours prior and only low-intensity exercise 24 hours prior to each visit. However, they were instructed to consume what they normally would consume before a race before each cycling visit to simulate their diet 24 hours before a race. A 24-hour dietary recall was completed before each visit to ensure diet replication for subsequent visits (See Appendix D). Additionally, cyclists were asked about their sleep quality (see Sleep Quality Questionnaire section). Visits 1, 2, and 3 also involved a 20-km TT. We asked participants

if there were any changes in their responses to the health-related, menstrual cycle, or injury questionnaires during each visit. Cyclists were allowed to listen to music during the visits, but we kept the music consistent each visit with the same playlist and volume; cyclists were allowed to choose their own music, but it was the same music for the subsequent visits. Participants were allowed to drink water *ad libitum.*

Participants recorded a 24-hour dietary recall and sleep questionnaire on a printed-out or an electronic version at each visit before the TT (See Appendix D and G). Participants were advised to follow their normal habitual dietary regimen, but they were asked to replicate what they consumed prior to each testing visit for 24 hours. Diet recalls were analyzed using an online food processor (version 11.1 ESHA research).

Visit 1 (V1)

Visit 1 consisted of completing the informed consent, health history/medical history questionnaire, 24-hr dietary recall, sleep index questionnaire, injury history questionnaire, PARQ+, Dual Energy X-ray Absorptiometry (DEXA) body composition scan, measurement of height and weight, and a 20-km TT familiarization bout. They were instructed to perform a maximal TT during this visit to ensure reliability between visits.

We educated the participant on the procedures, including the risks and benefits, and provided time for questions related to the methodology or any other questions the participant might have. Participants provided written consent if they chose to continue with the study. This took approximately 20-30 minutes.

Participants had their body composition (Lunar Prodigy encore: PR 510021), height, and weight measured before the familiarization session (see Bodyweight, Height, and Body

Composition section). The body composition scan was used for demographic purposes to measure weight, body fat percentage, fat mass, lean body mass, visceral fat, and bone concentration and density. We ensured proper bike set-up prior to the 20-km familiarization trial (for clarity, see 20-km Bike Time-Trial Set-Up). The purpose of the 20-km TT familiarization was to improve the reliability of the measurements (e.g., mean power) and individual familiarization of Zwift and the Wahoo trainer. The 20-km familiarization test took approximately 45-60 minutes [55, 421].

After the familiarization trial, we familiarized the participants with questionnaires used throughout the intervention (i.e., Weekly GI Distress Questionnaire, RPE, Supplement Compliance Dosing Diary, and ensure competency on the 24-hr. dietary recall). The questionnaire familiarization took approximately 10 minutes. Lastly, they were scheduled for visit 2 prior to leaving the laboratory. The initial visit occurred at GSU and took approximately ~2-2.5 hours to complete.

20-km Bike Time-Trial Set-Up

Cyclists wore their typical cycling attire. They brought their personal bike to attach to the Wahoo Kickr Core trainer to complete the 20-km TT. The bike seat was set at approximately the height of the cyclist's hips, allowing for a slight bend in their knees when riding. Clip-in or clipless pedals were allowed so cyclists could wear their own cycling shoes. The cyclist's preference was held consistent on all visits. Water was allowed *ad-libitum.* The set-up took approximately 10 minutes and was the same for all subsequent sessions.

Visit 2 (V2)

Visit 2 consisted of a baseline-TT performance bout prior to a four-week supplementation period. Prior to the TT, participants had their blood drawn for NO metabolite concentrations (see

Blood Collection and Assessments). Immediately following the TT, participants had their blood drawn again. Visit 1 and Visit 2 occurred about 72 hours apart. Participants were instructed to avoid strenuous exercise for at least 48 hours. After visit 2, individuals consumed a supplement for four weeks.

V2: Supplementation Condition Assignment/Instruction

Following the post-TT blood draw, all participants were randomly assigned to a supplementation group (using manual block formation based on rolling of dice). We instructed them to take their supplement daily for four weeks following meals before returning to the laboratory for visit 3. To ensure consistency, we required participants to track when they consumed the supplement and ensured it fell within the specified times (refer to Supplement Compliance section).

Supplement Conditions

Participants were randomized into one of four groups: (1) Q+CIT, (2) Q, (3) CIT, or (4) PL. Supplements were dissolved in 16 oz of water and consumed twice daily for 28 consecutive days at defined intervals (for clarity see Supplementation Conditions Strategy section). Participants were provided supplementation powders and instructed on how to dissolve them in 16 oz of water. The composition of these powders is as follows:

(1) Q+CIT (500 mg of quercetin dihydrate, 3.0 g of L-citrulline, 3.5 g orange crystal light), 2x/day
(2) Q (500 mg of quercetin dihydrate, 3.5 g orange crystal light), 2x/day
(3) CIT (3.0 g of L-citrulline and 3.5 g orange crystal light), 2x/day and
(4) PL (3.5 g orange crystal light), 2x/day.

The Q and CIT dosages were chosen based on previous research in which supplements were observed to positively improve performance [19, 95, 98, 102, 277, 298, 299, 364]. The placebo contained a zero-calorie orange-flavored crystal light package powder dissolved in 16 oz of water, like the treatment in the supplement.

Quercetin dihydrate and CIT powders were purchased from Bulk Supplements (Henderson, NV) and stored at room temperature. Bulk Supplements are quality and lab-tested, FDA registered, and contain pure raw ingredients. Crystal light (Kraft Heinz, Chicago, IL) was purchased in bulk from Amazon.

Supplementation Conditions Strategy

Participants consumed their supplement twice per day after meals for four weeks (Figure 13), as previously described [277, 298, 299]. Previous research shows that three weeks may not be adequate for aerobic adaptations [114, 277] since athletes typically consume more Q than non-athletes in their normal dietary intake [230]. Since aerobic adaptations can be detected after four weeks of training in trained individuals, the four-week supplementation period was chosen [93, 105, 193]. Additionally, athletes may benefit from Q's effects, but it may take longer to see a performance increase, possibly due to higher starting nutrient levels [209, 276]. Thus, we chose a four-week supplementation period to 1) investigate possible metabolic and cardiovascular changes (i.e., NO metabolite production) that may occur from these higher nutrient supplements and 2) observe how the supplement affects aerobic performance in trained cyclists.

A research team member measured the supplements and put them in identical, labeled bags. Another research member rolled a four-sided die to determine randomization. Then, the researcher distributed and tracked the supplement distribution to ensure a double-blind study; only two weeks

of supplements were given out at first. No more than two supplement powder packs were allowed during the day. The zero-calorie orange-flavored crystal light package was added to mask any taste and ensure that participants remained blinded to their group. The supplements were dissolved in a beverage form to enhance absorption [97, 249]. Further, black bottles were provided to mix the supplement to blind participants to any possible color difference from the dissolved supplements. We demonstrated the mixing during visit 2. We instructed individuals to consume their entire beverage within a 30-minute duration. In the case of variable meal schedules, we set the consumption times: 1st ingestion between 0600 to 1100 after their first meal, 2nd ingestion between 1730 to 2030 after their last meal. Participants were required to add only filtered or bottled room-temperature water to the bottle, but no other fluids were allowed in the mix. They were required to shake the bottle for a minimum of 20 seconds and a maximum of 30 seconds to allow for proper dissolving. To ensure consistency, we instructed participants to track what time of day they consumed the supplement (see Supplement Compliance section). During the supplementation period, participants received a weekly phone call or text check-ins with a research team member and logged physical activity, GI distress, and supplement compliance throughout the supplementation period. A research member delivered or had the cyclists pick up the remaining two weeks of supplements. If cyclists were not compliant and missed more than 10% (~5.6 supplement bags), we performed a sensitivity analysis to see to what extent they may or may not influence the primary outcomes of interest.

Visit 3 (V3)

Following the four-week supplementation period, cyclists returned to GSU for visit 3 for the post-20-km performance test. Prior to the TT test, participants had their blood drawn for NO

metabolite concentrations (see Blood Collection and Assessments section). Additionally, individuals completed a final Weekly GI Distress Questionnaire form (see Weekly GI Distress Questionnaire section). Immediately following the TT, participants had their blood drawn for nitric oxide metabolites.

20-km Time-Trial Stimulus Description

Cyclists completed a 10-minute warm-up at a self-selected pace and intensity [264, 387] before the 20-km TT (Figure 14). Cyclists performed the warm-up with their bottom on the saddle. The TT was our stimulus to measure our outcome variables. The TTs were performed on a Wahoo Core Kickr Smart Trainer using the Zwift system virtual training app. Zwift is a virtual training platform that simulated real outdoor races. The race consisted of a reproducible 20-km TT composed of flat terrain at a freely selected pedaling cadence allowing for the collection of mean power, as previously described [55, 176, 283, 345, 421] (Figure 14). Due to the lack of information on cycling TTs, the TT intensity was self-paced allowing cyclists to use the gear changers on their bike.

The Wahoo KICKR Trainer was set in open test mode during the TT, allowing cyclists to change gears and intensity freely. Participants self-selected their preferred pedal cadence. A self-selected pace allowed the resistance to increase or decrease as a function of cadence and pedal force. The participants were asked to produce their maximal power output for the TT and adopted their personal pacing strategies [52, 54, 251]. Cyclists were instructed to complete the total distance in the fastest time possible [345]. Respiratory exchange ratio in the range between ≥1.1 and ≥1.15 and RPE ≥17 or ≥18 indicated cyclists neared maximal effort [42, 50, 51]. RPE, HR, and

VO_2 were collected every 5 km (see Performance Variables section) (Figure 15). Once cyclists finished the 20-km, they performed a 5-minute cool-down at a self-selected intensity.

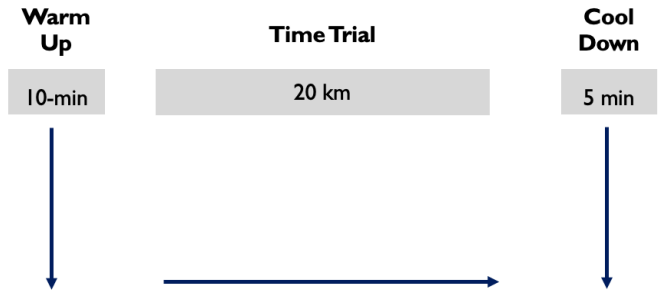

Figure 14. 20-km time trial protocol. Individuals completed these trials on visit 1, visit 2 (pre-supplementation), and visit 3 (post-supplementation) using a Wahoo KICKR Core trainer.

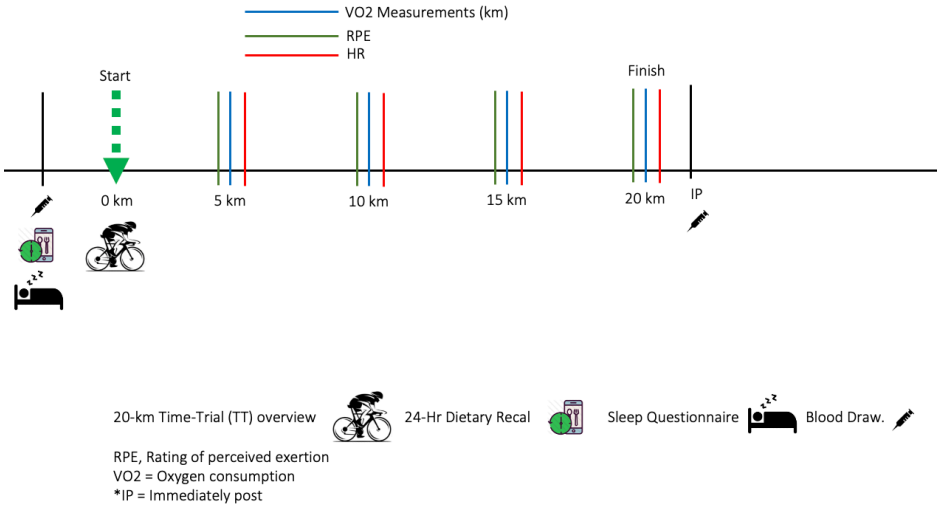

Figure 15. 20-km time trial overview. This overview shows the time points of RPE, HR, and VO2. Individuals completed these trials on visit 1, visit 2 (pre)- and visit 3 (post-supplementation) using a Wahoo KICKR Core trainer.

Reliability, Validity, and Reproducibility of Equipment

The Wahoo KICKR trainer measures power to a similar level of accuracy to the more reputable SRM power meter during an incremental exercise test [189]. The mean error across all KICKR units is -1.5% (range: -3.1% to 0.0%) compared to -1.6% reported by the SRM. An R^2 >0.999 was found for all KICKR and SRM compared to the Calibration RIG (CALRIG), the most accurate measure of reliability [189]. The KICKR still demonstrated an acceptable level of reliability for assessing cycling performance reporting a small mean bias and narrow limits of agreement in the measurement of power output between 250-700 W at cadences of 80-120 rpm with larger mean biases and wider limits of agreements observed at lower and higher set power outputs [189, 190, 418, 419]. The larger differences between the KICKR and CALRIG were observed at higher power ranges (900- 999 W) and lower ends of measured power at 80-120 rpm. However, these values were influenced by cadence selection. Compared to a CALRIG, the mean bias of the KICKR of - 1.1% (95% limits of agreement (LoA): -3.5-1.4%) over 250-700 W at cadences of 80-120 rpm falls within this recommended range for ergometer error [418, 419]. As reported [3, 419], the KICKR was consistent with the ergometer errors of the Velotron and SRM power meters of <1% in constant power trials of 250 W and 414 W compared with a CALRIG. When comparing the KICKR to the Monark, VO_2 and HR were slightly lower; however, VO_2 and HR they consistent when comparing the two Wahoo trials [152].

Measurements and Instruments

Screening Measures

The screening survey questionnaire collected demographic information (age, biological sex, gender identity, race, ethnicity, education level, women's menstrual cycle status, physical

activity, health, and disease) and assessed inclusion/exclusion criteria. The following variables: history of diseases (hypertension, diabetes, inflammation, respiratory, gout, hepatic, arthritis, gout), if they were a smoker or used THC or CBD products or used sports supplements were assessed with a single item delivered via questionnaire to assess whether candidates were eligible for the study. Then, cyclists indicated whether they were interested in improving their TT cycling performance introducing an experimental demand with a supplement. Additionally, we asked women about the regularity or irregularity of their menstrual cycle and if they were consuming an oral contraceptive.

Bodyweight, Height, and Body Composition

The DEXA measured body composition (Lunar Prodigy encore: PR 510021). The DEXA involved an X-ray analysis of the entire body to determine body composition (i.e., fat, bone, muscle, and total weight). During the DEXA scan, participants were asked to lie still on the exam table for approximately 6-12 minutes while two low-dose X-ray beams of different energy levels scanned their body for fat mass, body fat percentage, visceral fat, lean body mass, bone concentration, and density (t-score), and weight. Body composition measurements were expressed in pounds (lbs).

A second consent form (V1) detailing the DEXA test was completed before they completed the test. Height was measured on a stadiometer (cm). Individuals stood straight up with their back facing the wall with their feet together. The stadiometer arm was moved down to their head at a level height and height was recorded based on where the stadiometer arm is.

Blood Collection and Assessments

Blood was collected from the antecubital vein pre-exercise and immediately post-exercise. Approximately 20 mL of blood was drawn into two EDTA-treated Vacutainer® tubes. The EDTA-treated tubes were gently inverted 8-10 times and centrifuged. Samples were centrifuged for 10 minutes at 3600 RPMs at 4 ºC. Serum and plasma were aliquoted and stored in –20ºC until analyses of NO metabolites. Cayman's Nitrate/Nitrite Colorimetric Assay Kit provided an accurate and convenient method for measuring total nitrate/nitrite concentration in a simple two-step process [79]. NO metabolites were quantified per the manufacturer's instructions [90].

Performance Variables

We collected and analyzed the following performance variables: time-to-completion, mean power, oxygen consumption (VO_2), RER, heart rate (HR), and RPE (6-20 scale). These variables were defined and described below.

20-km Time-to-Completion

Time-to-completion was defined as the time the cyclist took to complete the 20-km TT. During warm-up, we asked the cyclists to remain seated in the saddle. The start position was a personal preference similar to what cyclists experience in races, typically a standing start with a big push. We manually started the race, after a 3-2-1-GO countdown. We immediately recorded the cyclist's time in minutes: seconds (min: secs) at the 20-km mark.

Reliability and Reproducibility of TT Performance Testing

In a study using a Velotron ergometer, 20 km TT performance was highly reproducible in competitive cyclists (r^2 = 0.96 between TT visit 1 [TT-1] and TT visit 2 [TT-2]; r^2 = 0.97 between TT visit 2 and TT visit 3 [TT-3]); a low coefficient of variance was demonstrated between trials for mean power (TT1 - TT2 = 2.1 %, CI = 1.6 % to 3.1 %; TT2 - TT3 = 1.9 %, CI = 1.4 % to 2.8 %) and is comparable to that expected during an actual performance race in elite athletes [359]. Furthermore, peak power and mean power were both correlated to performance time in TT1, accounting for most of the variance in performance time (r^2 = 0.993) [359]. Performance in a 20-km cycling TT using the Velotron is highly reproducible with familiarization trials [184, 359].

Familiarization trials increase the reliability of performance measurements such as average power output, time to completion, mean power, or speed [53, 359, 421]. In well-trained cyclists, there was a minimal decrease in the coefficient of variation (<1%) between familiarization sessions [367]. Additionally, pacing strategies in well-trained cyclists who completed three 20-km TTs were similar across [49, 382] repeat trials. Specifically, pacing strategies allow cyclists to perform the 20-km at their own pace and intensity, freely allowing them to change the gears on their bike. Self-paced races increase the reliability of power outputs (Figure 16) [49]. However, a higher degree of variability was detected at the start and end of the trial (typical error (TE) = 6.6 and 6.8% for the first and the last 1-km) and a trend for a progressively blunted start on repeat trials [49]. However, the reproducibility of performance, cardiorespiratory, and perceptual measures fell in a TE range of 1.0-4.0 % [382]. Evidence supports the efficacy of pacing strategies to increase the reliability of performance results compared to establishing a specific intensity [49]. Therefore, we implemented three TT visits with a Wahoo KICKR Core to anticipate the reliability of performance.

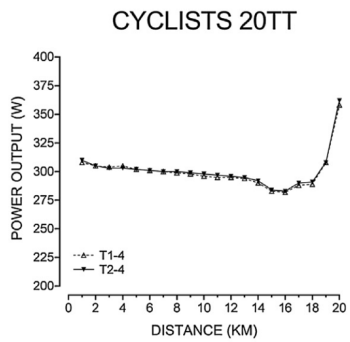

Figure 16. Cyclists group pacing strategy for 20-km race, as shown by mean power output per km. The dashed line indicates performance with no familiarization (i.e., time trials 1-4; T1-4), and the solid line indicates performance following a familiarization (i.e., time trials 2-4; T2-4) [49].

Mean power

Mean power was defined as the average power output during the performance tests. Mean power was expressed as watts (W). Mean power was collected through the Zwift software. After the race, a researcher downloaded the Zwift data as a CSV file, containing power measurements at each second. The researcher took the average of the power from 0- to 20-km to determine the mean power.

Oxygen consumption and Respiratory Exchange Ratio (RER)

All individuals wore a metabolic mask (Parvo Medics, TrueOne 2400, Salt Lake City, UT) for two to three minutes to capture oxygen consumption at each 5-km mark. VO_2 was defined as oxygen consumption per kg of body weight during performance tests. We collected VO_2 every 5-km (5, 10, 15, and 20 km) by indirect calorimetry (Parvo Medics, True One 2400 Metabolic unit, Salt Lake, City, UT) to detect their average oxygen consumption (VO_2) and RER. RER was the

measure of the volume of carbon dioxide an individual exhales (VCO_2) divided by the amount of oxygen they inhale (VO_2) at each 5-km mark [317]. The highest achieved RER indicated high-intensity effort during the whole 20-km at each 5 km mark.

Heart Rate

During all testing protocols, HR and HR_{max} were monitored by beats per minute (bpm) using a chest heart rate monitor (Polar heart rate monitors, model H10). HR was recorded every 5 km.

Measurement of Perceived Exertion

All individuals were instructed to report their perceived exertion (RPE) according to the 6-20 point Borg scale [50, 51] at each 5 km mark. 20 represents exhaustion, while 6 represents little to minimal effort (See Appendix H). The highest achieved RPE indicated high-intensity effort during the whole 20-km at each 5 km mark.

Manipulation Checks

We implemented manipulation checks to confirm the control efforts of our independent variable, supplement groups, and control variables. We used a weekly GI Distress questionnaire, physical activity log, supplement compliance, and a 24-hr. Diet Recall and Analysis to scrutinize other possible outside factors that could have influenced the study's outcomes.

Weekly GI Distress Questionnaire

The weekly GI Distress questionnaire was an electronic Qualtrics form that was delivered through email weekly (See Appendix C). It consists of 16 questions asking the cyclists about their gastrointestinal distress (GI distress) symptoms, and a prompt about their supplement intake, directing them to the Supplement Compliance Dosing Diary form. The questions and domains were derived from a validated and well-established methodology assessing gastrointestinal distress [78, 115]. The domains were abdominal pressure, abdominal distension, belching, difficult gas evacuation, flatulence, nausea, heartburn, and bowel movements. The domains and questions that were selected were based on previous research using Q and CIT [112, 204].

The questions consisted of subjective answers, short-answer responses, ranking symptoms (1-11 scale), or based on duration. However, some questions were based on a 1-11 scale. For example, one question asked, “Did you feel bloated? If so, what was the severity (11 represents the highest bloating, 1 represents minimal bloating but not normal.” Further, an example of a duration question is, “What was the duration of bloating (minutes or hours within a day).” A total summary score was represented by a ratio out of the total cyclists per group who experienced GI distress.

One question asked the cyclists a 'Yes' or 'No' question whether they have filled out the Supplement Compliance Dosing Diary yet (see Supplement Compliance section). This question prompts the completion of their Supplement Compliance Dosing Diary. Individuals were asked to fill out this questionnaire within one to two hours after their second dose. If they did not take their second dose, they were required to report it on the Supplement Compliance Dosing Diary. If cyclists experienced GI distress during the middle of the week before they filled out the weekly questionnaire, they were asked to report their symptoms immediately to the co-investigator.

Physical Activity

Cyclists were asked to answer one question about their physical activity on the Compliance Dosing Diary (See Appendix F), including their intensity, using a 6-20 RPE scale, mode, and duration. The following describes the question that was asked, "What was your physical activity today? Please include your mode, duration, and intensity (using the Borg 6-20 scale. 6 represents no exertion and 20 represents maximal exertion). They selected boxes to check for their mode, intensity, and duration.

Supplement Compliance Dosing Diary

Supplement compliance (see Appendix F) was recorded daily during the supplement period via a Qualtrics electronic dosing diary. The dosing diary consisted of one question documenting what time the cyclists consumed (AM and PM) or not consumed the supplement. If they missed a dose, they were asked to refer to the key to provide their reason: 1-6, 1 - Forgot, 2 - Physically Unable, 3 – Fell Asleep, 4 – Took More than directed, 5 - Consumed all supplements, 6 – Other. Cyclists were classified as non-compliant if they missed more than 10% (~5.6 supplement bags) of the total supplementation dosages. A total summary score was represented by a percentage per group who were compliant.

Sleep Quality Questionnaire

Sleep Quality was recorded at each visit from the previous night's sleep on a paper questionnaire (See Appendix G). The questionnaire included 5 questions derived from, The Pittsburg Sleep Quality Index (PSQI) [350], and inquired about a variety of factors, including: sleep quality, including estimates of sleep duration (how long it took them to fall asleep when they woke up),

latency, and of the frequency and severity of specific sleep-related problems (e.g., could not get to sleep within 30 minutes, felt too hot or too cold, had bad dreams, had pain). These five items were grouped into one of three categories: sleep latency, sleep duration, and habitual sleep efficiency, and were weighed equally on a 0-3 scale. The component scores were then summed to yield a global PSQI score, which had a range of 0-9; higher scores indicated worse sleep quality.

Weekly Communication

We checked in with participants by phone calls or text once a week to ask if they had experienced any additional GI symptoms, supplement regimen, menstrual cycle changes (for women only), or any changes in their supplement regimen, adherence, or negligence. Weekly phone calls or texts occurred once a week on the cyclist's chosen day.

Statistical Analyses

All data was reported as mean ± standard deviation or frequency (%). SPSS statistical software (V. 24.0, Chicago, IL, USA) was used for all analyses. A criterion alpha level of 0.05 was used to determine statistical significance. All data were tested for normality using the Shapiro–Wilk test. If normality assumptions were violated, an equivalent non-parametric test was performed. Descriptive statistics were reported for all study variables.

We performed a battery of preliminary analyses to check for potential covariates for our primary analyses. A series of one-way ANOVAs were performed to test for group differences in continuous anthropometric, demographic, and performance variables at baseline that were collected during visit 1. Chi-squared analyses were performed to test for group differences in categorical variables at baseline. Pearson’s correlation was computed for all continuous study

variables. Finally, one-way ANOVAs assessed differences in outcome variables according to categorical demographic variables (gender and ethnicity). Demographic, behavioral (i.e., sleep, physical activity), anthropometric, and nutrient variables that were observed to have significant differences according to group or in which there are significant differences in the outcome variables were entered into the final models as covariates.

Test re-test reliability was conducted on TT performance. The reproducibility was expressed using the coefficient of variation (CV [%]), and intraclass correlation coefficient (ICC) using a one-way random effects model and change in the mean between test and re-test was calculated from visit 1, visit 2, and visit 3. The variability of the NO metabolites was calculated using the coefficient of variation.

To address our research questions, we used a 2 (pre/post exercise bout) X 2 (pre-post supplement) X4 (condition) mixed model ANOVAs to assess mean differences in total NO metabolite production and a 2 (pre-post supplement) X 4 (condition) mixed model ANOVA to assess mean differences in time to completion. A 2 (pre/post) X 4 (condition) X 5 (repeated measures [every 5-km] within bouts) mixed model ANOVA was used to assess RPE, mean power, heart rate, and VO_2 (average oxygen utilization). Additionally, we repeated all mixed model analyses excluding women as sensitivity analyses to see to what extent the inclusion of women influenced the observed effects. Effect sizes were expressed as Cohen's *d*. Effect size thresholds were categorized and interpreted as small ($d = 0.20$), medium ($d = 0.50$), and large ($d = 0.80$) [88]. In the event of a significant F-ratio, the model was decomposed using a series of between-groups and repeated-measures ANOVAs with Bonferroni correction.

Tests for homogeneity of variances were conducted with Levene's test for heteroscedasticity. If the homogeneity of variances was violated, a Welch *F*-ratio was reported. Mauchly's

sphericity test was used to assess the assumption of equality of variance in the difference scores of repeated measures. In the case of unexpected protocol abnormalities (e.g., a female participant's menstrual cycle appears abnormal) or an extension of the duration between visits is unavoidable, we performed a sensitivity analysis to determine to what extent it may have affected or changed our observations.

RESULTS

All tables and figures referred to in the following text have been provided in Appendix A and B. We have provided an "A" or "B" following the figure or table number designation to help identify which appendix that item appears in.

Participant demographics and baseline anthropometry

Participants included 50 male (n = 42) and female (n = 8) cyclists (ages 18-55 years) who regularly competed in category 1-3 cycling races across several disciplines, including mountain, gravel, cross country, road, and cyclocross. Baseline anthropometric measures for cyclists randomized to Q+CIT (n = 11 males, 1 female), Q (n = 9 males, 4 females), CIT (n = 11 males, 1 female), and PL (n = 11 males, 2 females) groups are summarized in Table 1A. No significant differences were found for age, gender, ethnicity, or anthropometric measures ($p > 0.05$). There were no significant changes in menstrual cycles among women. All testing was performed during the participant's follicular phase. Three of the women were on mono or biphasic OCs. There was one women cyclist who did not have a regular cycle but there were no changes in performance between testing sessions ($p > 0.05$). Due to these similarities, sensitivity analyses eliminating participants based on menstrual variables were not required. All cyclists were encouraged to maintain

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training efforts during the supplementation period. Total weekly training distance ranged from 101.58(64.36) to 285.92(92.10) km represented as mean(SD), respectively. Cyclist's total weekly time spent training ranged from 11.16(5.08) to 18.55(7.41) hours. There were no group differences in training volume between or within groups at baseline ($p > 0.05$) (Table 1A).

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Performance Measures

Time-trial (TT) Performance

Average TT performance was 30.50(2.65) minutes, 30.46(2.26), and 30.57(2.49), at visits 1, 2, and 3, respectively. Total time trial performance did not differ among groups at all visits (Table 2-4A). After 28 days of supplementation, TT performance did not change due to supplementation in any group (Figure 17B). The main effect of visit was insignificant [F (1, 46) = 0.43, $p = 0.52$]. The main effect of the supplement was insignificant [F (3, 46) = 0.31, $p = 0.82$]. There were no significant interaction effects in the model [F (3, 46) = 0.84, $p = 0.48$)]. (Table 20A).

When excluding women, the main effect of visit was insignificant [F (1, 38) = 0.04, $p = 0.85$]. The main effect of the supplement was insignificant [F (3, 38) = 0.95, $p = 0.44$]. There were no significant interaction effects in the model [F (3, 38) = 0.90, $p = 0.45$)] (Table 21A).

There was low variability between TT performance between visits: visit 1 CV = 8.6% [CI: 29.74-31.23], visit 2 CV = 7.2% [29.83-31.08], and visit 2 CV = 8.0% [CI: 29.87-31.27] and a strong test-rest validity between visit 1-visit 2 (ICC= 0.78).

Average Oxygen Consumption (VO_2)

Cyclists maintained an average VO_2 of 39.96(6.75), 41.48(7.2), and 42.09(6.15) (mL/kg/min) and their highest achieved oxygen consumption was 45.31(6.69), 46.94(7.17), and

47.10(6.15) (mL/kg/min) at visits 1, 2, and 3 during the TTs, respectively (Tables 2-4A). The results did not achieve statistical significance in average VO_2 nor highest achieved VO_2 following supplementation (Figure 18B) ($p > 0.05$, Table 4A). The main effect of visit was insignificant [$F(1, 46) = 1.32$, $p = 0.26$]. The main effect of time was significant, indicating a large effect size [$F(4, 46) = 143.61$, $p < 0.01$, $d = 3.56$] such that VO_2 at 0-km was significantly lower than all other distance markers ($p < 0.00$) and VO_2 at 20-km was significantly higher than all other distance markers ($p < 0.00$) (Figure 35B). However, VO_2 at 5-10- and 15-km did not significantly differ from each other at pre- (visit 2) and post-supplementation (visit 3) ($p > 0.05$) (Figure 34B). The main effect of the supplement was insignificant [$F(3, 46) = 0.81$, $p = 0.49$]. The interaction effect of visit*time was insignificant [$F(1, 46) = 1.92$, $p = 0.17$]. The interaction effect of time*supplement was insignificant [F 12, 46) = 0.81, $p = 0.59$]. The interaction effect of visit*supplement was significant [$F(3, 46) = 3.24$, $p = 0.03$, $d = 0.91$] (Table 22A). Further, the magnitude of the visit*supplement interaction can be represented using the effect size statistic [87]. The effect size indicated a large effect, respectively (Table 22A). The average VO_2 differentially changed from V2 to V3 in Q and CIT groups, but not in the Q+CIT and PL groups (Table 24A). There was no difference in VO_2 across collapsed groups from pre-supplementation (visit 2) to post-supplementation (visit 3) (Table 25A, Figure 33B). However, Q and PL significantly differed on average VO_2 from each other ($p = 0.03$) (Table 26A). The interaction effect of visit*time*supplement was insignificant [$F(12, 46) = 0.93$, $p = 0.49$] (Table 22A).

When excluding women, the main effect of visit was insignificant [$F(1, 38) = 1.96$, $p = 0.17$]. The main effect of time was significant revealing a large effect size [$F(4, 38) = 121.28$, $p < 0.01$, $d = 3.56$] such that VO_2 at 0-km was significantly lower than all other distance markers ($p < 0.00$) and VO_2 at 20-km was significantly higher than all other distance markers ($p < 0.00$).

However, VO_2 at 5-10- and 15-km did not significantly differ from each other at pre- (visit 2) and post-supplementation ($p > 0.05$) (visit 3). The main effect of the supplement was insignificant [F (3, 38) = 0.86, $p = 0.47$]. The interaction effect of visit*time was insignificant [F (4, 38) = 1.87, $p = 0.12$]. The interaction effect of time*supplement was insignificant [F (12, 46) = 0.56, $p = 0.87$]. The interaction effect of visit*supplement was insignificant [F (3, 38) = 1.21, $p = 0.32$] (Figure 34B). The interaction effect of visit*time*supplement was insignificant [F (12, 46) = 1.07, $p = 0.39$] (Table 23A).

Average Power Output

Cyclists in all groups were able to maintain an average power output (Watts, W) of 251.61(48.35) (Q+CIT), 253.23(44.45) (Q), 232.53(52.55) (CIT), 255.67(52.97) (PL) at pre-supplementation (visit 2) (Table 3A) and 255.17(48.28) (Q+CIT), 263.46(52.73) (Q), 239.00(55.20) (CIT), and 248.08(50.80) (PL) at post-supplementation (Table 4A), respectively. There were no statistical differences in average power output with supplementation pre-to-post-supplementation (Figure 19B). The main effect of visit was significant [F (1, 46) = 8.89, $p = 0.01$, $d = 0.87$]. The main effect of time was significant, signifying a large effect size [F (4, 46) = 15.69, $p < 0.01$, $d = 1.15$]. The main effect of the supplement was insignificant [F (3, 46) = 0.65, $p = 0.59$]. The interaction effect of visit*time was significant with a moderate-large effect size [F (4, 46) = 3.12, $p = 0.02$, $d = 0.51$] (Table 29A). The interaction effect of time*supplement was insignificant [F 12, 46) = 0.59, $p = 0.85$]. The interaction effect of visit*supplement was insignificant [F (3, 46) = 1.55, $p = 0.22$]. At 0 and 20-km time points, there was a difference in average power collapsed across groups between visit 2 and visit 3 ($p = 0.01, 0.02$, $d = 0.77, 0.73$) (Table 31A, Figure

36B). The 3-way visit*time*supplement interaction was insignificant [F (12, 46) = 1.24, p = 0.29] (Table 29A).

When excluding women, the main effect of visit was significant with a large effect size [F (1, 38) = 9.28, $p < 0.01$, $d = 1.00$]. The main effect of time was significant with a large effect size [F (4, 38) = 11.30, $p < 0.01$, $d = 1.09$]. The main effect of the supplement was insignificant [F (3, 38) = 1.17, $p = 0.34$]. The interaction effect of visit*time was significant [F (4, 38) = 2.87, $p = 0.02$, $d = 0.55$] (Table 30A). At 0 and 20-km time points, there was a difference in average power collapsed across groups between visit 2 and visit 3 ($p = 0.01, 0.03$, $d = 0.87, 0.73$) (Table 32A). The interaction effect of time*supplement was insignificant [F 12, 38) = 0.45, $p = 0.90$]. The interaction effect of visit*supplement was insignificant [F (3, 38) = 1.81, $p = 0.16$]. The 3-way visit*time*supplement interaction was insignificant [F (12, 38) = 1.33, $p = 0.21$] (Table 30A).

Maximal Power

Cyclists in all groups exhibited a maximal power (W) during the trial at 462.17(9.92) (Q+CIT), 488.17(164.78) (Q), 560.17(148.40) (CIT), 459.54(127.03) (PL) at baseline testing (visit 1) (Table 2A), 514.17(129.97) (Q+CIT), 484.54(116.44) (Q), 511.92(128.99) (CIT), and 484.62(99.07) (PL) at pre-supplementation (visit 2) (Table 3A), and 529.00(150.33) (Q+CIT), 497.53(111.03) (Q), 533.67(190.02) (CIT), and 485.54(103.69) (PL) at post-supplementation (visit 3) (Table 4A), respectively. There were no statistical differences in maximal power output pre-to-post-supplementation (Figure 20B). There were no main or interactive effects for maximal power ($p > 0.05$).

Heart Rate (HR)

Average HR (beats· min^{-1}) during the trials were 164.49(11.86) (Q+CIT), 166.67(10.86) (Q), 170.08(8.16) (CIT), 162.27(12.31) (PL) at pre-supplementation (visit 2) (Table 3A) and 164.94(10.17) (Q+CIT), 168.08(7.21) (Q), 166.12(12.28) (CIT), and 164.09(11.37) (PL) at post-supplementation (Table 4A), respectively. Then, cyclists in all groups exhibited a maximal HR (beats· min^{-1}) during the trial at 182.08(12.91) (Q+CIT), 180.54(8.67) (Q), 183.17(9.87) (CIT), 177.23(11.80) (PL) at pre-supplementation (visit 2) (Table 3A) and 183.83(179.85) (Q+CIT), 179.85(7.00) (Q), 180.83(11.95) (CIT), and 178.31(12.78) (PL) at post-supplementation (Table 4A), respectively. The results did not achieve statistical significance in average, submaximal, or recovery HR pre-to-post supplementation (Figures 21, 23-27B).

The main effect of visit was insignificant [$F(1, 46) = 0.20$, $p = 0.61$]. However, the main effect of time was significant, indicating a large effect size [$F(4, 46) = 300.27$, $p < 0.01$, $d = 5.17$]. For example, HR was significantly different at all distance markers at pre- (visit 2) and post-supplementation (visit 3) ($p < 0.01$) (Figure 22B). The main effect of the supplement was insignificant [$F(3, 46) = 0.78$, $p = 0.51$]. The 3-way visit*time*supplement interaction was insignificant [$F(12, 46) = 0.90$, $p = 0.50$] (Table 33A).

When excluding women, the main effect of visit was insignificant [$F(1, 38) = 0.26$, $p = 0.61$]. The main effect of time was significant with a large effect size [$F(4, 38) = 268.97$, $p < 0.01$, $d = 5.42$] such that HR was significantly different at all distance markers at pre- (visit 2) and post-supplementation (visit 3) ($p < 0.01$). The main effect of the supplement was insignificant [$F(3, 38) = 0.64$, $p = 0.60$]. The 3-way visit*time*supplement interaction was insignificant [$F(12, 38) = 0.80$, $p = 0.57$] (Table 34A). There were no statistically significant results for 30-sec, 1-min, or 2-min recovery HR (Figures 25-27B).

Respiratory Exchange Ratio (RER)

The average RER at each visit was 0.98(0.07), 0.97(0.07), and 0.97(0.05), respectively. The respiratory exchange ratio (in the range between 1.10 and 1.15) indicated cyclists neared maximal effort [42, 50, 51]. The highest achieved RER at each visit was 1.06(0.08), 1.04(0.07), 1.04(0.07), respectively. Regardless of the group, there were no statistically significant results from pre-supplementation to post-supplementation for the highest achieved RER ($p > 0.05$) (Figure 28B). The main effect of visit was insignificant [$F(1, 46) = 0.09$, $p = 0.77$]. The main effect of time was significant revealing a large effect size [$F(4, 46) = 16.75$, $p < 0.00$, $d = 1.22$] such that RER at 0-km was significantly lower than 10- and 15-km ($p < 0.00$), and RER at 20-km was significantly higher than 10-and 15-km ($p < 0.00$). RER at 5-10- and 15-km significantly differed from each other at pre- (visit 2) and post-supplementation (visit 3) (Figure 37B). The main effect of the supplement was insignificant [$F(3, 46) = 0.32$, $p = 0.81$]. The 3-way visit*time*supplement interaction was insignificant [$F(12, 46) = 1.30$, $p = 0.24$] (Table 42A).

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When excluding women, the visit's main effect was insignificant [$F(1, 38) = 0.05$, $p = 0.83$]. The main effect of time was significant, demonstrating a large effect size [$F(4, 38) = 13.56$, $p < 0.00$, $d = 1.19$] such that RER at 0-km was significantly lower than 15-km ($p < 0.00$), and RER at 20-km was significantly higher than 10-and 15-km ($p < 0.00$). RER at 5-10- and 15-km significantly differed from each other at pre- (visit 2) and post-supplementation (visit 3). The supplement's main effect was insignificant [$F(3, 38) = 1.16$, $p = 0.34$]. The 3-way visit*time*supplement interaction was insignificant [$F(12, 38) = 1.61$, $p = 0.20$] (Table 42A).

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Ratings of Perceived Exertion (RPE)

The average RPE at each visit was 15.79(1.43), 15.82(1.42), and 15.90(1.29). An RPE greater than 17 indicated cyclists neared maximal effort [42, 50, 51]. The highest achieved RPE at each visit was 18.66(1.49), 19.08(1.24), and 19.22(0.91), respectively. Regardless of the group, the average RPE was insignificant (Figure 29B). The visit's main effect was insignificant [F (1, 45) = 0.50, $p = 0.49$]. The main effect of time was significant revealing a large effect size [F (4, 45) = 273.98, $p < 0.00$, $d = 4.96$] such that RPE was significantly different at all distance markers at pre- (visit 2) and post-supplementation (visit 3) ($p < 0.01$) (Figure 38B). The supplement's main effect was insignificant [F (3, 45) = 0.26, $p = 0.86$]. The 3-way visit*time*supplement interaction was insignificant [F (12, 46) = 1.71, $p = 0.11$] (Table 35A).

When excluding women, the visit's main effect was insignificant [F (1, 37) = 0.37, $p = 0.55$]. The main effect of time was significant [F (4, 38) = 221.67, $p < 0.00$, $d = 4.96$] such that RPE was significantly different at all distance markers at pre- (visit 2) and post-supplementation (visit 3) ($p < 0.01$). The main effect of the supplement was insignificant [F (3, 37) = 0.27, $p = 0.85$]. The 3-way visit*time*supplement interaction was significant with a large effect size [F (12, 38) = 2.19, $p = 0.02$, $d = 0.84$] (Table 36A). Pairwise comparisons revealed the source of this 3-way interaction to be a significant difference in the Q+CIT group at 0-km ($p = 0.15$) and at 20-km ($p = 0.01$) from pre-to-post-supplementation. (Table 40A). The interaction resulted in a higher RPE in the Q+CIT at 0-km and lower RPE at 20-km pre-supplementation (Figure 39B) and lower RPE at 0-km and higher RPE at 20-km at post-supplementation, compared to the other groups (Figure 40B). No other group differences were detected from pre-to-post supplementation (Table 38-39A, Figures 39-40B).

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Cadence/Speed

The average cadence (rpm) was 90.89(7.95), 89.89(8.55), and 90.10(8.90), respectively. Average speed (kph) was 63.60(17.04), 63.18(4.47), and 63.17(4.97) at visits 1, 2, and 3, respectively. There was no performance change in cadence nor average speed pre-to-post-supplementation (Tables 2-3A). There were no main or interactive effects for cadence or speed ($p > 0.05$).

Nitric Oxide Metabolites

Regardless of the group, NO metabolites were not significant pre-post supplementation (Figure 30-31B). The main effect of time for NO metabolites (μM) was significant revealing a large effect size [F (1, 46) = 5.52, $p = 0.03$, $d = 0.70$] such that NO metabolites were significantly different at pre-to-post-exercise time points at pre- (visit 2) and post-supplementation (visit 3) ($p < 0.01$) (Table 23A, Figure 32B). The visit's main effect was insignificant [F (1, 46) = 1.35, $p = 0.24$]. The supplement's main effect was insignificant [F (3, 46) = 1.18, $p = 0.33$]. Further, there were no significant interaction effects in the model [F (3, 46) = 2.21, $p = 0.10$] (Table 27A).

When women were excluded, the main effect of time for NO metabolites (μM) was significant with a large effect size [F (1, 46) = 4.42, $p = 0.04$, $d = 0.70$] such that NO metabolites were significantly different at pre-to-post-exercise time points at pre- (visit 2) and post-supplementation (visit 3) ($p < 0.05$). The visit's main effect was insignificant [F (1, 46) = 1.09, $p = 0.30$]. The supplement's main effect was insignificant [F (3, 46) = 1.06, $p = 0.38$]. Further, there were no significant interaction effects in the model [F (3, 46) = 1.59, $p = 0.21$] (Table 28A). NO metabolite concentration was increased albeit not significantly (Figure 29-30 B). There was high variability of NO metabolites between visits: visit 2 pre-exercise (CV = 67.3% [CI: 1.72-2.56]), visit 2 post-

exercise (CV = 68.4% [CI: 1.57-4.07]), visit 3 pre-exercise (CV = 154.0% [CI: 1.57-4.07]), and visit 3 post-exercise (CV = 142.4% [CI: 1.77-4.26]).

Statistical Correlations

Correlations at baseline

For clarity, all correlations presented below have p values < 0.05 and are presented in Appendix A. Group macronutrient and micronutrient differences are presented in Table 9A.

Demographic Correlations

Age was significantly correlated to average VO_2, maximal, and submaximal HR. Body mass was significantly correlated to average VO_2 and average HR. Height was significantly correlated to time-trial performance and average power. Lean tissue was significantly correlated to time-trial performance, average power, and average HR. Fat mass was significantly correlated to TT performance, average power, average VO_2, and average HR. Visceral adipose tissue was significantly correlated to average VO_2, average HR, maximum HR, and submaximal HR. Body fat percentage was significantly correlated to time-trial performance, average power, and average VO_2 (Table 5A).

Performance and Nutrient Correlations

Saturated fat was significantly correlated to TT performance and average power. Carbohydrates and omega-3 were significantly correlated to the highest achieved RER. Omega-6 was significantly correlated to the highest achieved RER (Table 6A). There were no differences in macronutrients or micronutrients among supplement groups at baseline (p > 0.05) (Table 9A).

Correlations at Visit 2

For clarity, all correlations presented below have p values < 0.05 and are presented in Appendix A. Group macronutrient and micronutrient differences are presented in Table 14A.

Demographic Correlations

Age was significantly correlated to average VO_2 and average HR. Body mass was significantly correlated to TT performance and average power. Height was significantly correlated to TT performance, average power, and average HR. Lean tissue was significantly correlated to TT performance, average power, and average VO_2. Fat mass was significantly correlated to average VO_2, average HR, maximum HR, and submaximal HR. Visceral adipose tissue was significantly correlated to TT performance, average power, and average VO_2. Sleep quality was significantly correlated to average power (Table 10A).

Performance and Nutrient Correlations

Carbohydrates were significantly correlated to TT performance and average power. Fat was significantly correlated to average power. Total cholesterol was significantly correlated to NO metabolite's pre-exercise (Table 11A). Vitamin A was significantly correlated to TT performance. Vitamin B6 was significantly correlated to TT performance and average power. (Table 12A). Iron was significantly correlated to TT performance (Table 13A) There were significant differences in vitamin B3 among supplement groups at baseline ($p < 0.05$) (Table 14A).

Correlations at Visit 3

For clarity, all correlations presented below have p values < 0.05 and are presented in Appendix A. Group macronutrient and micronutrient differences are presented in Table 19A.

Demographic Correlations

Age was significantly correlated to average HR, maximum HR, submaximal HR, and highest achieved RER. Body mass was significantly correlated to average VO_2 and average HR. Height was significantly correlated to TT performance and average power. Lean tissue was significantly correlated to TT performance, average power, and average HR. Fat mass was significantly correlated to TT performance, average power, average VO_2, average HR, and highest achieved RER. Visceral adipose tissue was significantly correlated to average VO_2, average HR, and highest achieved RER. Body fat percentage was significantly correlated to TT performance, average power, and average VO_2. Sleep quality was significantly correlated to TT performance and average power. Sleep quality was significantly correlated with average power (Table 15A).

Performance and Nutrient Correlations

Total fiber intake was significantly correlated to pre- and post-exercise NO metabolites. Omega-3 was significantly correlated to pre- and post-exercise NO metabolites. Total cholesterol was significantly correlated to NO metabolite's pre-exercise (Table 16A). Vitamin A was significantly correlated to pre- and post-exercise NO metabolites. Vitamin B2 was significantly correlated to the highest achieved RER. Vitamin C was significantly correlated to pre- and post-exercise NO metabolites. Vitamin D was significantly correlated to TT performance (Table 17A). Calcium was significantly correlated to average VO_2. Iron was significantly correlated to average HR,

highest achieved RER, and the highest achieved RPE. Potassium was significantly correlated to average VO_2. Magnesium was significantly correlated to pre- and post-exercise NO metabolites (Table 18A). There were significant differences in total daily calories, vitamin B1, and folate among supplement groups at baseline ($p < 0.05$) (Table 19A).

Physical Activity (PA)

Physical activity (PA) is expressed as arbitrary units and is calculated as RPE*total daily minutes/ total exercised days out of 28 (Table 1A). There were no differences in PA across supplement groups ($p > 0.05$). All subjects were training to compete in competitive races (A, B, or C) and were instructed to maintain their current training volume. Physical activity had no statistically significant correlation to TT performance (Table 13A).

Sleep Quality

At baseline testing, cyclists' sleep quality was not correlated to any of the performance variables (Table 5A). At pre-supplementation, visit 2, cyclists' sleep quality was related to average power ($p = 0.03$) (Table 10A). At post-supplementation, cyclists' sleep quality was related to TT performance ($p = 0.02$) and average power (Table 15A).

Supplement Compliance

There was a 92% supplement compliance rate with all cyclists. Four cyclists were non-compliant, missing more than 10% of the supplement dosages (~5.6 supplement dosages) (Figure 41B). Non-compliant individuals were removed for sensitivity analyses. After removal, there were no observed changes in the outcome of the analyses.

GI distress

Cyclists from each group reported some degree of GI distress (Q+CIT: n = 8, Q: n = 11, CIT: n = 10, PL: n = 6) (Figure 42B). Minor symptoms reported include bloating, heaviness in the stomach, belching, abdominal pain, and difficulty with gas evacuation. However, only 29 cyclists completed all four weekly GI surveys thus making it difficult for us to determine the true impact of supplementation on GI distress.

DISCUSSION

The role of supplementation in cycling is of high interest to increase performance in competition and metabolic adaptations associated with training [130]. Here, we have conducted the first double-blinded study to examine the combined effects of Q+CIT on 20-km TT performance in trained cyclists compared to cyclists receiving either Q or CIT alone. Our results demonstrated that Q+CIT does not increase NO synthesis and cycling performance in contrast to our hypotheses. Specifically, our data suggest no potential benefits of Q+CIT on NO metabolite production and cycling performance (as measured by TT performance, mean power, VO_2, submaximal and maximal heart rate, RER, RPE, and GI distress. Further, Q+CIT does not seem beneficial for 20-km TT performance. Nevertheless, the novelty of our experimental design provides a strong base for systematic replication (e.g., an increase in cycling duration or the addition of other polyphenols).

Performance Effects

Time-trial (TT) Performance

To our knowledge, we were the first to examine the influence of Q+CIT, Q, and CIT on a single maximal 20-km TT. In our study, participants in the Q group received 1000 mg/day for 4 weeks which did not result in an improvement in cycling TT performance. In contrast, a 6-week supplementation of Q (600 mg) combined with vitamins and minerals improved 30-km TT performance in similarly trained male athletes [266]. However, there were no differences between the performance times of Q compared to the placebo although Q exhibited better performance compared to the baseline TT [266]. It is confounding to interpret Q's effects on 30-km TT performance since the formulations contained caffeine which is a powerful ergogenic aid to improve aerobic performance [154]. Alternatively, a longer duration (> 4 weeks) of Q supplementation may have been required to improve cycling performance [74]. Further, Q (1000 mg) was combined with epigallocatechin 3-gallate (EGCG), (120 mg) and resulted in an enhanced granulocyte oxidative burst activity, and resisted inflammatory markers (CRP, IL-6, and IL-10) in Q–EGCG after three days of heavy exercise (cycling for three hours at ~57% Wmax) [299]. However, there was no difference in mRNA expression for genes related to skeletal muscle mitochondrial biogenesis [299]. Q may exhibit its effects with the appropriate mixtures of vitamins and minerals for optimal absorption. Vitamin mixtures are suggested for optimal absorption of Q in humans [48]. In contrast to our findings, 2.4 g/day CIT alone improved 4-km TT performance in trained males by 1.5% after 7 days of consumption then 2.4 g 1 hr. before the TT implying that CIT supplementation may reduce the time to complete a TT with shorter distance durations [373]. This suggests that Q may increase cycling TT performance to a greater extent when combined with

antioxidants, polyphenols, nutrients, flavonoids, fish oil, or isoquercetin than an amino acid (CIT) [299].

Our results are in concert with previous results obtained from 13 trained cyclists who were supplemented with 1000 mg/day of Q+ 820 mg Vitamin C, 40 mg Vitamin B3, or the same vitamin supplement without Q for 28 days. These cyclists did not improve their cycling time trial performance after performing a defined amount of work [398]. Similarly, we controlled for physical activity and diet to confirm the reliability of TT performance after 28 days of supplementation. The lack of findings may be due to our strict adherence to trained cyclists, the type of exercise test utilized, the duration of the supplementation, and/or the training status of our participants.

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The trained subjects in our study reported longer durations of riding (>5 hours per week), including at least two hours of zone two, higher intensity threshold, tempo, and anaerobic training. Thus, the 20-km TT may have not provided a significant stimulus to detect changes in completion times and longer durations may have revealed impacts of the supplement [361, 368]. Future research needs to examine longer durations of testing to match cyclists' specific training loads [368]. Further, it is important to track the cyclists' season/offseason, demands of the race, and specificity for greater responses of variables related to endurance performance. The principle of sport specificity predicts that the closer the training or testing routine is to the demands of the desired outcome (i.e., a cycling race), the greater the likelihood that adaptations will occur [175, 324]. It is possible that the 20-km TT did not reach the cyclist's 'biological ceiling,' implying that the regulatory control mechanisms signaling adaptive responses may have been inadequate. This notion is supported by studies that report increased endurance capacity in untrained individuals supplemented with Q [102, 390]. Future research needs to utilize cycling protocols in

consideration of the complex interplay and interactions of racing demands to investigate the effects of Q+CIT on endurance exercise performance.

It seems reasonable to consider cyclists' training volume for improving the physiological determinants of cycling performance with a longer duration test. Additionally, the use of greater or lesser training volume will be affected by several factors (e.g., training phases over a season, A, B, or C races, training status) when incorporating a supplement to enhance cycling performance [145]. Cycling includes many hours of competition at moderate and high intensities and future Q+CIT, Q, or CIT cycling research must examine the demands of the cycling season, specificity, and design a cycling test that is similar to training and racing demands. Even though our study resulted in null exercise performance findings, it allowed us to measure VO_2, mean power, HR, RPE, and RER at specific intervals and control environmental factors that would normally affect race performances. Future studies are needed to examine the effect of Q+CIT with greater bouts of intensity, longer duration, and more specific measures of performance or biochemical markers.

Average Oxygen Consumption (VO_2)

To our knowledge, we are the first to investigate Q+CIT, Q, and CIT on average oxygen consumption during a 20-km TT. Here, our findings suggest that Q supplementation did not enhance TT performance, but the ergogenic aid did increase VO_2 from pre-to-post supplementation (Table 24A). The performance results are in contrast with a published study that investigated Q supplementation (600 mg) with essential vitamins for six weeks in trained cyclists which reported increased 30-km TT performance but saw no effects of Q on the change in VO_{2max} percentage [266]. The mechanisms behind the performance changes are unknown in the Q group

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since they did not perform blood analyses. Further, they implemented a hilly 30-km TT at sustained higher intensities, compared to our flat TT. The different course terrain could have allowed for the recruitment of different muscle fibers, muscular activity, power, and energetics which Q could have targeted [137]. However, our results reveal that Q is capable of enhancing muscle oxidative potential but the mechanisms are not understood. Nevertheless, the impact of Q on oxygen consumption in trained athletes still remains controversial [98, 266, 422, 423]. A study of 40 trained cyclists provided 1000 mg/day of Q for three weeks failed to show any group differences in measures of cycling efficiency, substrate utilization, power output, or skeletal muscle mRNA expression for PGC-1α or SIRT1 – key factors known to regulate mitochondrial function and, by extension, impact oxygen consumption [114]. The three weeks of Q supplementation may have been insufficient to detect the biological effect of Q. Similar to our study, the administration of a higher dosage, longer duration, or specific Q supplementation timing may be required.

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The increase in VO_2 across distance markers (time) agrees with previous cycling studies [210, 218, 243, 322]. However, this may indicate that the trained cyclists have quicker and adapted VO_2 kinetics and can increase their VO2 rapidly, thus possibly minimizing the oxygen debt [132, 229]. Future research needs to examine the effects of Q+CIT and Q on VO_2 kinetics during various TTs. Four weeks of 6 g of CIT supplementation was shown to increase average oxygen consumption (VO_2) (Table 24A). The effect seen by CIT is likely due to its ability to stimulate NO synthesis and plays a critical role in mitochondrial biogenesis and respiration, glucose uptake, and calcium handling [14, 301, 401]. Similar to previous research, CIT improved VO_2 kinetics during a strenuous cycling bout in recreationally active men [19]. The increased synthesis of NO can ultimately result in multiple performance effects such as enhancing oxygen

and nutrient delivery thus improving exercise tolerance and recovery mechanisms [38]. CIT supplementation is shown to enhance cycling performance [364, 374]. In contrast, previous research has used different doses, supplementation periods, or training intensities that make the impact of CIT supplementation on cycling performance or physiological variables difficult to reconcile [146]. For example, 6 g of CIT was administered for 7 days in trained cyclists [364], and 2.4 g/day of CIT was administered for 7 days in trained athletes [374] and showed performance improvements. Chronic supplementation of CIT for at least 7 days seems to improve long-lasting high-intensity exercise performance during cycling tests consistently. Trained individuals have an enhanced blood flow which could be a proposed mechanism for the ergogenic potential of CIT [135]. However, evidence supporting acute improvements (< 7 days) in vasodilation, vascular conductance, and skeletal muscle tissue perfusion after supplementation remains unknown.

When all cyclists were considered, there was a significant interaction between visit and supplement. However, when women were entered into a sensitivity analysis, there was no interaction effect, suggesting the significant improvement in VO_2 from pre-to-post supplementation in the Q and CIT groups was driven by women. Even though the follicular phase has low concentrations of estrogen and progesterone, our findings suggest there may be a menstrual cycle influence (i.e., follicular phase) on oxygen consumption with the ingestion of a supplement. Our results are in contrast to those previously investigated in that there was an increase in oxygen consumption during the luteal phase [23, 99, 113]. However, these studies did not examine the role of Q and CIT during the menstrual cycle phases. Further, when Q and CIT were ingested alone, this may suggest the combination of Q+CIT may not be necessary to improve VO_2. It is possible that Q and CIT may play a role in the different phases of the menstrual cycle to improve oxygen consumption, but future research needs to test these supplements with a larger female

sample size compared to the other menstrual cycle phases. Thus, gender and hormone variations need to be considered when examining the influence of a supplement on oxygen consumption.

Average Power

In the current study, average power increased from pre-to post-supplementation as cyclists pedaled higher average power outputs at the beginning (0-km) and end (20-km) of the time trial challenge during V3 compared to V2. The higher power at the beginning of a race is often seen at the start line to accelerate the bike and establish a riding pace. Additionally, the higher power at the end of a race can be attributed to a final sprint, often performed by many cyclists, to reach the finish line [125]. Further, gender differences need to be considered when examining the power profile in TT performance.

There was an improvement in average power over the 20-km TT marks from pre-to-post-supplementation in men across all groups (i.e., 0-, 5-, 20-, 15-, 20-km). However, our sample size of women may not have been large enough to detect sex differences and power changes with the supplement. Future research needs to explore Q+CIT's biological and power effects in men and women with a larger sample size.

Even though all women were tested during their luteal phase, this suggests hormonal considerations when testing power production. It has previously been shown that the luteal phase can affect strength and thus power production [75]. Therefore, when including women and men when testing power, future research needs to consider the different menstrual phases and their effect on power performance. Further, limited research exists examining the influence of Q+CIT, Q, and CIT on power performance but this was the first study to look at power-specific outcomes on 20-km TT performance.

Heart Rate (HR)

Average heart rate increased over time at pre- and post-supplementation for all cyclists (Tables 33-34A). However, there were no changes in maximal, submaximal, or recovery HR with Q+CIT, CIT, or PL. An HR increase with an increase in exercise intensity is expected and is similar to previous studies [244, 322, 391]. Our results show that the increase in average HR is an indication of achieving maximal effort. The findings of the current study support the notion that HR can be used for setting intensity and optimal pacing strategies [40, 368].

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Respiratory Exchange Ratio (RER)

Our findings show that RER increased over time across all cyclists (Tables 40-41A) which aligns with a previous finding [322]. The observation of RER >1.00 in our cyclists supports the conclusion that their performance indicated maximal effort which could serve as a performance threshold marker for 20 km TT performance. However, the 20-km maximal TT may not be an accurate measurement to detect fuel utilization over time due to intermittent collection of RER (i.e., 0-, 5-, 10-, 15-, 20-) and the maximal effort required for the test. As previously seen, measuring RER in steady-state aerobic conditions is commonly performed to indirectly determine the relative contribution of carbohydrates and lipids to the overall energy expenditure [320, 330]. Thus, if future research aims to acquire fuel utilization indications for cyclists, the methodological design of exercise intensity needs to be considered.

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Ratings of perceived exertion (RPE)

Ratings of perceived exertion increased across distance markers (time) in the TTs (Tables 35-36A) which were contrary to previously seen with well-trained male subjects who performed

20-km trials in normoxia and hyperoxia [391]. However, our results are similar to a previous study investigating the effects of Q combined with essential vitamins on 30-km TT performance in trained cyclists [266]. Our results suggest there was an increase in exercise intensity which indicated maximal effort and an increase in physiological cost [42, 50, 51, 229]. Considering this, a practical implication to coaches and athletes is that average power, RPE, and RER are important metrics to detect maximal exertion and fatigue.

The time*visit*supplement interaction for RPE when excluding women modifies perceived effort with Q+CIT. Our results suggest that combining Q and CIT may alter the perceived effort at the start (0-km) and the end of a race (20-km), indicating that the supplement may have increased the level of effort the cyclists could maintain during the TT. Similarly, three weeks of Q supplementation did not alter RPE in trained male and female ultramarathoners did not alter RPE before the Western States endurance run [393]. In contrast, 6 weeks of Q supplementation combined with antioxidants, at any given RPE, power output was higher in trained male cyclists suggesting that Q supplementation supports a perceptual effect in trained males [266]. Future research needs to establish the reliability of perceived exertion changes at 0-km and 20-km with Q+CIT in trained males and females and investigate the mechanisms causing the changes. Based on the present findings and conflicting previous results, it is our contention that it is premature to preclude any beneficial effects of Q supplementation on perceptual responses during a self-paced, competitive TT event.

Performance Conclusions

Similar to previous research, VO_{2max} or oxygen kinetics of already accomplished world-class trained endurance cyclists were not improved with a four-week supplementation of

citrulline malate [260]. Successful improvements in cycling performance involve the manipulation of training intensity, duration, and frequency, with implicit and specific goals of maximizing performance and recovery. 80% of cyclists' training sessions are performed at low intensity (i.e., 2 mM blood lactate), with about 20% dominated by periods of high-intensity work, such as interval training at approximately 90% $VO2_{max}$ [348]. For example, most cyclists implement training approaches that are characterized by several intervals of low-intensity training performed below the ventilatory threshold, but with different contributions of threshold training (i.e., between the first and second ventilatory threshold) coupled with intervals of high-intensity training (i.e., above the ventilatory threshold) [56]. Together, the intensity of the training and its distribution over time are important determinants of the outcome of adaptation and the influence of supplementation on performance improvement [348]. Thus, these results suggest effectively designing the volume and intensity of a cycling bout around the cyclist's training season may be necessary to observe sufficient evidence to increase aerobic improvements or metabolic effects from Q+CIT. Accordingly, future supplementation and cycling research should focus on low-intensity, long-duration training, in combination with fewer, high-intensity bouts to optimize and increase adaptive signaling and performance improvements.

Nitric Oxide Metabolites

Nitric oxide can modulate skeletal muscle function by regulating blood flow, contractility, glucose-calcium homeostasis, mitochondrial respiration, and biogenesis [363]. Our results showed that Q+CIT, Q, and CIT did not improve NO metabolites or aerobic performance in trained cyclists (Figures 29-30B). To our knowledge, we were the first to examine Q+CIT, Q, and CIT on NO metabolite production after a 20-km maximal TT. This significant effect of time

may be explained by the large within-individual or biological variation (CV) between visits. As previously seen, the large variability in NO metabolites may be due to a substantial inter-individual variability in plasma nitrate and nitrite pharmacokinetics before and after CIT administration [198]. The nitrate–nitrite–NO pathway may influence muscle function and exercise performance, but research is limited, especially in humans. We did not find any significant NO metabolite or aerobic improvements in our findings which are similar to those previously investigated in that no performance effects were found with NO donors on performance in trained individuals [39, 372] suggesting that NO supplements may increase redox intermediates and metabolism in untrained individuals; however, limited research exists investigating the effects of NO metabolites on aerobic performance in humans [39].

We did not control the cyclist's diet to adequately measure NO metabolites in plasma or urine. Certainly, diet has been previously shown to affect intra- and inter-variability of circulating NO metabolite concentration levels [9, 254]. Controlling for diet can account for intra-subject metabolic variation and metabolic modulations against relatively low doses of bioactive food supplements [162]. The baseline concentrations of nitrite and nitrate from the cyclist's diet could have altered the redox state. Thus, there could have been a blunted serum NO metabolite response to the supplementation in these trained cyclists, compared to lesser-trained endurance athletes [212].

Another possibility for not detecting a change in NO metabolites is that trained athletes may not respond to additional supplementation due to having sufficient Q and CIT in their diet and/or training-induced NOS upregulation [209, 276]. The training-induced NOS adaptations may have allowed the cyclists to maintain their NO concentrations and upregulation of pyruvate dehydrogenase and increase mitochondrial membrane potential, allowing for the steady supply of

oxygen, oxygen utilization, and generation of high ATP concentrations lowering oxidative stress and muscle damage during our 20-km TT [310].

Future research must consider the biological variation of NO metabolites in combination with analytical error to calculate the critical difference which is defined as the change from baseline NO metabolites to post-exercise before a meaningful biological difference can be claimed [254]. The critical difference can provide a single criterion threshold which, if exceeded, can conclude a significant change has occurred in response to Q+CIT, Q, or CIT supplementation. Further, future research is warranted to re-examine if there is validity in the large variability of NO metabolites in trained cyclists and establish a criterion threshold for "normal" NO metabolite concentration levels.

Biochemical Conclusions

The human body has protective mechanisms for maintaining redox homeostasis to cope with excess free radicals produced by oxidative stress. Endurance athletes adapt to mitochondrial biogenesis and antioxidant capacities to maintain redox homeostasis. These adaptations include activation of the Kelch-like ECH-associated protein 1-Nrf2 pathway and the nuclear factor κB (NFκB) signaling [12, 275]. Q+CIT supplement may not have been needed to regulate redox status, nor required to improve VO_2 kinetics due to upregulation and adaptations of mitochondrial and antioxidant enzymes from training [182, 271, 380]. Q+CIT could have shifted the redox status of the cyclist's muscles toward a more reduced state, thus limiting the acute fatiguing effects (i.e., RPE) of exercise-induced ROS production on NO production and oxygen consumption [248]. However, in our current study, we found no effects of Q+CIT lowering RPE post-supplementation (Figure 28B).

Interestingly, antioxidants provided in addition to an endurance athlete's normal diet may not enhance mitochondrial capacity [230]. In parallel, due to the cyclist's high training volume, the 20-km TT may not have provided sufficient evidence to detect metabolic or redox changes from the supplement. However, without a measure of oxidative stress, it cannot be concluded if the lack of changes can be linked to changes in oxidative status. Previously, the redox status was assessed in a group of professional athletes at the beginning and the end of the season. A new static oxidation-reduction potential marker and total antioxidant capacity were significantly increased, and glutathione was decreased at the end of the season, compared to the beginning [358]. This indicates the activation of adaptive response for counteracting oxidative stress was greater with a higher volume of training. Similar to our dosage of Q, 1,000 mg of daily Q ingestion for three weeks did not improve antioxidant status before the 160-km Western States Endurance Run [329]. Additionally, 40 athletes consumed either 1000 mg of Q or the placebo every day for 3 weeks before and during 3 days of cycling at 57% work maximum for 3 h. Quercetin supplementation increased the circulating plasma values of Q; however, the increase in plasma Q metabolites did not affect oxidative stress, inflammation, or plasma antioxidant capacity [277]. Likewise, Q failed to reduce oxidative stress via the inhibition of xanthine oxidase after 1 week of Q (1,000 mg) in a repeated sprint performance [2]. The physiological stress experienced by the cyclists was likely inadequate to elicit oxidative damage or immune dysfunction in which Q may play a role. Future studies may need to examine Q combined with other antioxidants or anti-inflammatory molecules which may yield greater effectiveness due to possibly yielding greater synergistic absorption and its proposed mechanisms on oxidative stress and inflammation.

Anti-oxidant effects due to Q may be increased by the co-ingestion of EGCG or polyunsaturated fatty acids [71, 196, 288]. However, 7 days of Q (1,000 mg) enhanced antioxidant

capacity and resulted in decreased oxidative stress after cycling for 60 minutes at 70% VO_{2max} in healthy adults [390]. Similar to our study's testing stimulus, we found a lack of performance improvement at visit 3 compared to visit 2. Since these athletes are trained and cycle at higher intensities for longer durations, this may be explained by the large recruitment and adaptation of antioxidant mechanisms and their ability to maintain homeostasis leaving no room for redox disturbances from the relatively short duration of the 20-km TT. It is possible that the longer duration could have caused the downregulation of endogenous antioxidant enzymes and lowered the activation of ROS-mediated pathways of cellular adaptation [398]. Previously, glutathione was shown to have an inverse relationship with the amount of superoxide and hydrogen peroxide production which may be a reflection of the lack of significance we found with Q+CIT [171]. Future research needs to consider the individuals' training status and volume for Q+CIT, Q, or CIT supplementation and its effects on oxidative stress and additional biochemical markers.

Exercise of sufficient volume, intensity, and duration accelerating damage/ fatigue can lead to an alteration in immune function increasing ROS production which may lead to the oxidation of several biological molecules [17, 28]. Therefore for a performance improvement to occur with the supplement, the stimulus applied may need to exceed a certain minimal threshold, effectively overloading the system to cause redox changes [166]. If the overload is achieved, the physiological capacity of the body will increase and adapt and the synergistic effects from the supplement could be attained; ultimately leading to improvements in health, oxidative stress, and cycling performance. Therefore, the mode of a testing stimulus is crucial to detect redox or biochemical changes, physiological adaptations, or performance improvements. Further, future research needs to examine mitochondrial protein expressions, metabolomic markers, and oxidative stress markers pre- and post-supplementation.

The Half-Life of Quercetin and Citrulline

The half-life of Q ranges between 11-28 hr [31, 32, 341] with plasma Q at 6-12 hr [116] and peak Q concentrations occurring at 1-3 hours post-ingestion [116]. Thus, the cyclist's peak Q level may have dropped by the start of the TT. Additionally, the half-life of CIT was reported to be approximately 60 minutes following ingested concentrations between 0.8 and 1.0 L/kg [331]. Some cyclists consumed their last supplement on the day of the last TT, while others finished their last supplement 24-48 hours before the last TT so this may suggest future research is warranted to investigate the acute effects of Q+CIT. Together, these half-life ranges may provide future research with the ability to detect the effects of the supplement [230, 268, 285]. In parallel, there also may be an upper limit of Q or CIT storage and thus providing further supplementation may have only provided negligible, additional bioavailability [233]. Due to the variability of consumption, future studies that control supplementation windows and diet are needed to elucidate the effects of Q+CIT's rate of absorption, peak concentration, storage, clearance levels, and acute vs chronic supplementation with varying dosages and forms to detect possible effects on cycling performance.

Dosage Conclusions

In the standard US diet, consumption of Q equivalents (i.e., flavanol glycosides), was estimated to be approximately 107 mg/d [174, 379]. However, 'higher-end' Q consumption intake is reported as high as 226 mg/d (90th percentile) [174]. Athletes still may benefit from Q+CIT or Q, but it may take longer to see a performance increase, possibly due to higher starting nutrient levels [209, 276]. Since some athletes may fall in the higher percentile and could have had higher

starting levels of Q or CIT, there could be an upper limit of absorption for Q based on the food matrix [249, 335]. In addition, the consumption of other dietary components such as fiber, fat, and polyphenols may impact the synergistic effects of Q and CIT when provided to improve cycling performance. Future studies need to assess baseline Q and CIT levels on various training stimuli to precisely analyze any impact on cycling performance [300].

GI Distress

Importantly, GI distress symptoms were reported in all groups and were not severe enough for the participants to stop consuming their daily supplement (Figure 39A). Because GI distress was also reported in the placebo group, it is unlikely that these symptoms could be attributed solely to the supplement. By extension, this may suggest that the vehicle provided (here, Crystal Lite) should be considered when designing supplementation strategies. Further, future studies should provide a daily GI survey to document GI distress. Additionally, future GI studies need to include dietary tracking to reason if the onset of symptoms causing GI distress was resulting from the supplement or normal dietary intake.

Demographic and Nutritional Intake Correlations

Correlations were analyzed to detect any possible covariates and their influence on the primary variables. Similar to previous research, age, and HR are significantly correlated to VO_2 ($p < 0.05$) [242, 246]. There is a considerable decline in VO_2 with age, typically reporting declines between 0.3 and 0.5 mL/kg/min per year [246]. In line with previously seen, it is important to consider age and HR (i.e., submaximal, maximal, and recovery) when assessing cycling performance. Our results are in line with previous findings that lean tissue and body fat percentage are significantly correlated to cycling performance (e.g., power, TT, HR, cycling economy) [289,

399]. For example, competitive female cyclists with greater lower lean body mass tend to have ~4-9% higher maximum average power per kg lean body mass over 1 second to 10 minutes [289]. This suggests that a sufficient lean of lean body mass and a reduction in body fat percentage can contribute to a cyclist's ability to generate more force and power in a specific period of time. Resistance training off the bike may be particularly useful for cyclists who want to improve their power performance. Further, VAT appears to trigger a cascade of metabolic disturbances that seem to coexist with an increase in ectopic fat storage in the muscle, liver, and heart which can lead to an increased risk of metabolic disorders such as type 2 diabetes mellitus and cardiovascular diseases and a decrease in exercise performance [228, 402]. Our significant findings of VAT to average VO_2, average HR, maximum HR, and submaximal HR suggest that exercise training can improve VAT, thereby contributing to improved cycling performance.

An adequate nutritional profile of trained cyclists allows for a high level of performance and recovery. Previously seen, a high level of carbohydrates is traditionally seen to be shown during high competition which is in line with our findings that carbohydrates were significantly correlated to TT performance and average power at pre-supplementation (visit 2) (Table 11A) [292]. A higher level of carbohydrates helps cyclists maintain optimal blood glucose levels, perform at a higher intensity, and the prevention of "hitting the wall." Saturated fat and total fat were inversely correlated to average power and our findings agree that a higher fat consumption is often seen in endurance athletes [142, 292, 293]. It is suggested that a reduction in fat intake during the competitive season will allow for a greater carbohydrate intake, enabling cyclists to perform at a higher capacity.

An adequate intake of vitamins and minerals is essential to improve cycling performance and optimal physiological functioning. Significant correlations were found between omega-3,

omega-6, calcium, iron, vitamin B6, vitamin A, vitamin D, magnesium, and vitamin C to cycling performance (Tables 13A, 16-18A). Our findings align with those previously discussed [180, 409] suggesting the potential benefits of a high nutrient density may improve cycling performance. Additionally, iron and magnesium are shown to have the strongest evidence to athletic performance. Further, there is evidence to suggest the combination of sodium, potassium, and magnesium loading may improve aspects of prolonged endurance performance [108]. The current study used dietary recall, in large part, to quantify basal intake levels of foods known to contain our supplements of interest, Q and CIT. Thus, the design of our study was not focused on identifying the impact nor making dietary recommendations about other specific nutrients. Nevertheless, future studies may consider including these nutrients in concert with Q+CIT as research has suggested bioavailability may improve with select nutrients [343]. Further, research is still limited to the potential benefits of some minerals and trace elements to athletic performance [180]. Future research needs to continue to evaluate the impact of vitamins and minerals on cycling performance, efficiency, and fatigue.

Commented [JK14]: Do I need to discuss every single nutrient?? It kind of seems beyond the scope of my study.

Limitations/Delimitations

A limitation of the current study is that the Q, CIT, and placebo powders were not analyzed for nutrient composition and therefore derived from the nutritional information from Bulk Supplements. To allow for comparison of the nutrient density scores based on the analyzed data of the supplement. Though we collected dietary recall information to control for variance in dietary intake, we did not quantify the specific concentrations of Q and CIT. This was beyond the scope of our study. Future studies need to control for nutrient compositions and density scores by controlling with design or through statistical control. Future studies need to examine the overall

nutrient density scores and quality aspects of the supplement to avoid associated risks and obtain the greatest possible benefit from consumption [337]. For future research, it is suggested to test the quality of these supplements, analyze the content, and verify if there is any difference between the information provided and the actual content, such as the presence of other undeclared ingredients.

Moreover, we did not standardize fluid intake before coming into the lab or tested hydration levels which could have affected the cardiovascular measurements. Future studies need to standardize and/or track fluid intake and hydration levels pre- and post-exercise [25, 282]. Further, by asking if the cyclists wanted to improve their performance there could have been an experimental demand artifact on TT time to completion and/or RPE [278]. Future research needs to assess this potential confounding variable on TT performance. However, we tried to control this by implementing daily physical activity tracking. Even though we measured GI distress, it was challenging to interpret the findings since all groups experienced symptoms. Additionally, we did not measure GI distress on testing days which could have affected performance results. Future GI studies need to include daily dietary tracking to reason if the onset of symptoms causing GI distress resulted from the supplement or normal dietary intake.

The main delimitation of the current study is that we restricted our participant pool to Tier 2 athletes. Unfortunately, defining a Tier 2 athlete is still ambiguous for trained cyclists [279]. Future research needs to continue to develop a classification framework for trained cyclists. Even though all cyclists were training to compete, the training volume and cycling seasons varied amongst subjects. However, the varied training volume did not affect the results, future cycling studies should aim to keep the cycling volume and training season consistent amongst cyclists or implement supplementation strategies along with progressive training as previously

alluded to but not overly stated. As such, the present study results cannot be generalized to other people, such as sedentary, clinical individuals, or regularly active non-cyclists; however, it is possible Q+CIT could provide a performance advantage in untrained individuals or recreationally trained cyclists.

Another delimitation of the current work is that all cycling performance measures (e.g., power, cadence, speed, time) were recorded using the Wahoo Kickr. Despite its use in other studies, future studies need to compare the power production from a Kickr device to validated, lab-based cycle ergometers. As such, the present study may not be generalizable to recordings acquired on other cycling equipment.

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Conclusions and Future Research

The novel aspect of this research is that this was the first study to examine the effects of Q (1000 mg/day) + CIT (6 g/day), Q (1000 mg/day), or CIT (6 g/day) four-week supplementation in trained cyclists' metabolic, cardiovascular, and performance changes during a 20-km TT. Although Q+ CIT provided insufficient evidence impacting TT cycling performance or NO metabolites, Q and CIT improved VO_2.

Commented [JO16]: This last clause is confusing and seems to mask a positive result. Flesh this idea out.

Discrepancies with the impact of Q and CIT within the existing literature and our current work may be due to several factors, including population selection, dosing strategy (i.e., amount, duration, and timing), supplement combinations, and testing protocols. Indeed, this diversity adds a level of noise to our ability to draw firm conclusions about the efficacy of Q+CIT, Q, and CIT supplementation on cycling TT performance. However, athletes wishing to explore NO enhancers are reminded there is greater existing evidence for CIT to improve exercise performance and therefore may consider the use of this supplement while the intricacies of Q+CIT and Q are

discovered [156]. Additional research is required with either Q+CIT, Q, or CIT with additional polyphenols or anti-inflammatory molecules to determine the effectiveness of producing notable improvements to endurance performance.

It is of further interest to investigate how this unique combination would impact inflammatory and recovery markers post-exercise after a prolonged training session or race combining high-intensity and low-intensity intervals. Ultimately, future challenges will be identifying a mixture of flavonoids that deliver optimal, quantifiable benefits on cycling performance. Failure to find a significant improvement in cycling performance indices in trained individuals in the current study could be reasoned that a supplement of Q+CIT may improve endurance performance in untrained people. Future Q+CIT research needs to continue to determine the proper outcome measures, dosing regimen, and duration that may amplify any perceived bioactive or metabolic effects on cycling performance. The results from the present study suggest that Q+CIT does not improve TT performance and NO metabolites in trained cyclists.

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APPENDICES

Appendix A: Chapter 2 Tables

Table 1A. Cyclist demographic characteristics at baseline familiarization testing, visit 1

m(SD)	Total Suppl. Groups	Q+CIT (n = 12)	Q (n =13)	CIT (n = 12)	PL (n = 13)
Age (yr)	30.37(8.81)	33. 43(9.12)	34.59(8.94)	36.54(8.11)	36.86(9.56)
Body Mass (kg)	77.43(10.60)	78.23(12.29)	74.61(13.70)	79.75(8.29)	77.36(7.44)
Ht (cm)	175.75(9.45)	175.57(12.70)	172.96(8.88)	177.79(8.19)	176.84(7.89)
Lean Tissue (cm)	58.16(7.90)	59.42(7.46)	55.47(10.15)	58.97(7.51)	58.96(6.21)
Fat Mass (kg)	16.49(5.87)	15.98(6.50)	16.41(6.00)	17.9(5.86)	15.75(5.65)
VAT (kg)	0.41(0.46)	0.41(0.57)	0.36(0.50)	0.38(0.38)	0.49(5.65)
Body Fat (%)	21.96(6.18)	20.87(5.61)	22.85(5.93)	23.28(6.89)	20.89(6.58)
Total Weekly Cycling Volume (AU)	1460.28(580.48)	1415.74(368.13)	1447.21(419.10)	1346.66(813.88)	1619.34(587.02)
N(%)					
Females	8(16)	1(12.5)	4(50)	1(12.5)	2(25)
Ethnicity N(%)					
White	40(80)	11(27.5)	10(25)	9(22.5)	10(25)
African American	3(6)	0(0)	0(0)	2(67)	1(33)
Asian	2(4)	1(50)	1(50)	0(0)	0(0)
Hispanic	5(10)	0(0)	2(40)	1(20)	2(40)

Data is presented as mean(SD). Ht = height, VAT = visceral adipose tissue.

Table 2A. Cyclist performance measures at baseline familiarization testing, visit 1

m(SD)	Total Suppl. Groups	Q+CIT (n = 12)	Q (n =13)	CIT (n = 12)	PL (n = 13)
Average Power (W)	241.56(49.42)	245.94(42.92)	249.36(49.08)	238.13(53.70)	232.88(55.26)
Max Power (W)	491.82(137.84)	462.17(89.92)	488.17(164.78)	560.17(148.40)	459.54(127.03)
Power (W/kg)	3.15(0.71)	3.17(0.65)	3.38(0.65)	3.03(0.72)	3.02(0.83)
Average HR (beats·min^{-1})	164.37(13.16)	163.64(15.66)	168.38(10.30)	167.84(10.62)	157.39(13.95)
Max HR (beats·min^{-1})	179.14(12.54)	180.42(15.30)	180.62(8.61)	182.58(10.56)	172.83(14.10)
SubMax HR 70% (beats·min^{-1})	125.27(8.67)	126.29(10.71)	126.43(6.03)	127.28(6.99)	120.98(9.87)
SubMax HR 75% (beats·min^{-1})	134.22(9.28)	135.31(11.47)	135.46(6.46)	136.38(7.49)	129.63(10.58)
30-sec Recovery HR	173.19(13.78)	176.77(14.88)	177.05(9.27)	172.72(17.45)	166.21(10.77)
1-Min Recovery HR	150.53(14.19)	153.00(14.53)	159.93(12.95)	146.94(11.66)	142.23(12.39)
2-Min Recovery HR	140.51(14.59)	139.83(12.79)	152.35(16.43)	135.34(10.56)	134.50(11.93)
Average VO_2 (mL-min/L)	39.93(6.64)	40.01(6.72)	40.50 ±7.18)	38.56(5.66)	40.56(7.43)
Highest Achieved VO_2 (mL- min/L)	45.33(6.69)	46.38(6.86)	45.69(7.42)	43.40(4.85)	45.78(7.61)
Average RER	0.98(0.07)	1.00(0.48)	0.97(0.08)	0.96(0.07)	1.00(0.08)
Highest Achieved RER	1.06(0.08)	1.08(0.08)	1.04(0.08)	1.03(0.06)	1.08(0.11)
Average RPE	15.79(1.43)	15.72(1.53)	15.82(1.42)	16.00(1.57)	15.63(1.37)
Highest Achieved RPE	18.66(1.49)	18.58(1.88)	18.31(1.49)	19.08(1.44)	18.69(1.18)
Average Cadence (rpm)	90.87(8.02)	89.53(9.93)	89.95(9.05)	93.33(7.87)	90.76 ±4.95)
Average Speed (kph)	39.51(3.11)	39.78(2.80)	39.96(2.98)	39.45(3.36)	38.87(3.52)
Max Speed (kph)	56.91(2.87)	56.50(1.98)	57.44(2.48)	57.00(2.40)	56.70(2.41)
Time Trial (min.)	30.49(2.62)	30.27(2.35)	29.96(2.36)	30.93(2.69)	30.82(3.19)

Data is presented as mean(SD). HR = heart rate, SubMax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion.

Table 3A. Cyclist performance measures at pre-supplementation period, visit 2

Variable	Total Suppl. Groups	Q+CIT (n = 12)	Q (n =13)	CIT (n = 12)	PL (n = 13)
Body Mass (kg)	77.55(10.55)	78.14(12.24)	74.81(13.55)	79.86(8.23)	77.62(7.57)
Average Power (W)	248.51(48.99)	251.61(48.35)	253.23(44.45)	232.53(52.55)	255.67(52.97)
Max Power (W)	491.82(137.84)	514.17(129.97)	484.54(116.44)	511.92(128.99)	484.62(99.07)
Power (W/kg)	3.23(0.71)	3.27(0.67)	3.41(0.62)	2.89(0.64)	3.34(0.84)
Average HR (beats·min^{-1})	165.82(11.00)	164.49(11.86)	166.67(10.86)	170.08(8.16)	162.27(12.31)
Max HR (beats·min^{-1})	180.68(10.81)	182.08(12.91)	180.54(8.67)	183.17(9.87)	177.23(11.80)
SubMax HR 70% (beats·min^{-1})	126.60(7.51)	127.46(9.04)	126.38(6.07)	128.22(6.91)	124.06(8.26)
SubMax HR 75% (beats·min^{-1})	135.65(8.05)	136.56(9.68)	135.40(6.50)	137.38(7.40)	132.92(8.85)
30-sec Recovery HR	176.42(10.84)	178.19(12.13)	175.41(9.53)	180.02(10.35)	172.48(11.06)
1-Min Recovery HR	151.76(12.76)	153.96(14.07)	153.46(11.02)	152.27(11.60)	147.57(14.53)
2-Min Recovery HR	141.73(12.58)	145.12(15.10)	145.11(11.44)	139.49(19.72)	137.28(13.00)
Average VO_2 (mL-min/L)	41.52(7.29)	42.61(7.38)	41.92(7.59)	38.35(5.87)	43.04(8.00)
Highest Achieved VO_2 (mL-min/L)	46.98(7.38)	48.50(7.23)	47.40(7.68)	43.36(5.24)	48.50(8.51)
Average RER	0.97(0.07)	0.98(0.43)	0.96(0.06)	0.97(0.07)	0.97(0.07)
Highest Achieved RER	1.04(0.07)	1.05(0.07)	1.03(0.08)	1.04(0.09)	1.04(0.06)
Average RPE	15.82(1.42)	15.80(1.66)	15.51(1.26)	15.97(1.49)	16.01(1.40)
Highest Achieved RPE	19.08(1.24)	18.83(1.85)	18.77(1.24)	19.50(0.90)	19.23(0.73)
Average Cadence (rpm)	89.86(8.44)	88.97(9.23)	88.53(9.46)	92.27(8.39)	89.80± 7.11)
Average Speed (kph)	39.26(2.71)	39.30(2.85)	39.71(2.69)	38.79(2.89)	39.22(2.69)
Max Speed (kph)	57.19(2.00)	56.85(2.41)	57.28(2.25)	56.35(2.56)	56.35(2.47)
Time Trial (min.)	30.46(2.20)	30.48(2.330	30.15(2.10)	30.83(2.26)	30.39(2.34)

Data is presented as mean(SD). HR = heart rate, SubMax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion.

Table 4A. Cyclist performance measures post-supplementation period, visit 3

Variable	Total Suppl. Groups	Q+CIT (n = 12)	Q (n =13)	CIT (n = 12)	PL (n = 13)
Body Mass (kg)	77.40(10.41)	78.00(12.06)	74.52(13.16)	79.99(8.72)	77.36(10.41)
Average Power (W)	251.60(51.02)	255.17(48.28)	263.46(52.73)	239.00(55.20)	248.08(50.80)
Max Power (W)	510.64(138.77)	529.00(150.33)	497.54(111.03)	533.67(190.02)	485.54(103.69)
Power (W/kg)	3.29(0.74)	3.33(0.74)	3.59(0.78)	3.00(0.66)	3.24(0.74)
Average HR (beats·min^{-1})	165.82(10.19)	164.94(10.17)	168.08(7.21)	166.12(12.28)	164.09(11.37)
Max HR (beats·min^{-1})	180.64(10.56)	183.83(179.85)	179.85(7.00)	180.833(11.95)	178.31(12.78)
SubMax HR 70% (beats·min^{-1})	126.45(7.39)	128.68(7.14)	125.89(4.90)	126.58(8.37)	124.82(8.95)
SubMax HR 75% (beats·min^{-1})	135.48(7.92)	137.88(7.65)	134.88(5.25)	135.63(8.96)	133.73(9.59)
30-sec Recovery HR	176.89(12.05)	181.12(10.33)	176.25(8.22)	178.98(10.99)	171.71(16.28)
1-Min Recovery HR	151.32(12.03)	153.95(13.65)	153.32(8.57)	150.44(13.45)	142.23(12.39)
2-Min Recovery HR	139.28(12.08)	141.10(10.96)	141.43(8.23)	138.87(14.54)	135.83(14.25)
Average VO_2 (mL-min/L)	42.10(6.19)	43.00(6.02)	43.96(6.89)	40.31(5.12)	41.08(6.56)
Highest Achieved VO_2 (mL-min/L)	47.09(6.31)	49.61(5.83)	47.96(6.13)	45.04(5.18)	45.78(7.47)
Average RER	0.97(0.05)	0.97(0.06)	0.96(0.06)	0.99(0.05)	0.96(0.05)
Highest Achieved RER	1.04(0.07)	1.04(0.08)	1.02(0.07)	1.07(0.07)	1.02(0.08)
Average RPE	15.90(1.29)	15.88(1.33)	15.65(1.13)	16.05(1.28)	16.02(1.52)
Highest Achieved RPE	19.22(0.91)	19.58(0.67)	19.08(0.95)	19.17(0.94)	19.08(1.04)
Average Cadence (rpm)	90.05(8.95)	89.76(10.00)	88.00(9.05)	92.97(8.82)	89.66(7.73)
Average Speed (kph)	39.25(3.10)	39.75(2.86)	39.83(3.17)	38.35(3.24)	39.05(3.10)
Max Speed (kph)	56.71(2.38)	56.85(2.41)	57.28(2.25)	56.35(2.56)	56.35(2.47)
Time Trial (min.)	30.57(2.46)	30.17(2.23)	30.19(2.59)	31.19(2.56)	30.74(2.59)

Data is presented as mean(SD). HR = heart rate, SubMax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion.

Table 5A. Anthropometric correlations to primary and secondary variables at baseline, visit 1

Variable	Age (yr)	Body Mass (kg)	Ht (cm)	Lean tissue (kg)	Fat Mass (kg)	VAT (kg)	Body Fat (%)	Sleep Quality
Time Trial (min.)	0.02	-0.19	-0.41**	0.50**	0.35*	0.21	0.56	0.18
Average Power (W)	-0.05	0.19	0.42**	0.47**	-0.31*	-0.18	-0.51**	-0.21
Average VO_2 (mL-min/L)	-0.26	-0.45**	0.05	-0.12	-0.62**	-0.44**	-0.57**	0.20
Average HR (beats·min^{-1})	-0.34**	-0.37**	0.04	-0.03**	-0.61	-0.47**	-0.60	-0.12
Max HR (beats·min^{-1})	-0.45**	-0.46	-0.22	-0.38	-0.28	-.37**	-0.12	-0.24
SubMax HR 70% (beats·min^{-1})	-0.58**	-0.24	-0.21	-0.16	-0.19	-.41**	-0.13	-0.02
Highest Achieved RER	-0.58	-0.25	-0.22	-0.17	-0.18	-0.41	-0.12	-0.12
Highest Achieved RPE	-0.13	0.10	-0.05	0.02	0.17	0.01	0.14	0.04
Time Trial (min.)	-0.13	0.10	-0.05	0.02	0.16	0.01	0.13	-0.12

Values are expressed in *r* values. HR = heart rate, SubMax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, VAT = visceral adipose tissue. *$p < 0.05$.

Table 6A. Macronutrient correlations to primary and secondary variables at baseline, visit 1

Variable	Avg Calories (kcal/day)	CHOs (g)	PRO (g)	Fiber (g)	FAT (g)	SAT Fat (g)	TC (mg)	Omega-3 (g)	Omega-6 (g)
Time Trial (min.)	-0.26	-0.22	-0.13	0.04	-0.19	-0.28*	0.01	-0.02	-0.07
Average Power (W)	0.23	0.14	0.10	-0.08	0.22	0.34*	0.10	0.00	0.05
Average VO_2 (mL- min/L)	0.02	-0.04	0.06	-0.14	0.06	0.09	0.21	-0.18	-0.04
Average HR (beats·min-1)	0.11	0.09	0.01	-0.11	0.10	0.12	0.20	-0.12	-0.05
Max HR (beats·min-1)	-0.17	-0.12	-0.12	-0.02	-0.13	-0.10	-0.02	-0.18	-0.15
SubMax HR 70% (beats·min-1)	-0.06	0.01	-0.12	0.01	-0.06	-0.07	0.02	-0.05	-0.09
Highest Achieved RER	-0.03	0.02	-0.10	0.02	-0.03	-0.04	0.03	-0.04	-0.08
Highest Achieved RPE	0.14	0.33*	-0.17	0.29	0.02	-0.10	-0.19	0.41**	0.01
Time Trial (min.)	-0.15	-0.07	-0.12	-0.06	-0.14	-0.08	-0.20	-0.15	-0.16

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, CHOS = carbohydrates, PRO = protein, SAT FAT = saturated fat, TC = total cholesterol. *$p < 0.05$.**$p < 0.01$.

Table 7A. Vitamin and Mineral correlations to primary and secondary variables at baseline, visit 1

Variable	VITA (mcg)	VITB1 (mg)	VITB2 (mg)	VITB3 (mg)	VITB6 (mg)	VITB12 (mcg)	VITC (mg)	VITD (mcg)
Time Trial (min.)	-0.20	-0.18	-0.16	-0.18	-0.15	-0.11	0.00	-0.06
Average Power (W)	0.10	0.20	0.20	0.14	0.14	0.16	-0.05	0.13
Average VO_2 (mL-min/L)	0.20	0.17	-0.11	0.08	0.10	0.16	0.11	0.10
Average HR (beats·min-1)	0.18	0.24	-0.01	0.07	0.10	0.11	0.07	0.10
Max HR (beats·min-1)	0.10	0.01	-0.08	-0.05	0.02	0.04	0.15	0.04
SubMax HR 70% (beats·min-1)	0.09	0.12	0.04	-0.05	0.00	-0.02	0.12	-0.01
Highest Achieved RER	0.07	0.12	0.04	-0.04	0.01	-0.01	0.14	-0.01
Highest Achieved RPE	0.12	0.04*	-0.06	-0.04	0.11	-0.17	-0.06	0.04
Time Trial (min.)	-0.12	0.09	0.05	-0.15	-0.06	-0.09	-0.06	-0.15

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, VIT = vitamin. *$p < 0.05$.**$p < 0.01$

Table 8A. Vitamin and mineral correlations to primary and secondary variables at baseline, visit 1

Variable	Na^+ (mg)	Folate (mcg)	Ca^{2+} (mg)	Fe (mg)	PO_4 (mg)	K^+ (mg)	Mg^{2+} (mg)	Zn^{2+} (mg)
Time Trial (min.)	-0.12	-0.11	-0.09	-0.04	-0.11	-0.05	0.02	-0.05
Average Power (W)	0.06	0.01	0.05	-0.03	0.09	0.02	-0.03	0.07
Average VO_2 (mL- min/L)	0.02	0.02	-0.04	0.08	0.02	0.01	-0.15	0.13
Average HR (beats·min-1)	0.02	0.06	-0.10	0.04	-0.06	-0.01	-0.20	0.05
Max HR (beats·min-1)	-0.19	-0.14	0.03	-0.30	0.01	-0.11	-0.09	0.06
SubMax HR 70% (beats·min-1)	-0.09	-0.18	0.01	-0.34	-0.02	-0.15	-0.11	0.03
Highest Achieved RER	-0.07	-0.18	0.02	-0.33	-0.01	-0.13	-0.11	0.04
Highest Achieved RPE	-0.02	0.08	-0.18	-0.16	-0.05	0.27	0.15	-0.16
Time Trial (min.)	-0.19	0.13	-0.07	-0.27	-0.14	-0.12	-0.09	-0.03

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, Ca^{2+} = calcium, Fe = iron, PO_4 = phosphate, K^+ = potassium, Mg^{2+} = magnesium, Zn^{2+} = zinc. *$p < 0.05$.**$p < 0.01$.

Table 9A. Baseline testing daily total average caloric intake of supplement groups

Nutrient								
Total calories (kcal)	**Supplement Group**	***m***	***SD***	***df***	***MS***	***F***	***p***	***d***
	Q+CIT	2312.06	667.23					
	Q	2233.22	609.07					
	CIT	2176.55	1045.53					
	PL	2261.78	731.19					
	Total Between Groups	2245.96	754.64	3.00	38533.91	0.06	0.98	0.00
Carbohydrates (g)								
	Q+CIT	252.13	94.32					
	Q	244.07	98.19					
	CIT	219.78	123.09					
	PL	242.51	102.36					
	Total Between Groups	239.77	102.31	3.00	2321.63	0.21	0.89	0.20
Protein (g)								
	Q+CIT	115.74	51.74					
	Q	120.08	48.75					
	CIT	96.00	42.40					
	PL	103.07	37.75					
	Total Between Groups	108.84	45.06	3.00	1540.94	0.75	0.53	0.46
Fiber (g)								
	Q+CIT	29.25	17.58					
	Q	16.87	9.37					
	CIT	23.78	13.77					
	PL	29.49	23.23					
	Total Between Groups	24.78	17.13	3.00	451.45	1.60	0.20	0.63
Fat (g)								
	Q+CIT	94.86	30.56					
	Q	89.13	30.70					
	CIT	103.91	67.45					
	PL	100.01	45.44					
	Total Between Groups	96.88	44.71	3.00	517.09	0.25	0.86	0.87
Saturated Fat (g)								
	Q+CIT	26.67	13.70					
	Q	29.69	14.90					
	CIT	34.94	29.19					
	PL	26.80	10.22					

	Total Between Groups	29.47	18.01	3.00	182.03	0.55	0.65	0.35
Cholesterol (mg)								
	Q+CIT	508.51	437.71					
	Q	516.12	352.55					
	CIT	338.18	205.00					
	PL	522.91	465.43					
	Total Between Groups	473.35	376.63	3.00	96603.25	0.67	0.58	1.70
Vitamin A (mcg)								
	Q+CIT	727.77	623.53					
	Q	521.73	410.96					
	CIT	656.30	654.11					
	PL	598.72	577.88					
	Total Between Groups	623.49	558.85	3.00	95339.42	0.29	0.83	0.29
Vitamin B1 (mg)								
	Q+CIT	1.27	1.59					
	Q	0.90	0.55					
	CIT	0.84	0.52					
	PL	0.97	0.48					
	Total Between Groups	0.99	0.89	3.00	0.44	0.54	0.66	0.35
Vitamin B2 (mg)								
	Q+CIT	1.46	1.54					
	Q	2.54	4.71					
	CIT	1.18	0.78					
	PL	0.97	0.59					
	Total Between Groups	1.55	2.56	3.00	6.25	0.95	0.43	0.51
Vitamin B3 (mg)								
	Q+CIT	20.40	13.43					
	Q	22.87	15.31					
	CIT	13.08	7.36					
	PL	14.23	7.25					
	Total Between Groups	17.68	11.84	3.00	282.39	2.16	0.11	0.74
Vitamin B6 (mg)								
	Q+CIT	1.74	1.51					
	Q	1.86	1.45					
	CIT	1.06	0.58					

	PL	1.36	0.87					
	Total Between Groups	1.51	1.18	3.00	1.66	1.21	0.32	0.55
Vitamin B12 (mg)								
	Q+CIT	2.76	3.27					
	Q	3.72	5.81					
	CIT	1.45	1.51					
	PL	1.27	1.47					
	Total Between Groups	2.31	3.57	3.00	17.11	1.37	0.26	0.59
Vitamin C (mg)								
	Q+CIT	149.62	195.95					
	Q	67.90	107.96					
	CIT	75.15	86.44					
	PL	97.10	77.71					
	Total Between Groups	96.85	125.10	3.00	16656.36	1.07	0.37	0.55
Vitamin D (mcg)								
	Q+CIT	5.29	8.50					
	Q	2.85	3.19					
	CIT	1.57	1.94					
	PL	2.38	3.08					
	Total Between Groups	3.01	4.88	3.00	30.89	1.33	0.28	0.59
Folate (mcg)								
	Q+CIT	177.82	103.93					
	Q	218.34	167.60					
	CIT	197.81	104.63					
	PL	546.12	964.28					
	Total Between Groups	288.91	513.23	3.00	390808.95	1.53	0.22	0.63
Calcium (mg)								
	Q+CIT	825.48	441.93					
	Q	776.13	382.35					
	CIT	871.94	446.62					
	PL	584.96	261.64					
	Total Between Groups	761.26	391.80	3.00	201145.61	1.34	0.27	0.59
Iron (mg)								
	Q+CIT	16.48	8.17					
	Q	12.87	8.13					
	CIT	11.13	4.42					
	PL	37.21	82.21					

	Total Between Groups	19.65	42.49	3.00	1865.99	1.04	0.39	0.51
Phosphate (mg)								
	Q+CIT	990.84	592.59					
	Q	948.64	495.80					
	CIT	771.89	481.27					
	PL	706.87	451.90					
	Total Between Groups	853.49	505.37	3.00	234481.34	0.91	0.44	0.51
Potassium (mg)								
	Q+CIT	2894.42	2044.66					
	Q	2130.22	810.73					
	CIT	1772.68	1096.31					
	PL	2778.67	1553.74					
	Total Between Groups	2396.41	1474.47	3.00	3488397.48	1.67	0.19	0.67
Magnesium (mg)								
	Q+CIT	168.59	161.25					
	Q	177.38	69.17					
	CIT	199.99	150.23					
	PL	224.46	155.69					
	Total Between Groups	192.94	136.00	3.00	7925.13	0.41	0.74	0.35
Zinc (mg)								
	Q+CIT	7.00	4.57					
	Q	8.70	9.05					
	CIT	6.57	5.35					
	PL	6.20	4.46					
	Total Between Groups	7.13	6.09	3.00	15.84	0.41	0.75	0.35
Omega-3 (g)								
	Q+CIT	1.53	1.30					
	Q	1.20	1.07					
	CIT	0.94	1.29					
	PL	1.31	1.33					
	Total Between Groups	1.24	1.23	3.00	0.72	0.46	0.71	0.35
Omega-6 (g)								
	Q+CIT	10.80	8.02					
	Q	8.51	3.87					
	CIT	10.81	9.73					
	PL	9.27	7.51					
	Total Between Groups	9.81	7.36	3.00	16.51	0.29	0.83	0.29

Sodium (mg)								
	Q+CIT	3693.42	1131.15					
	Q	3203.30	1030.01					
	CIT	3131.28	2015.71					
	PL	3760.49	2261.38					
	Total Between Groups	3448.51	1671.13	3.00	1324809.57	0.46	0.71	0.35

*$p < 0.05$.

Table 10A. Anthropometric correlations to primary and secondary variables pre-supplementation, visit 2

Variable	Age (yr)	Body Mass (kg)	Ht (cm)	Lean tissue (kg)	Fat Mass (kg)	VAT (kg)	Body Fat (%)	Sleep Quality
Time Trial (min.)	-0.01	-0.12**	-0.42**	-0.41*	0.33	0.20**	0.51	0.04
Average Power (W)	-0.09	0.14**	0.44**	0.46**	-0.36	-0.23**	-0.56	0.03*
Average VO_2 (mL-min/L)	-0.22**	-0.42	0.07	-0.08**	-0.60**	-0.40**	-0.57	-0.06
Average HR (beats·min^{-1})	-0.36**	-0.36	0.09**	0.04	-0.65*	-0.45	-0.67	-0.03
Max HR (beats·min^{-1})	-0.37	-0.42	-0.14	-0.39	-0.18*	-0.29	-0.02	-0.06
SubMax HR 70% (beats·min^{-1})	-0.54	-0.24	-0.21	-0.22	-0.11*	-0.32	-0.03	-0.06
Highest Achieved RER	-0.54	-0.24	-0.21	-0.22	-0.11	-0.32	-0.03	-0.06
Highest Achieved RPE	-0.30	0.02	-0.07	0.09	-0.10	-0.18	-0.12	0.12
NO Metabolites Pre	-0.14	-0.14	-0.17	-0.15	-0.04	-0.14	0.02	0.04
NO Metabolites Post	-0.17	0.09	0.21	0.12	0.01	0.02	-0.05	-0.00
Time Trial (min.)	-0.06	0.19	0.15	0.11	0.17	0.20	0.11	-0.05

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, VAT = visceral adipose tissue. *$p < 0.05$. **$p < 0.01$.

Table 11A. Macronutrient correlations to primary and secondary variables at pre-supplementation, visit 2

Variable	Avg Calories (kcal/day)	CHOs (g)	PRO (g)	Fiber (g)	FAT (g)	SAT Fat (g)	TC (mg)	Omega-3 (g)	Omega-6 (g)
Time Trial (min.)	-0.21	-0.08*	-0.29	0.03	-0.18	-0.25	-0.22	-0.19	-0.24
Average Power (W)	0.31	0.18*	0.32	0.02	0.25	0.36**	0.33	0.17	0.19
Average VO_2 (mL- min/L)	0.10	0.01	0.12	-0.02	0.15	0.21	0.27	-0.06	-0.06
Average HR (beats·min-1)	0.14	0.04	0.17	-0.05	0.12	0.20	0.24	-0.07	-0.07
Max HR (beats·min-1)	-0.03	-0.09	-0.14	0.01	0.15	0.16	-0.07	-0.05	-0.03
SubMax HR 70% (beats·min-1)	-0.02	-0.06	-0.10	-0.13	0.12	0.20	-0.13	-0.08	-0.04
Highest Achieved RER	-0.02	-0.06	-0.10	-0.13	0.12	0.20	-0.13	-0.08	-0.04
Highest Achieved RPE	-0.19	-0.08	-0.22	-0.13	-0.24	-0.14	-0.16	-0.02	-0.13
NO Metabolites Pre	0.17	-0.02	0.09	0.11	0.21	0.09	0.14	-0.12	-0.05
NO Metabolites Post	-0.11	0.03	-0.23	0.16	-0.10	-0.14	-0.31	0.14	0.07
Time Trial (min.)	-0.06	0.07	-0.17	0.17	-0.08	-0.05	-0.20	-0.01	-0.05

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, CHOS = carbohydrates, PRO = protein, SAT FAT = saturated fat, TC = total cholesterol, RER = respiratory exchange ratio, RPE = rating of perceived exertion, NO = nitric oxide. *$p < 0.05$.**$p < 0.01$

Table 12A. Vitamin and mineral correlations to primary and secondary variables at pre-supplementation, visit 2

Variable	VITA (mcg)	VITB1 (mg)	VITB2 (mg)	VITB3 (mg)	VITB6 (mg)	VITB12 (mcg)	VITC (mg)	VITD (mcg)
Time Trial (min.)	-0.17*	-0.33	-0.25	-0.27	-0.16*	-0.31	0.02	-0.17
Average Power (W)	0.08	0.33	0.25	0.29	0.20**	0.38	-0.04	0.27
Average VO_2 (mL-min/L)	0.10	0.06	0.03	0.09	-0.06	0.14	0.00	0.25
Average HR (beats·min-1)	0.11	0.15	0.03	0.11	-0.02	0.09	0.02	0.15
Max HR (beats·min-1)	-0.22	-0.24	-0.14	-0.08	-0.14	-0.10	-0.01	0.06
SubMax HR 70% (beats·min-1)	-0.23	-0.01	-0.24	-0.16	-0.21	-0.20	-0.09	-0.12
Highest Achieved RER	-0.23	-0.01	-0.24	-0.16	-0.21	-0.20	-0.09	-0.12
Highest Achieved RPE	-0.11	-0.13	-0.20	-0.22	-0.14	-0.11	-0.15	0.03
NO Metabolites Pre	0.07	-0.02	-0.13	-0.16	-0.26	-0.12	0.07	0.07
NO Metabolites Post	0.11	-0.13	-0.14	-0.11	-0.03	-0.21	0.24	-0.15
Time Trial (min.)	0.11	-0.16	-0.15	-0.09	-0.04	-0.13	0.16	-0.11

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, VIT = vitamin. *$p < 0.05$.**$p < 0.01$.

Table 13A. Vitamin and Mineral correlations to primary and secondary variables at pre-supplementation, visit 2

Variable	Na^+ (mg)	Folate (mcg)	Ca^{2+} (mg)	Fe (mg)	PO_4 (mg)	K^+ (mg)	Mg^{2+} (mg)	Zn^{2+} (mg)
Time Trial (min.)	-0.13	-0.07	-0.15	-0.03*	-0.31	0.03	-0.17	-0.21
Average Power (W)	0.27	0.11	0.24	0.01	0.27	0.06	0.10	0.18
Average VO_2 (mL- min/L)	0.06	-0.14	0.11	0.05	0.02	-0.11	-0.10	-0.03
Average HR (beats·min-1)	0.09	-0.08	0.06	0.03	0.04	-0.11	-0.10	0.02
Max HR (beats·min-1)	-0.12	-0.04	0.16	-0.26	-0.06	-0.10	-0.08	0.07
SubMax HR 70% (beats·min-1)	-0.01	-0.02	0.05	-0.27	-0.11	-0.14	-0.11	0.09
Highest Achieved RER	-0.01	-0.02	0.05	-0.27	-0.11	-0.14	-0.11	0.09
Highest Achieved RPE	-0.07	0.00	-0.10	-0.10	-0.16	-0.02	-0.10	-0.13
NO Metabolites Pre	-0.02	0.09	0.01	-0.12	-0.04	0.05	0.09	-0.03
NO Metabolites Post	-0.16	0.05	-0.11	-0.03	-0.13	0.11	0.09	0.03
Time Trial (min.)	-0.08	-0.04	-0.05	-0.05	-0.17	0.10	0.01	0.02

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, Ca^{2+} = calcium, Fe = iron, PO_4 = phosphate, K^+ = potassium, Mg^{2+} = magnesium, Zn^{2+} = zinc. *$p < 0.05$.**$p < 0.01$.

Table 14A. Pre-supplementation, visit 2, daily total average caloric intake of supplement groups

Nutrient								
Total calories (kcal)	**Supplement Group**	***m***	***SD***	***df***	***MS***	***F***	***p***	***d***
	Q+CIT	2122.48	689.34					
	Q	2325.39	993.17					
	CIT	1708.90	648.18					
	PL	2470.48	714.77					
	Total Between Groups	2166.46	806.63	3.00	1355152.44	2.24	0.10	0.77
Carbohydrates (g)								
	Q+CIT	250.94	102.74					
	Q	276.30	124.36					
	CIT	172.40	94.39					
	PL	264.13	105.18					
	Total Between Groups	242.11	111.87	3.00	26919.15	2.33	0.09	0.77
Protein (g)								
	Q+CIT	119.96	48.88					
	Q	110.27	44.10					
	CIT	81.49	29.80					
	PL	104.40	36.16					
	Total Between Groups	104.16	41.58	3.00	3215.87	1.97	0.13	0.70
Fiber (g)								
	Q+CIT	27.87	18.02					
	Q	22.87	16.45					
	CIT	18.95	7.07					
	PL	32.81	24.60					
	Total Between Groups	25.71	18.07	3.00	455.12	1.43	0.25	4.76
Fat (g)								
	Q+CIT	94.18	38.71					
	Q	92.90	49.86					
	CIT	82.08	42.04					
	PL	99.41	38.42					
	Total Between Groups	92.30	41.76	3.00	652.39	0.36	0.78	0.29
Saturated Fat (g)								

	Q+CIT	26.72	15.32					
	Q	28.50	17.76					
	CIT	21.58	13.89					
	PL	29.38	10.07					
	Total Between Groups	26.64	14.40	3.00	149.68	0.71	0.55	0.41
Cholesterol (mg)								
	Q+CIT	414.44	371.65					
	Q	392.77	302.61					
	CIT	405.63	412.01					
	PL	504.26	327.99					
	Total Between Groups	430.04	346.30	3.00	33247.71	0.27	0.85	0.29
Vitamin A (mcg)								
	Q+CIT	627.41	693.41					
	Q	489.29	409.94					
	CIT	514.53	663.50					
	PL	834.83	1442.26					
	Total Between Groups	618.34	881.39	3.00	318694.56	0.40	0.76	0.35
Vitamin B1 (mg)								
	Q+CIT	1.32	1.62					
	Q	0.94	1.26					
	CIT	0.56	0.31					
	PL	0.98	0.80					
	Total Between Groups	0.95	1.11	3.00	1.15	0.94	0.43	0.51
Vitamin B2 (mg)								
	Q+CIT	1.04	0.76					
	Q	1.09	0.72					
	CIT	1.00	0.70					
	PL	1.24	0.80					
	Total Between Groups	1.10	0.73	3.00	0.14	0.24	0.87	0.29
Vitamin B3 (mg)								
	Q+CIT	22.26	16.06					
	Q	22.49	15.17					

	CIT	10.26	4.87					
	PL	14.68	7.45					
	Total Between Groups	17.47	12.66	3.00	442.29	3.12	0.04*	0.91
Vitamin B6 (mg)								
	Q+CIT	1.97	1.75					
	Q	1.48	0.67					
	CIT	1.02	0.64					
	PL	1.55	0.84					
	Total Between Groups	1.51	1.08	3.00	1.81	1.61	0.20	0.67
Vitamin B12 (mg)								
	Q+CIT	3.71	3.89					
	Q	2.38	2.02					
	CIT	2.67	3.75					
	PL	1.50	1.26					
	Total Between Groups	2.54	2.93	3.00	10.43	1.23	0.31	0.55
Vitamin C (mg)								
	Q+CIT	165.53	265.44					
	Q	68.49	62.73					
	CIT	57.49	58.81					
	PL	89.13	93.58					
	Total Between Groups	94.51	146.49	3.00	28716.46	1.37	0.26	0.59
Vitamin D (mcg)								
	Q+CIT	4.28	8.70					
	Q	2.39	3.08					
	CIT	2.17	3.94					
	PL	2.36	2.03					
	Total Between Groups	2.78	4.95	3.00	11.97	0.47	0.70	0.35
Folate (mcg)								
	Q+CIT	176.20	104.31					
	Q	234.74	282.40					
	CIT	164.18	98.10					
	PL	303.69	255.29					

	Total Between Groups	221.68	207.94	3.00	51386.65	1.20	0.32	0.55
Calcium (mg)								
	Q+CIT	707.26	459.47					
	Q	897.19	573.90					
	CIT	590.39	317.28					
	PL	667.56	518.03					
	Total Between Groups	718.27	479.15	3.00	215771.99	0.94	0.43	0.51
Iron (mg)								
	Q+CIT	17.01	14.54					
	Q	14.22	8.15					
	CIT	10.32	5.62					
	PL	37.64	81.59					
	Total Between Groups	20.04	42.63	3.00	1903.14	1.05	0.38	0.51
Phosphate (mg)								
	Q+CIT	1071.74	564.32					
	Q	902.91	656.21					
	CIT	791.46	457.53					
	PL	925.70	814.23					
	Total Between Groups	922.61	629.33	3.00	159484.36	0.39	0.76	0.35
Potassium (mg)								
	Q+CIT	2631.27	1480.56					
	Q	2032.94	814.06					
	CIT	1823.20	1079.62					
	PL	2723.44	1542.24					
	Total Between Groups	2305.73	1283.57	3.00	2433798.51	1.53	0.22	0.63
Magnesium (mg)								
	Q+CIT	253.60	217.04					
	Q	180.51	109.62					
	CIT	207.80	100.48					
	PL	267.09	201.54					
	Total Between Groups	227.11	164.30	3.00	20633.20	0.75	0.53	0.46
Zinc (mg)								
	Q+CIT	7.84	4.78					
	Q	6.48	4.42					

	CIT	7.26	5.95					
	PL	6.89	5.16					
	Total Between Groups	7.10	4.97	3.00	4.18	0.16	0.92	0.20
Omega-3 (g)								
	Q+CIT	1.59	1.44					
	Q	0.80	0.62					
	CIT	1.23	1.45					
	PL	1.20	1.24					
	Total Between Groups	1.20	1.22	3.00	1.31	0.88	0.46	0.46
Omega-6 (g)								
	Q+CIT	11.96	8.83					
	Q	6.62	5.60					
	CIT	10.07	8.14					
	PL	10.27	9.44					
	Total Between Groups	9.68	8.11	3.00	63.36	0.96	0.42	0.51
Sodium (mg)								
	Q+CIT	3318.93	1717.77					
	Q	3265.84	2031.97					
	CIT	2467.50	1296.03					
	PL	3515.84	1477.31					
	Total Between Groups	3151.98	1656.88	3.00	2615462.19	0.95	0.42	0.51

*****$p < 0.05$.

Table 15A. Anthropometric correlations to primary and secondary variables post-supplementation, visit 3

Variable	Age (yr)	Body Mass (kg)	Ht (cm)	Lean tissue (kg)	Fat Mass (kg)	VAT (kg)	Body Fat (%)	Sleep Quality	Physical Activity (AU)
Time Trial (min.)	0.04	-0.16	-0.42**	-0.44**	0.31*	0.18	0.51**	0.33*	-0.12
Average Power (W)	-0.05	0.13	0.42**	0.40**	-0.30*	-0.20	-0.47**	-0.35**	0.13
Average VO_2 (mL- min/L)	-0.28	-0.42**	0.06	-0.08	-0.62**	-0.39**	-0.58**	-0.24	0.14
Average HR (beats·min-^{1})	-0.37**	-0.32*	0.10	0.02**	-0.58	-0.41*	-0.59	-0.29	0.06
Max HR (beats·min-^{1})	-0.44**	-0.35	-0.20	-0.39	-0.08	-0.31	0.07	0.00	0.23
SubMax HR 70% (beats·min-^{1})	-0.51**	-0.11	-0.10	-0.15	0.02	-0.24	0.04	0.02	0.04
Highest Achieved RER	-0.51**	-0.11	-0.10	-0.15	0.02	-0.24**	0.04	0.02	0.04
Highest Achieved RPE	-0.38	-0.16	0.08	-0.02	-0.28	-0.41	-0.24	0.25	-0.04
NO Metabolites Pre	-0.19	-0.14	-0.16	-0.20	0.01	-0.15	0.09	0.05	0.01
NO Metabolites Post	-0.03	-0.03	0.04*	0.04	-0.08	-0.03	-0.12	-0.10	0.06
Time Trial (min.)	-0.01	-0.04	-0.01*	0.03	-0.06	0.02	-0.10	-0.12	0.03

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, VAT = visceral adipose tissue. Physical activity is expressed as arbitrary units (average total daily activities over 28-days). *$p < 0.05$. **$p < 0.01$.

Table 16A. Macronutrient correlations to primary and secondary variables at post-supplementation, visit 3

Variable	Avg Calories (k/cal/day)	CHOs (g)	PRO (g)	Fiber (g)	FAT (g)	SAT Fat (g)	TC (mg)	Omega-3 (g)	Omega-6 (g)
Time Trial (min.)	-0.22	-0.18	-0.18	0.14	-0.13	-0.19	-0.20	0.12	0.03
Average Power (W)	0.18	0.13	0.15	-0.16	0.08	0.12	0.24	-0.19	-0.07
Average VO_2 (mL- min/L)	-0.16	-0.14	-0.08	-0.27	-0.16	-0.11	0.17	-0.24	-0.21
Average HR (beats·min^{-1})	-0.03	-0.06	0.04	-0.24	-0.04	0.01	0.28	-0.26	-0.21
Max HR (beats·min-^{1})	-0.13	-0.09	-0.11	0.03	-0.07	0.03	0.08	-0.14	-0.26
SubMax HR 70% (beats·min^{-1})	0.05	0.09	0.02	0.07	0.06	0.16	0.15	-0.12	-0.22
Highest Achieved RER	0.05	0.09	0.02	0.07	0.06	0.16	0.15	-0.12	-0.22
Highest Achieved RPE	-0.12	-0.01	-0.11	-0.00	-0.19	-0.08	-0.05	-0.12	-0.16
NO Metabolites Pre	0.09	0.19	0.01	0.11	-0.01	0.03	0.05	0.10	0.04
NO Metabolites Post	-0.06	0.03	-0.09	0.31*	-0.05	-0.11	-0.16	0.35*	0.25
Time Trial (min.)	-0.05	0.02	-0.07	0.30*	-0.03	-0.09	-0.14	0.35*	0.24

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, CHOS = carbohydrates, PRO = protein, SAT FAT = saturated fat, TC = total cholesterol, RER = respiratory exchange ratio, RPE = rating of perceived exertion, NO = nitric oxide. *$p < 0.05$. **$p < 0.01$.

Table 17A. Vitamin and mineral correlations to primary and secondary variables at post-supplementation, visit 3

Variable	VITA (mcg)	VITB1 (mg)	VITB2 (mg)	VITB3 (mg)	VITB6 (mg)	VITB12 (mcg)	VITC (mg)	VITD (mcg)
Time Trial (min.)	0.10	-0.08	-0.14	-0.07	-0.19	-0.19	0.02	0.31*
Average Power (W)	-0.15	0.05	0.12	0.10	0.27	0.22	-0.09	-0.25
Average VO_2 (mL- min/L)	-0.07	-0.12	-0.09	-0.17	0.09	-0.06	-0.01	-0.20
Average HR (beats·min-1)	-0.09	-0.12	-0.11	-0.13	0.10	0.04	-0.04	-0.21
Max HR (beats·min-1)	0.14	-0.21	-0.13	-0.23	0.19	-0.19	0.04	-0.03
SubMax HR 70% (beats·min-1)	0.13	-0.19	-0.19	-0.20	0.12	-0.14	0.03	-0.08
Highest Achieved RER	0.13	-0.19	-0.19*	-0.20	0.12	-0.14	0.03	-0.08
Highest Achieved RPE	-0.05	-0.20	-0.30	-0.11	0.19	-0.33	-0.20	-0.12
NO Metabolites Pre	0.12	0.02	-0.20	0.05	0.11	-0.19	0.08	-0.03
NO Metabolites Post	0.64**	-0.04	0.00	-0.04	-0.00	-0.10	0.83**	0.03
Time Trial (min.)	0.62**	-0.05	0.01	-0.03	-0.01	-0.07	0.81**	0.04

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, VIT = vitamin. *$p < 0.05$.**$p < 0.01$.

Table 18A. Vitamin and mineral correlations to primary and secondary variables at post-supplementation, visit 3

Variable	Na^+ (mg)	Folate (mcg)	Ca^{2+} (mg)	Fe (mg)	PO_4 (mg)	K^+ (mg)	Mg^{2+} (mg)	Zn^{2+} (mg)
Time Trial	-0.04	0.05	0.08	0.03	-0.00	0.25	0.06	-0.02
Average Power (W)	0.01	-0.06	-0.15	-0.05	-0.02	-0.23	-0.08	0.01
Average VO_2 (mL-min/L)	-0.10	-0.16	-0.39**	0.02	-0.23	-0.32*	-0.24	-0.18
Average HR (beats·min^{-1})	-0.02	-0.19	-0.37	-0.03	-0.19	-0.31	-0.27	-0.10
Max HR (beats·min^{-1})	-0.18	-0.15	-0.05	-0.31	-0.12	-0.06	-0.13	-0.17
SubMax HR 70% (beats·min^{-1})	-0.03	-0.18	-0.07	-0.27	-0.13	-0.06	-0.22	-0.11
Highest Achieved RER	-0.03	-0.18	-0.07	-0.27	-0.13	-0.06	-0.22	-0.11
Highest Achieved RPE	-0.06	-0.16	-0.05	-0.30*	-0.23	-0.07	-0.26	-0.26
NO Metabolites Pre	0.00	0.11	0.02	-0.32*	0.17	0.02	0.10	0.02
NO Metabolites Post	-0.15	0.02	0.08	0.11	0.29	0.17	0.48**	0.10
Time Trial	-0.11	0.00	0.09	0.19	0.28	0.17	0.47**	0.12

Values are expressed in *r* values. Nutrients are expressed as total daily values amongst cyclists. HR = heart rate, Submax = submaximal, VO_2 = oxygen consumption, RER = respiratory exchange ratio, RPE = rating of perceived exertion, Ca^{2+} = calcium, Fe = iron, PO_4 = phosphate, K^+ = potassium, Mg^{2+} = magnesium, Zn^{2+} = zinc. *$p < 0.05$.**$p < 0.01$.

Table 19A. Post-supplementation, visit 3, daily total average caloric intake of supplement groups

Nutrient								
Total calories (kcal)	**Supplement Group**	***m***	***SD***	***df***	***MS***	***F***	***p***	***d***
	Q+CIT	2428.26	847.49					
	Q	2005.20	858.59					
	CIT	1947.89	769.05					
	PL	2734.76	717.58					
	Total Between Groups	2282.67	841.39	3.00	1752373.11	2.74	0.05*	0.84
Carbohydrates (g)								
	Q+CIT	281.96	123.22					
	Q	224.60	87.89					
	CIT	204.88	95.29					
	PL	296.96	114.55					
	Total Between Groups	252.45	109.78	3.00	24483.93	2.18	0.10	0.74
Protein (g)								
	Q+CIT	126.81	59.15					
	Q	98.18	45.62					
	CIT	95.81	51.55					
	PL	110.72	26.86					
	Total Between Groups	107.74	47.11	3.00	2459.03	1.12	0.35	0.55
Fiber (g)								
	Q+CIT	28.44	20.98					
	Q	19.15	9.26					
	CIT	26.56	13.22					
	PL	29.70	26.10					
	Total Between Groups	25.90	18.53	3.00	287.76	0.83	0.49	0.46
Fat (g)								
	Q+CIT	96.08	42.90					
	Q	81.22	48.16					
	CIT	87.16	60.05					
	PL	123.23	45.99					
	Total Between Groups	97.13	50.81	3.00	4452.30	1.81	0.16	0.70

Saturated Fat (g)								
	Q+CIT	31.18	25.64					
	Q	27.35	22.77					
	CIT	20.71	17.97					
	PL	31.62	10.17					
	Total Between Groups	27.79	19.78	3.00	310.57	0.78	0.51	0.46
Cholesterol (mg)								
	Q+CIT	526.62	343.53					
	Q	354.66	257.37					
	CIT	285.37	224.07					
	PL	544.27	293.36					
	Total Between Groups	428.60	295.70	3.00	202162.52	2.53	0.07	0.81
Vitamin A (mcg)								
	Q+CIT	562.53	763.67					
	Q	250.36	238.08					
	CIT	518.18	686.68					
	PL	554.97	437.61					
	Total Between Groups	468.76	561.19	3.00	283834.13	0.90	0.45	0.51
Vitamin B1 (mg)								
	Q+CIT	0.58	0.34					
	Q	0.47	0.30					
	CIT	0.61	0.33					
	PL	1.20	1.08					
	Total Between Groups	0.72	0.67	3.00	1.38	3.62	0.02*	0.97
Vitamin B2 (mg)								
	Q+CIT	0.87	0.58					
	Q	3.68	11.09					
	CIT	0.94	0.68					
	PL	1.52	1.20					
	Total Between Groups	1.78	5.66	3.00	22.08	0.68	0.57	0.41
Vitamin B3 (mg)								

	Q+CIT	14.55	11.23					
	Q	11.28	11.55					
	CIT	10.41	7.40					
	PL	19.18	13.33					
	Total Between Groups	13.91	11.36	3.00	201.23	1.62	0.20	0.67
Vitamin B6 (mg)								
	Q+CIT	1.18	1.12					
	Q	2.36	5.91					
	CIT	1.02	0.58					
	PL	1.52	1.31					
	Total Between Groups	1.53	3.10	3.00	4.55	0.46	0.71	0.35
Vitamin B12 (mg)								
	Q+CIT	2.35	2.17					
	Q	1.26	1.95					
	CIT	1.22	1.49					
	PL	1.57	1.54					
	Total Between Groups	1.59	1.81	3.00	3.35	1.02	0.39	0.51
Vitamin C (mg)								
	Q+CIT	130.61	244.02					
	Q	23.18	27.11					
	CIT	80.94	76.83					
	PL	50.81	52.64					
	Total Between Groups	70.01	130.99	3.00	26268.89	1.59	0.21	0.63
Vitamin D (mcg)								
	Q+CIT	1.82	1.92					
	Q	3.73	5.90					
	CIT	1.77	2.26					
	PL	14.90	44.53					
	Total Between Groups	5.71	22.96	3.00	505.65	0.96	0.42	0.51
Folate (mcg)								
	Q+CIT	146.44	108.71					
	Q	118.58	82.61					
	CIT	184.40	123.48					

	PL	335.45	318.01					
	Total Between Groups	197.45	199.74	3.00	120569.57	3.48	0.02*	0.97
Calcium (mg)								
	Q+CIT	799.61	487.49					
	Q	723.02	493.24					
	CIT	644.35	417.45					
	PL	666.24	475.37					
	Total Between Groups	707.76	459.30	3.00	58304.14	0.26	0.85	0.29
Iron (mg)								
	Q+CIT	17.97	15.66					
	Q	11.11	6.05					
	CIT	9.86	5.96					
	PL	38.64	81.38					
	Total Between Groups	19.61	42.82	3.00	2273.64	1.26	0.30	0.59
Phosphate (mg)								
	Q+CIT	810.51	566.13					
	Q	493.81	289.69					
	CIT	693.03	426.89					
	PL	943.44	672.58					
	Total Between Groups	734.54	522.48	3.00	470202.40	1.81	0.16	0.70
Potassium (mg)								
	Q+CIT	2488.71	1917.41					
	Q	1847.38	1250.21					
	CIT	1635.97	791.92					
	PL	2533.07	1795.55					
	Total Between Groups	2128.84	1514.60	3.00	2541093.19	1.12	0.35	0.55
Magnesium (mg)								
	Q+CIT	196.21	225.99					
	Q	143.53	115.51					
	CIT	205.62	150.63					
	PL	268.42	225.38					
	Total Between Groups	203.54	185.34	3.00	34077.60	0.99	0.41	0.51
Zinc (mg)								
	Q+CIT	7.30	5.77					

	Q	3.26	1.86					
	CIT	5.62	4.99					
	PL	7.32	5.73					
	Total Between Groups	5.85	4.99	3.00	47.19	2.02	0.13	0.74
Omega-3 (g)								
	Q+CIT	1.01	1.02					
	Q	0.74	0.67					
	CIT	1.15	1.50					
	PL	1.35	1.51					
	Total Between Groups	1.06	1.21	3.00	0.84	0.56	0.64	0.41
Omega-6 (g)								
	Q+CIT	8.59	6.88					
	Q	4.78	4.16					
	CIT	10.72	9.59					
	PL	13.74	12.04					
	Total Between Groups	9.45	9.07	3.00	183.60	2.43	0.08	0.81
Sodium (mg)								
	Q+CIT	4084.41	2023.10					
	Q	3154.99	1808.92					
	CIT	3118.17	1592.45					
	PL	4065.52	2030.20					
	Total Between Groups	3605.95	1877.01	3.00	3663894.48	1.04	0.38	0.51

*$p < 0.05$.

Table 20A. Represents mixed model repeated measures within-and-between subjects ANOVA for TT Completion

Variable	*F*	*df*	*p*	*d*	*Observed Power*
TT Completion					
Visit	0.43	1.00	0.52	0.00	0.10
Supplement Condition	0.31	3.00	0.82	0.29	0.11
Visit*Supplement Condition	0.84	3.00	0.48	0.46	0.22

TT = time trial.

Table 21A. Mixed model excluding women repeated measures within-and-between subjects ANOVA for TT completion

Variable	*F*	*df*	*p*	*d*	*Observed Power*
TT Completion					
Visit	0.04	1.00	0.85	0.00	0.05
Supplement Condition	0.95	3.00	0.43	0.55	0.24
Visit*Supplement Condition	0.9	3.00	0.45	0.55	0.23

TT = time trial.

Table 22A. Mixed model repeated measures within-and-between subjects ANOVA for VO_2

Variable	*F*	*df*	*p*	*d*	*Observed Power*
VO_2					
Visit	1.32	1.00	0.26	0.35	0.20
Time	143.61	4.00	0.00**	3.56	1.00
Supplement	0.81	3.00	0.49	0.46	0.21
Visit*Time	1.92	4.00	0.17	0.41	0.27
Visit*Supplement	3.24	3.00	0.03*	0.91	0.71
Time*Supplement	0.81	12.00	0.59	0.46	0.35
Visit*Time*Supplement	0.93	12.00	0.49	0.59	0.38

VO_2 = oxygen consumption.

Table 23A. Mixed model excluding women repeated measures within-and-between subjects ANOVA for VO_2

Variable	*F*	*df*	*p*	*d*	*Observed Power*
VO_2					
Visit	1.96	1.00	0.17	0.46	0.28
Time	121.28	4.00	0.00**	3.56	1.00
Supplement	0.86	3.00	0.47	0.51	0.22
Visit*Time	1.87	4.00	0.12	0.46	0.56
Visit*Supplement	1.21	3.00	0.32	0.63	0.30
Time*Supplement	0.56	12.00	0.87	0.41	0.31
Visit*Time*Supplement	01.07	12.00	0.39	0.59	0.60

VO_2 = oxygen consumption.

Table 24A. Average VO_2 between groups, comparing pre-supplementation (visit 2) to post-supplementation (visit 3). VO_2 = oxygen consumption. Data is presented as mean(SD)

Supplement Group	V2 Average VO_2 (mL/kg/min)	V3 Average VO_2 (mL/kg/min)	*Pairwise Comparisons*	*df*	*p*
Q+CIT	42.61(7.38)	42.99(6.02)	-0.40	11	0.70
Q	41.92(7.59)	43.96(6.89)	-2.21	12	0.05*
CIT	38.35(5.87)	40.31(5.12)	-2.33	11	0.04*
PL	43.04(8.00)	41.08(1.82)	1.42	12	0.10

VO_2 = oxygen consumption.

Table 25A. One-way ANOVA comparing across collapsed groups

Visit	*df*	*F*	*p*
2	3	1.05	0.38
3	3	0.92	0.44

Table 26A. VO_2 pairwise comparisons between supplement groups

Supplement Group	Group	Mean Difference	*p*
Q+CIT	Q	-0.74	1.00
	CIT	-0.22	1.00
	PL	1.11	0.54
Q	Q+CIT	0.74	1.00
	CIT	0.52	1.00
	PL	1.85	0.03*
CIT	Q+CIT	0.22	1.00
	Q	-0.52	1.00
	PL	1.33	0.30
PL	Q+CIT	-1.11	0.54
	Q	-1.85	0.03*
	CIT	-1.33	0.30

VO_2 = oxygen consumption.

Table 27A. Mixed model repeated measures within-and-between subjects ANOVA for NO metabolites

Variable	*F*	*df*	*p*	*d*	*Observed Power*
NO Metabolites					
Visit	1.41	1.00	0.24	0.35	0.21
Time	5.22	1.00	0.03*	0.70	0.61
Supplement	1.18	3.00	0.33	0.59	0.29
Visit*Time	0.82	1.00	0.37	0.29	0.14
Visit*Supplement	1.08	3.00	0.37	0.55	0.27
Time*Supplement	1.83	3.00	0.16	0.70	0.44
Visit*Time*Supplement	2.21	3.00	0.10	0.77	0.52

NO = nitric oxide.

Table 28A. Mixed model excluding women repeated measures within-and-between subjects ANOVA for NO metabolites

Variable	*F*	*df*	*p*	*d*	*Observed Power*
NO Metabolites					
Visit	1.09	1.00	0.30	0.35	0.18
Time	4.42	1.00	0.04*	0.70	0.54
Supplement	1.06	3.00	0.38	0.59	0.26
Visit*Time	0.12	1.00	0.73	0.00	0.06
Visit*Supplement	0.90	3.00	0.45	3.06	0.23
Time*Supplement	1.58	3.00	0.21	0.70	0.38
Visit*Time*Supplement	1.59	3.00	0.21	0.70	0.38

NO = nitric oxide.

Table 29A. Represents mixed model repeated measures within-and-between subjects ANOVA for average power

Variable	*F*	*df*	*p*	*d*	*Observed Power*
Average Power					
Visit	8.89	1.00	0.01*	0.87	0.83
Time	15.69	4.00	0.00**	1.15	1.00
Supplement	0.65	3.00	0.59	0.41	0.18
Visit*Time	3.12	4.00	0.02*	0.51	0.66
Visit*Supplement	1.55	3.00	0.22	0.63	0.38
Time*Supplement	0.59	12.00	0.85	0.41	0.27
Visit*Time*Supplement	1.24	12.00	0.29	0.59	0.53

Table 30A. Mixed model excluding women repeated measures within-and-between subjects ANOVA for average power

Variable	***F***	***df***	***p***	***d***	***Observed Power***
Average Power					
Visit	9.28	1.00	0.00**	1.00	0.84
Time	11.3	4.00	0.00**	1.09	1.00
Supplement	1.17	3.00	0.34	0.59	0.29
Visit*Time	2.89	4.00	0.02*	0.55	0.61
Visit*Supplement	1.81	3.00	0.16	0.77	0.43
Time*Supplement	0.45	12.00	0.90	0.41	0.21
Visit*Time*Supplement	1.33	12.00	0.24	0.67	0.55

Table 31A. Dependent samples t-test comparing average power across collapsed groups pre-supplementation (visit 2) to post-supplementation (visit 3)

Time Point (km)	***F***	***df***	***p***	***d***	***Observed Power***
0	6.90	1.00	0.01**	0.77	0.73
5	2.82	1.00	0.10	0.5-	0.38
10	0.99	1.00	0.33	0.29	0.16
15	0.18	1.00	0.68	0.13	0.07
20	6.13	1.00	0.02**	0.73	0.68

Table 32A. Dependent samples t-test excluding women comparing average power across collapsed groups pre-supplementation (visit 2) to post-supplementation (visit 3)

Time Point (km)	***F***	***df***	***p***	***d***	***Observed Power***
0	7.13	1.00	0.01**	0.87	0.74
5	1.83	1.00	0.18	0.44	0.26
10	1.39	1.00	0.25	0.38	0.21
15	0.10	1.00	0.76	0.11	0.06
20	5.02	1.00	0.03**	0.73	0.59

Table 33A. Mixed model repeated measures within-and-between subjects ANOVA for HR

Variable	*F*	*df*	*p*	*d*	*Observed Power*
HR					
Visit	0.12	1.00	0.73	0.00	0.06
Time	300.27	4.00	0.00**	5.17	1.00
Supplement	0.78	3.00	0.51	0.46	0.20
Visit*Time	1.88	4.00	0.16	0.41	0.37
Visit*Supplement	0.26	3.00	0.86	0.29	0.10
Time*Supplement	0.88	12.00	0.52	0.46	0.35
Visit*Time*Supplement	0.9	12.00	0.50	0.51	0.33

HR = heart rate.

Table 34A. Mixed model excluding women repeated measures within-and-between subjects ANOVA for HR

Variable	*F*	*df*	*p*	*d*	*Observed Power*
HR					
Visit	0.26	1.00	0.61	0.20	0.08
Time	268.97	4.00	0.00**	5.42	1.00
Supplement	0.64	3.00	0.60	0.46	0.17
Visit*Time	1.13	4.00	0.33	0.35	0.23
Visit*Supplement	0.20	3.00	0.89	0.29	0.08
Time*Supplement	0.71	12.00	0.67	0.51	0.29
Visit*Time*Supplement	0.80	12.00	0.57	0.51	0.29

HR = heart rate.

Table 35A. Mixed model repeated measures within-and-between subjects ANOVA for RPE

Variable	*F*	*df*	*p*	*d*	*Observed Power*
RPE					
Visit	0.50	1.00	0.49	0.20	0.11
Time	273.98	4.00	0.00**	4.96	1.00
Supplement	0.26	3.00	0.86	0.29	0.10
Visit*Time	2.34	4.00	0.09	0.46	0.52
Visit*Supplement	0.09	3.00	0.97	0.20	0.06
Time*Supplement	0.16	12.00	0.98	0.20	0.09
Visit*Time*Supplement	1.71	12.00	0.11	0.67	0.70

RPE = rating of perceived exertion.

Table 36A. Mixed model excluding women repeated measures within-and-between subjects ANOVA for RPE

Variable	*F*	*df*	*p*	*d*	*Observed Power*
RPE					
Visit	0.37	1.00	0.55	0.20	0.09
Time	221.67	4.00	0.00**	4.96	1.00
Supplement	2.16	4.00	0.11	0.51	0.49
Visit*Time	0.27	3.00	0.85	0.29	0.10
Visit*Supplement	0.07	3.00	0.98	0.20	0.06
Time*Supplement	0.23	12.00	0.96	0.27	0.14
Visit*Time*Supplement	2.19	12.00	0.02*	0.84	0.51

RPE = rating of perceived exertion.

Table 37A. Ttest for average RPE between groups, comparing pre-supplementation (visit 2) to post-supplementation (visit 3)

Supplement Group	V2 Average RPE	V3 Average RPE	*t*	*df*	*p*
Q+CIT	16.00±1.58	16.05±1.25	-0.19	10	0.86
Q	15.42±1.51	15.52±1.35	-0.33	8	0.75
CIT	15.84±1.49	16.00±1.33	-0.90	10	0.39
PL	15.98±1.51	15.98±1.64	0.00	10	1.00

RPE = rating of perceived exertion.

Table 38A. One-way ANOVA for RPE showing the difference of time points at pre-supplementation (visit 2)

Time point (km)	*F*	*df*	*p*	*d*
0	0.67	3.00	0.58	0.46
5	0.46	3.00	0.71	0.41
10	0.40	3.00	0.76	1.31
15	0.04	3.00	0.99	0.00
20	0.91	3.00	0.45	0.55

RPE = rating of perceived exertion.

Table 39A. One-way ANOVA for RPE showing the difference of time points at post-supplementation (visit 3)

Time point (km)	*F*	*df*	*p*	*d*
0	0.26	3.00	0.85	0.29
5	0.36	3.00	0.78	0.35
10	0.22	3.00	0.89	0.29
15	0.25	3.00	0.86	0.29
20	0.83	3.00	0.49	0.51

RPE = rating of perceived exertion.

Table 40A. RPE pairwise comparisons between supplement groups pre (visit 2)-to-post (visit 3) supplementation

Supplement Condition	**Time Points (km)**	**Mean Difference**	***p***
Q+CIT	0	1.55	0.02*
	5	0.18	0.66
	10	-0.46	0.20
	15	-0.73	0.06
	20	-0.82	0.01*
Q	0	0.25	0.73
	5	-0.25	0.61
	10	-0.38	0.36
	15	0.13	0.78
	20	-0.38	0.29
CIT	0	1.78E-15	1.00
	5	-0.36	0.38
	10	-0.46	0.20
	15	-0.36	0.34
	20	0.36	0.23
PL	0	-0.27	0.66
	5	0.18	0.66
	10	0.00	1.00
	15	-0.19	0.81
	20	0.18	0.54

Table 41A. Mixed model repeated measures within-and-between subjects ANOVA for RER

Variable	***F***	***df***	***p***	***d***	***Observed Power***
RER					
Visit	0.09	1.00	0.77	0.00	0.06
Time	16.75	4.00	0.00**	1.22	1.00
Supplement	0.32	3.00	0.81	0.29	0.11
Visit*Time	1.25	4.00	0.30	0.35	0.32
Visit*Supplement	0.53	3.00	0.66	0.35	0.15
Time*Supplement	1.09	12.00	0.37	0.55	0.47
Visit*Time*Supplement	1.30	12.00	0.24	0.59	0.60

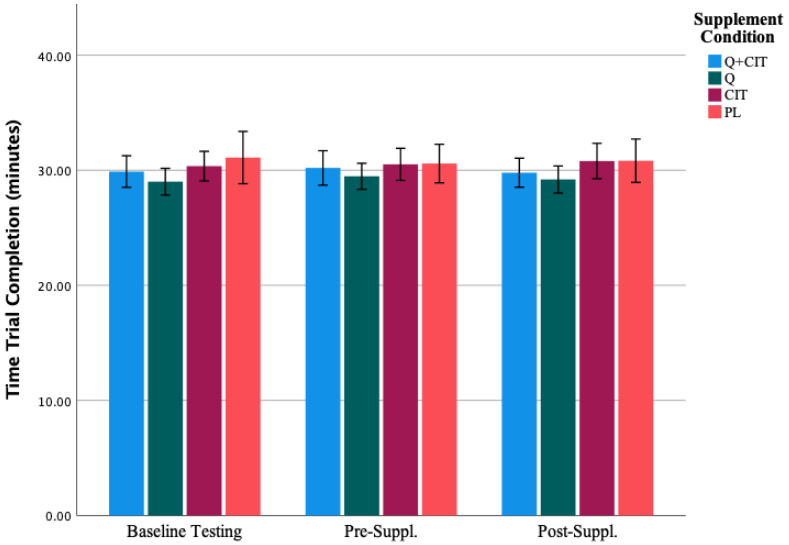
RER = respiratory exchange ratio.

Table 42A. Mixed model excluding women repeated measures within-and-between subjects ANOVA for RER

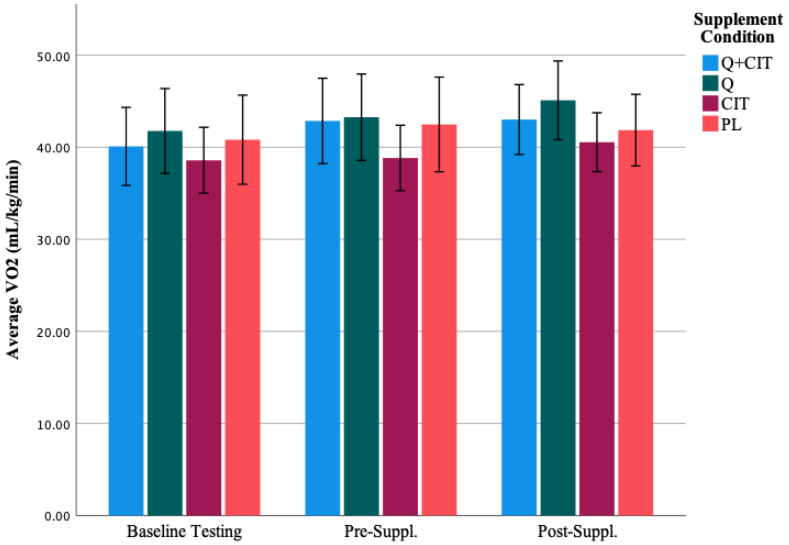
Variable	*F*	*df*	*p*	*d*	*Observed Power*
RER					
Visit	0.05	1.00	0.83	0.00	0.06
Time	13.56	4.00	0.00**	1.19	1.00
Supplement	1.16	3.00	0.34	0.59	0.29
Visit*Time	0.83	1.00	0.51	0.29	0.26
Visit*Supplement	0.94	3.00	0.43	0.55	0.24
Time*Supplement	0.83	12.00	0.62	0.51	0.47
Visit*Time*Supplement	1.46	12.00	0.15	0.67	0.77

RER = respiratory exchange ratio.

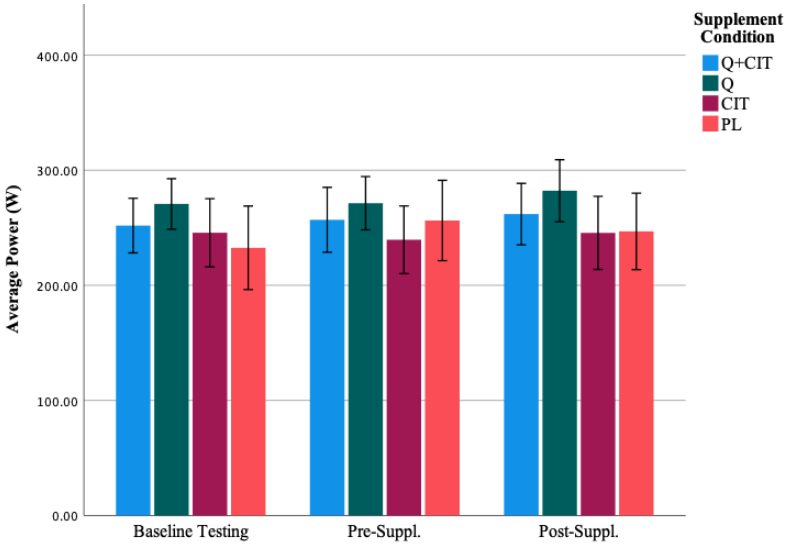
Appendix B: Chapter 2 Figures



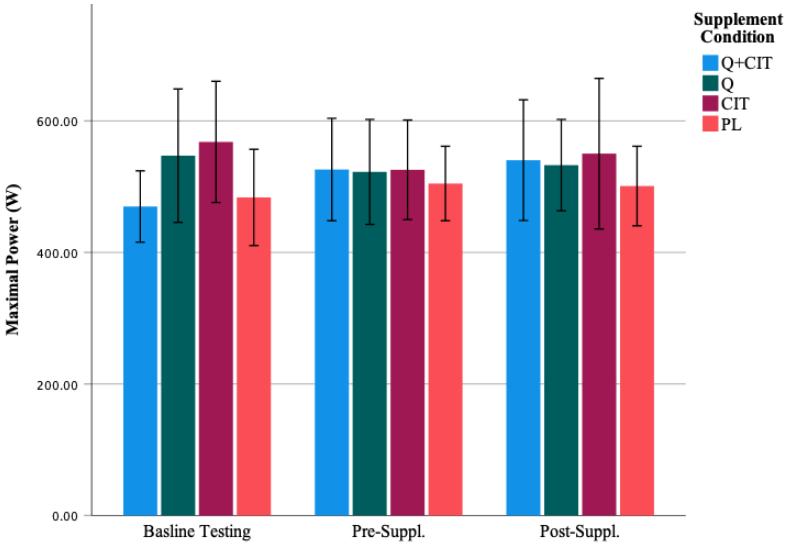
*Data are displayed as means ± SEM.
Figure 17B. Time-trial completion at baseline testing, before and after 28 days of supplementation.



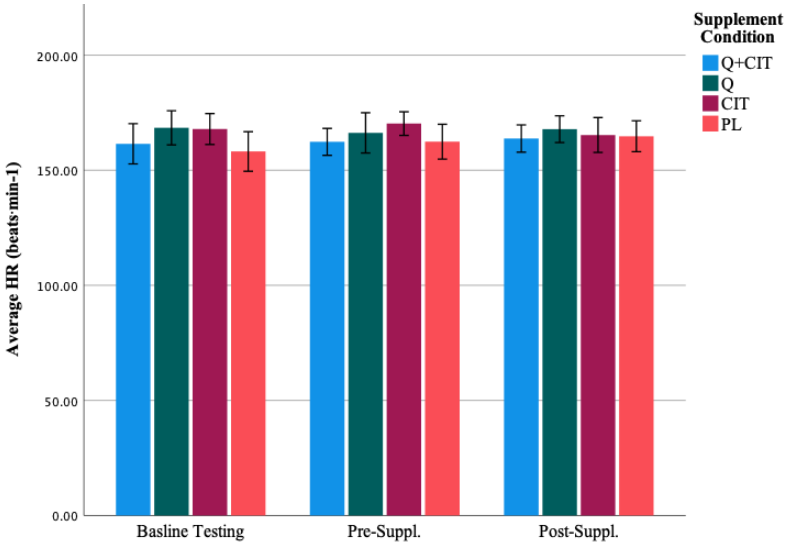
*Data are displayed as means ± SEM.
Figure 18B. Average VO_2 at baseline testing, before and after 28 days of supplementation.



*Data are displayed as means ± SEM.
Figure 19B. Average power at baseline testing, pre and post 28 days of supplementation.



*Data are displayed as means ± SEM.
Figure 20B. Maximal power at baseline testing, pre and post 28 days of supplementation.



*Data are displayed as means ± SEM.
Figure 21B. Average HR (beats·min-[1]) at baseline testing, before and after 28 days of supplementation.

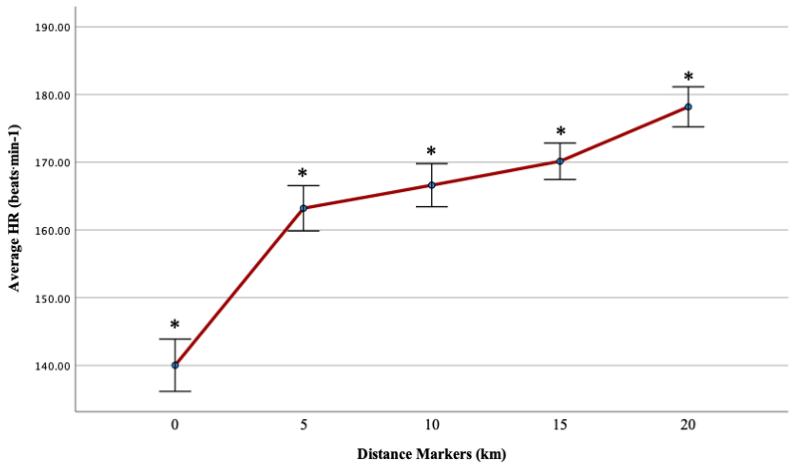
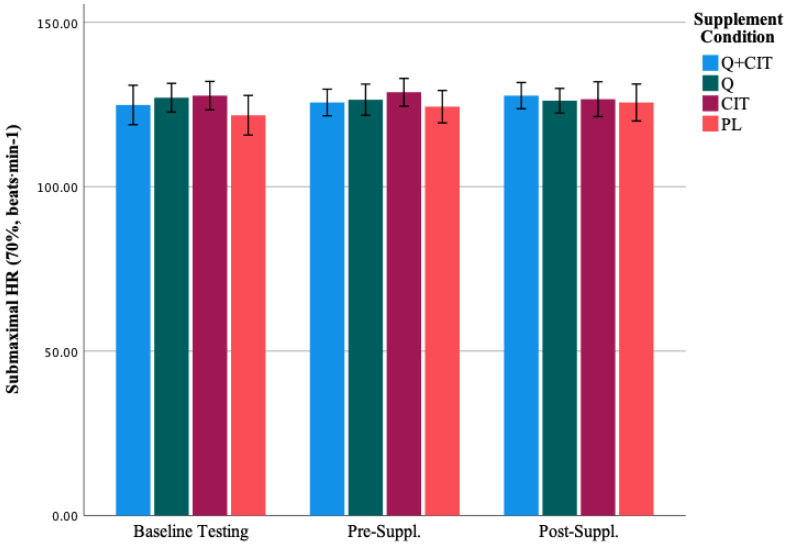
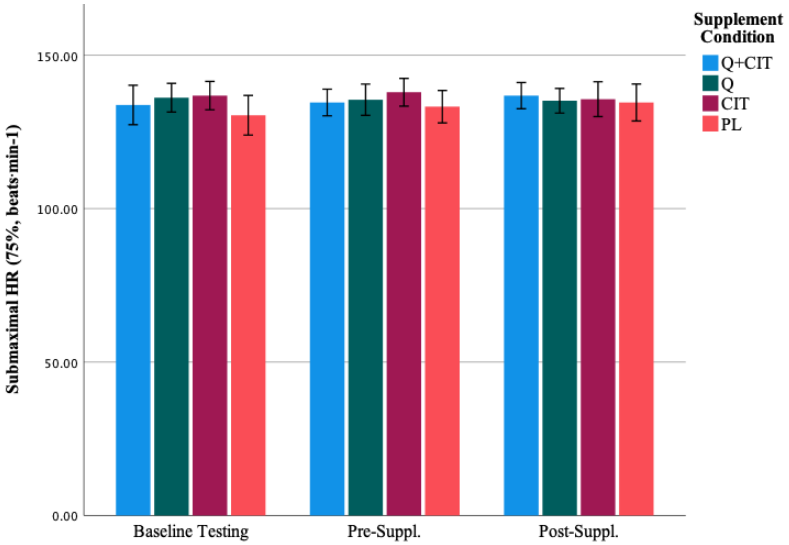


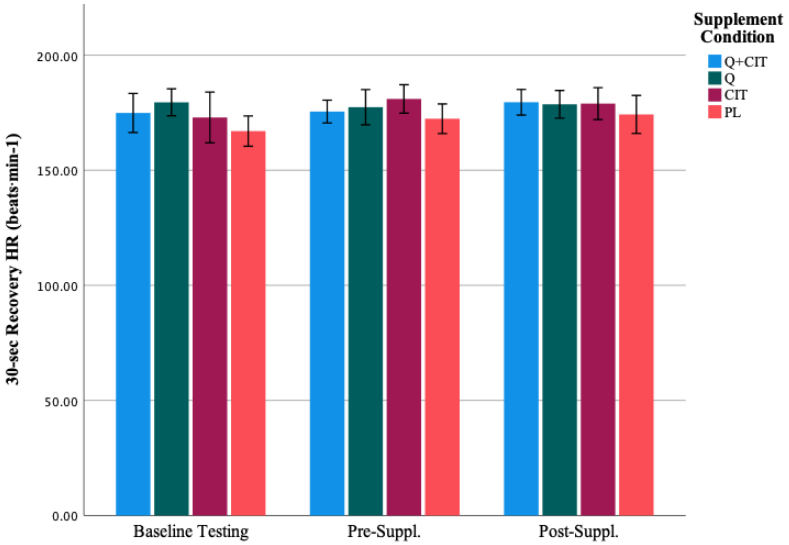
Figure 22B. Average HR across distance markers (km) at pre-supplementation (visit 2) and post-supplementation (visit 3).



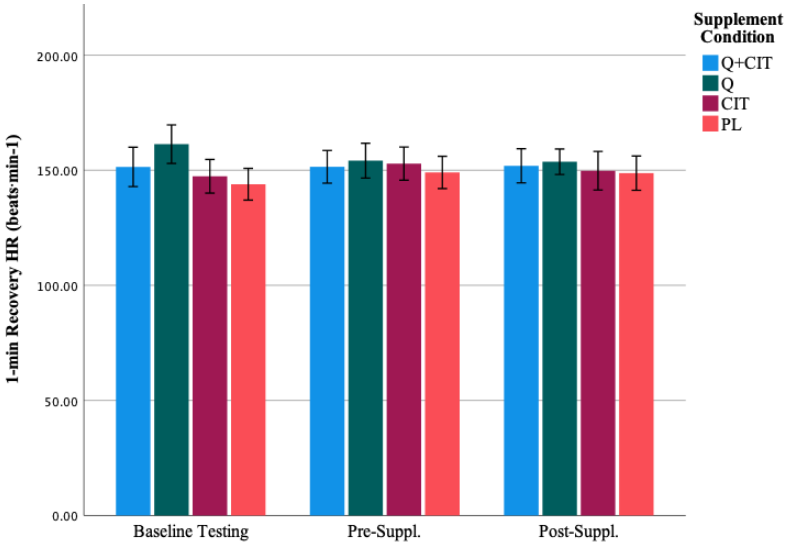
*Data are displayed as means ± SEM.
Figure 23B. Average submaximal HR (70%, beats·min-^{1}) at baseline testing, before and after 28 days of supplementation.



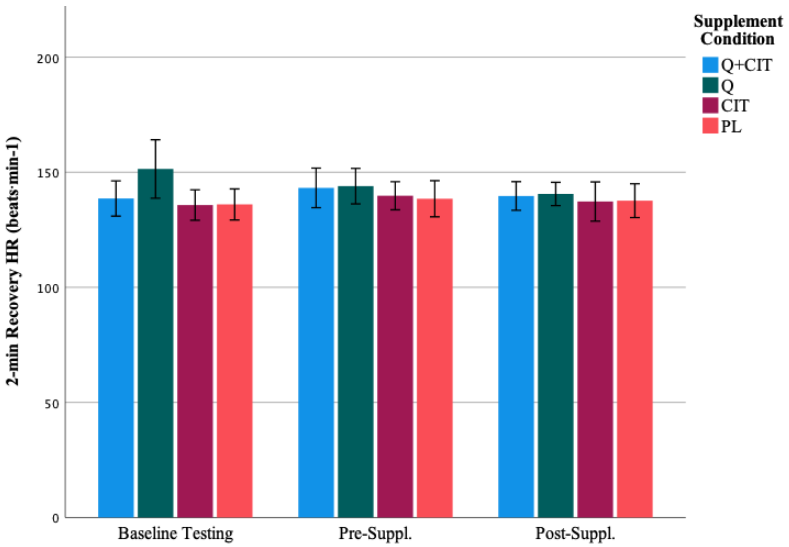
*Data are displayed as means ± SEM.
Figure 24B. Average submaximal HR (75%, beats·min-^{1}) at baseline testing, before and after 28 days of supplementation.



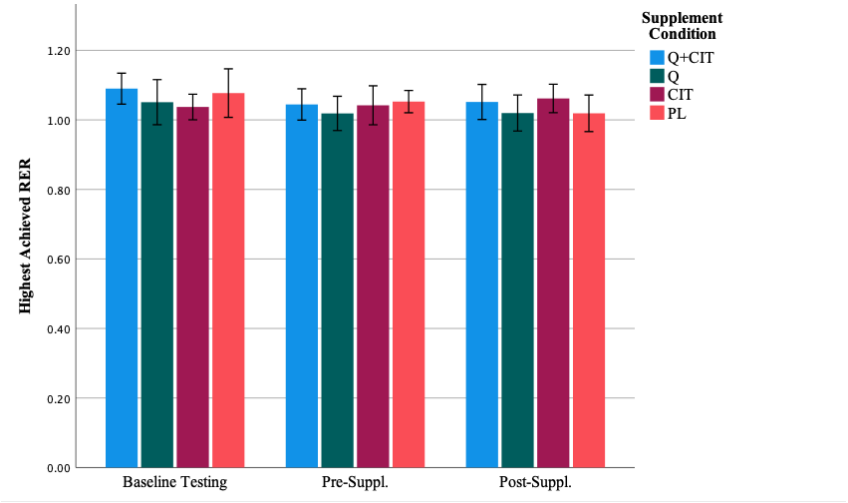
*Data are displayed as means ± SEM.
Figure 25B. Average recovery HR (beats·min-[1]) at 30-sec post exercise at baseline testing, before and after 28 days of supplementation.



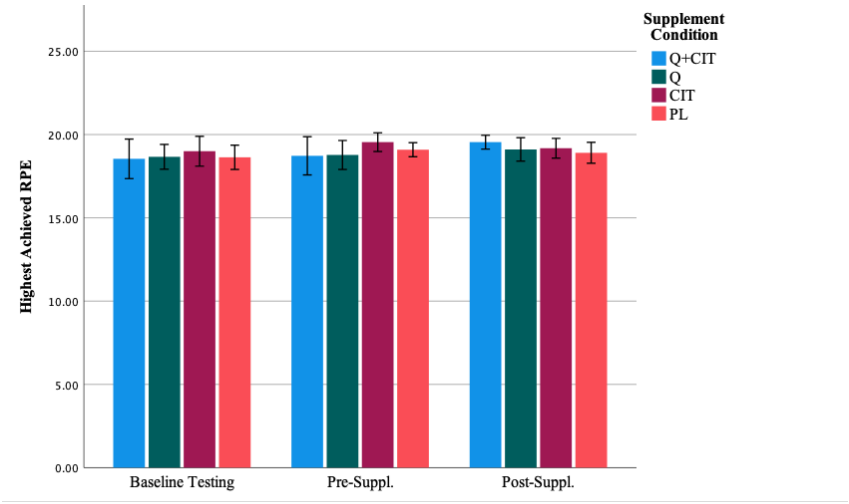
*Data are displayed as means ± SEM.
Figure 26B. Average recovery HR (beats·min-[1]) at 1-min post exercise at baseline testing, before and after 28 days of supplementation.



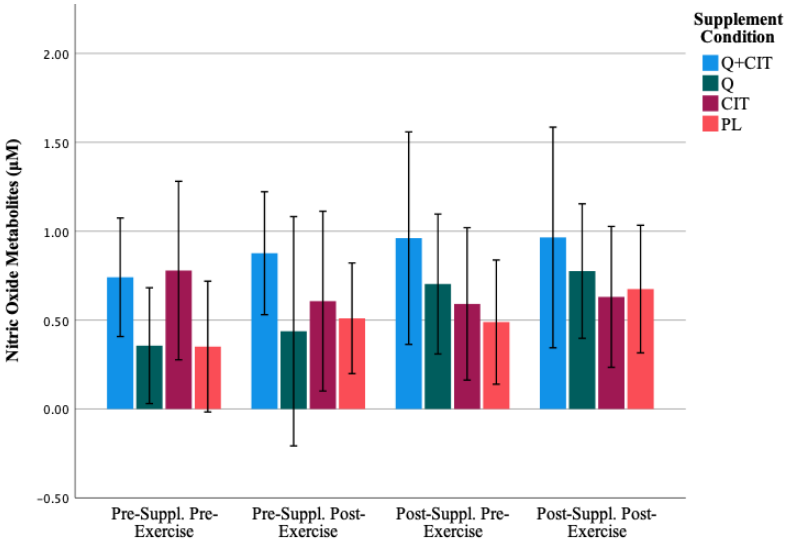
*Data are displayed as means ± SEM.
Figure 27B. Average recovery HR (beats·min-[1]) at 2-min post exercise at baseline testing, before and after 28 days of supplementation.



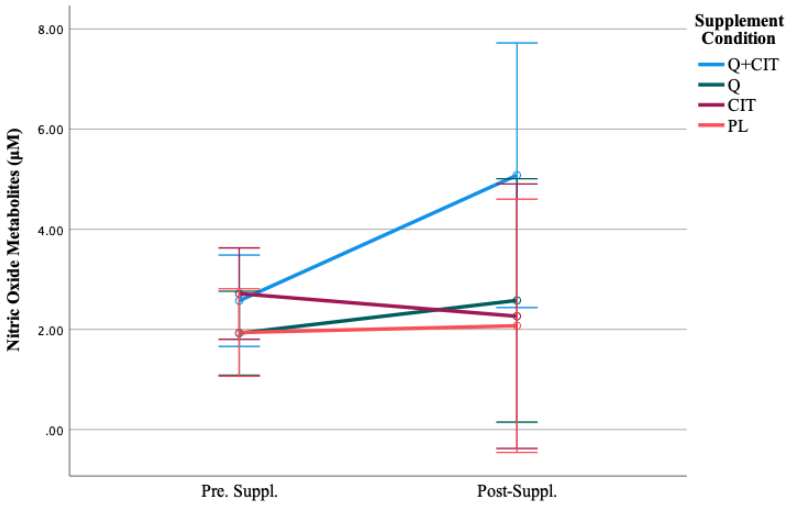
*Data are displayed as means ± SEM.
Figure 28B. Highest Achieved Respiratory Exchange Ratio (RER) at baseline testing, before and after 28 days of supplementation



*Data are displayed as means ± SEM.
Figure 29B. Highest Achieved Rating of Perceived Exertion (RPE) at baseline testing, before and after 28 days of supplementation.



*Data are displayed as means ± SEM.
Figure 30B. Nitric Oxide Metabolites before and after 28 days of supplementation.



*Data are displayed as means ± SEM.
Figure 31B. Nitric Oxide metabolites comparisons pre-to-post supplementation.

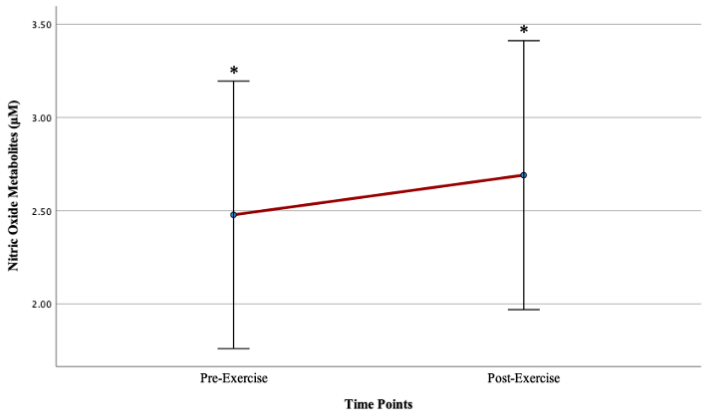
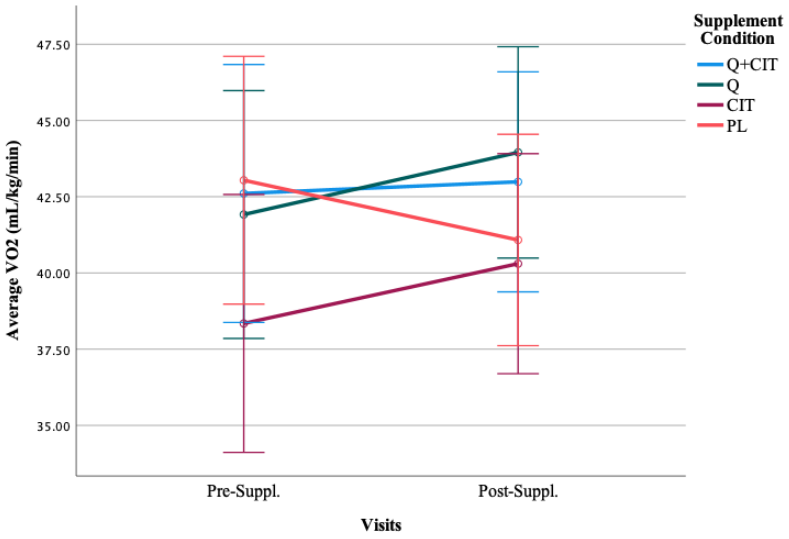
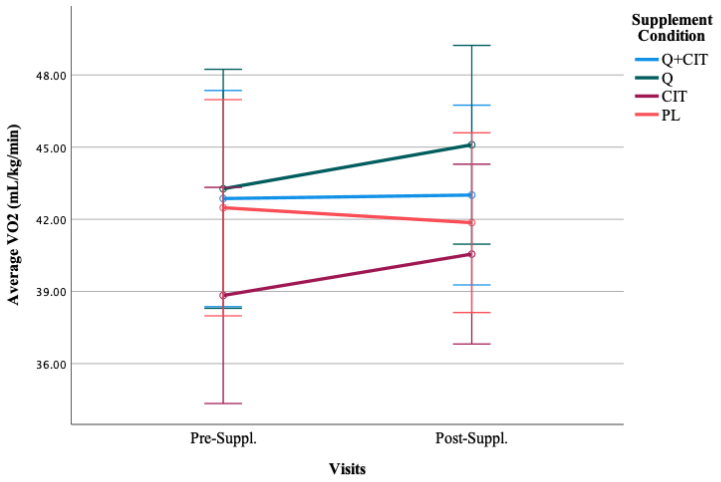


Figure 32B. Nitric Oxide metabolites across time points at pre-supplementation (visit 2) and post-supplementation (visit 3).



*Data are displayed as means ± SEM.
Figure 33B. Average VO_2 differences between supplement groups before (visit 2) and after 28 days of supplementation (visit 3).



*Data are displayed as means ± SEM.
Figure 34B. Average VO_2 differences between supplement groups excluding women before (visit 2) and after 28 days of supplementation (visit 3).

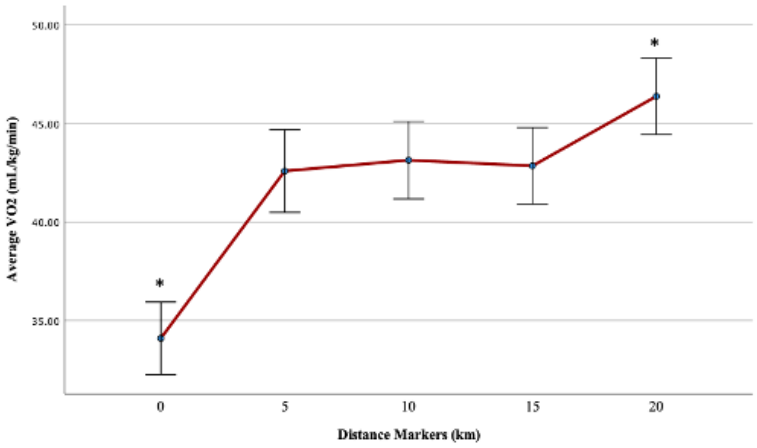
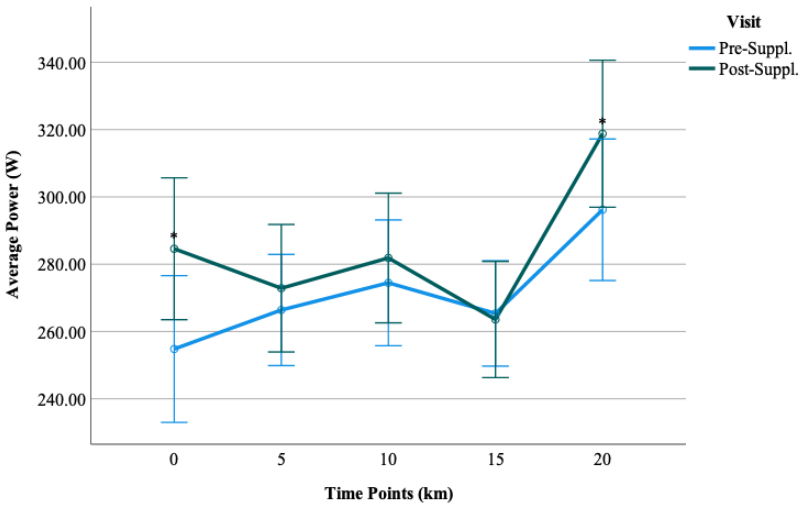


Figure 35B. Average VO_2 across distance markers (km) at pre-supplementation (visit 2) and post-supplementation (visit 3).



*Data are displayed as means ± SEM.
Figure 36B. Comparisons of average power between visit 2 and visit 3 time-points.

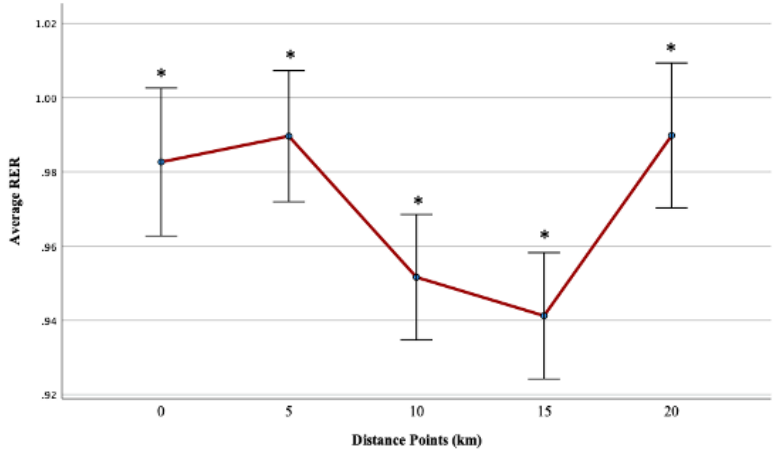


Figure 37B. Average RER across distance markers (km) at pre-supplementation (visit 2) and post-supplementation (visit 3).

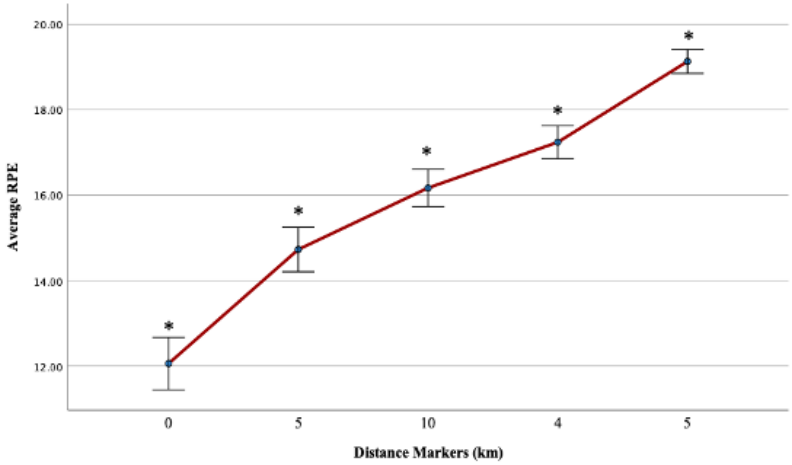
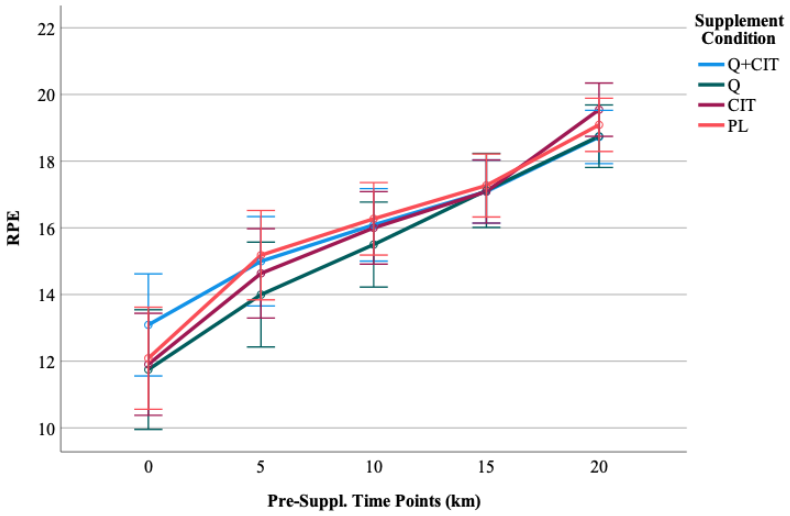
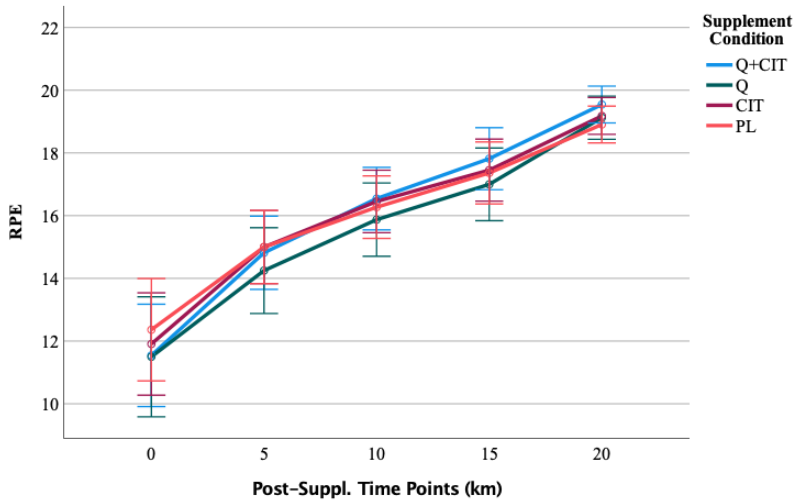


Figure 38B. Average RPE across distance markers (km) at pre-supplementation (visit 2) and post-supplementation (visit 3).



*Data are displayed as means ± SEM.
Figure 39B. RPE time points at pre-supplementation, visit 2.



*Data are displayed as means ± SEM.
Figure 40B. RPE time points at post-supplementation, visit 3.

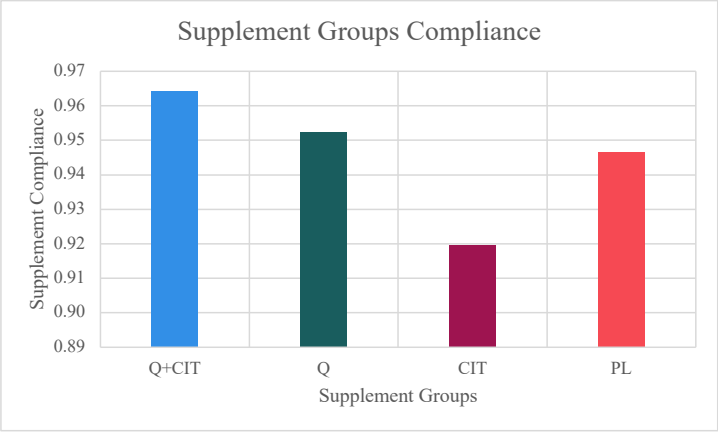


Figure 41B. Supplement groups' compliance during the four-week supplementation period.
*Compliance is denoted as a fraction out of 100.

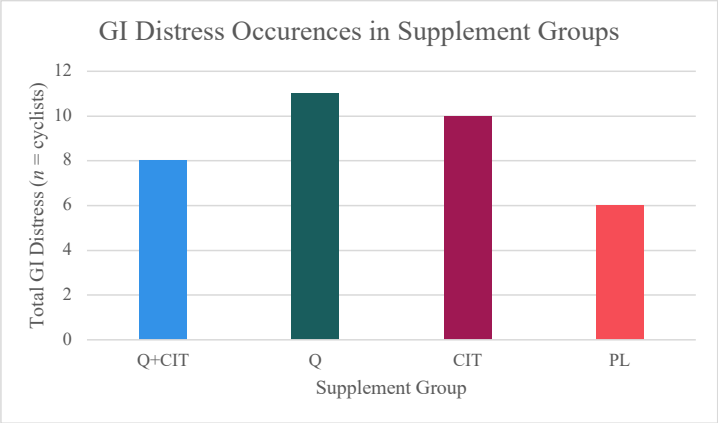


Figure 42B. Supplement groups gastrointestinal (GI) distress during the four-week supplementation period.
*GI distress is denoted as the total number of cyclists who experienced GI distress in their respective groups.

Appendix C: Weekly Gastrointestinal Questionnaire

Weekly Gastrointestinal Questionnaire
This will take less than 10 minutes.
These questions and domains are derived, adapted, and modified from a validated and well-established methodology assessing gastrointestinal distress [78, 115].

What is your DOB?

What is your study ID Number?

Please enter today's date.

After taking the supplement this week, please answer the following questions:

Abdominal Pressure

1. **Did you feel bloated?**
   If you did not have bloating, please skip questions A-C.
   a. *If so, what was the severity (11 represents the highest bloating, 1 represents minimal bloating but not normal)*
   b. *What was the duration of bloating (minutes or hours within a day)?*
   c. *How many times per week?*
      - 1
      - 2
      - 3
      - 4
      - 5
      - 6
      - 7

2. **Did you have a feeling of fullness?**
   If you did not have feelings of fullness, please skip questions A-B.
   b. If so, what was the severity (11 represents the fullest, 1 represents minimal fullness but not normal)?
   c. What was the duration of fullness (minutes or hours within a day)?
   a. *How many times per week?*
      - 1
      - 2
      - 3
      - 4
      - 5
      - 6
      - 7

3. **Did you have a feeling of heaviness in your tummy this week?**
   If you did not have feelings of heaviness, please skip questions A-C.

b. *If so, what was the severity (11 represents the heaviest, 1 represents minimal heaviness but not normal)*
c. *What was the duration of a feeling of heaviness in your tummy (minutes or hours within a day)?*
d. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

4. Did you have a feeling of tightness in your tummy?
If you did not have feelings of tightness, please skip questions A-C.

a. *If so, what was the severity (11 represents the most tightness, 1 represents minimal bloating but not normal)*
b. *What was the duration of tightness in your tummy (minutes or hours within a day)?*
c. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

5. Did you have abdominal pain in your tummy?
If you did not have feelings of pain, please skip questions A-C.

a. *If so, what was the severity (11 represents the most painful, 1 represents minimal pain but not normal)*
b. *What was the duration of abdominal pain (minutes or hours within a day)?*
c. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

6. Did you have constipation?
If you did not have constipation, please skip questions A-C.

a. *If so, what was the severity (11 represents the high constipation, 1 represents minimal constipation but not normal)*

b. *What was the duration of constipation (minutes or hours within a day)?*
c. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

7. Did your stomach look big and round (like a balloon/ being pregnant)?
If your stomach was not big and round, please skip questions A-B.

a. *If so, what was the duration of your stomach looking big and round (minutes or hours within a day)?*
b. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

Abdominal Distension (swollen beyond its normal size) – *sensation of feeling pregnant*

8. Did you have abdominal distension?
If you did not have abdominal distension, please skip questions A-C.

a. *If so, what was the severity (11 represents the highest distension, 1 represents minimal bloating but not normal)*
b. *What was the duration of abdominal distension (minutes or hours within a day)?*
c. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

Belching

9. Did you have belching after you took your supplement?
If you did not have flatulence, please skip questions A-C.

a. *If so, what was the quantity of gas passed per mouth: after meals?*
b. If so, what was the quantity of gas passed per mouth: apart from meals?
c. *How many times per week?*
    - 1

- 2
- 3
- 4
- 5
- 6
- 7

Difficult Gas Evacuation

10. Did you have difficult gas evacuation?

If you did not have difficult gas evacuation, please skip questions A-D.

a. If so, what was the duration of difficult gas evacuation (minutes or hours per day)?

b. What was the severity (11 represents the most severe, 1 represents minimal severity)?

c. What was the difficultly (11 represents the most difficult) getting rid of it?

d. *How many times per week?*

- 1
- 2
- 3
- 4
- 5
- 6
- 7

Flatulence

11. Did you have flatulence?

If you did not have flatulence, please skip questions A-F.

a. If so, what was the severity (11 represents high flatulence and 1 represents little flatulence but not normal)?

b. What was the number of gas evacuations per anus within a day?

c. What was the duration of flatulence (minutes or hours per day)?

d. Did this happen after you consumed the supplement?

e. What was the severity of odor of gas passed per anus (11 represents high odor and 1 represents minimal odor but not normal)?

f. *How many times per week?*

- 1
- 2
- 3
- 4
- 5
- 6
- 7

Nausea

12. Did you have nausea?

If you did not have nausea, please skip questions A-C.

a. *If so, what was the duration of nausea (minutes or hours per day)?*
b. *What was the severity (11 represents high nausea, 1 represents minimal nausea but not normal)?*
c. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

Heartburn

13. Did you have heartburn?

If you did not have heartburn, please skip questions A-C.

a. *If so, what was the duration of heartburn (minutes or hours per day)?*
b. *What was the severity (11 represents high heartburn, 1 represents minimal heartburn but not normal)?*
c. *How many times per week?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7

Bowel Movements

14. Did you have any abnormal bowel movements?

If you did not have normal bowel movements, please skip questions A-B.

a. *How many times per week did you not have normal bowel movements?*
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6
    - 7
b. *Were they normal color?*

15. Did you have diarrhea after taking the supplement?

If you did not have diarrhea, please skip questions A-B.

a. *What was the severity (11 represents high diarrhea, 1 represents minimal diarrhea but not normal)?*
b. *How many times per week did you have diarrhea?*
    - 1
    - 2

- 3
- 4
- 5
- 6
- 7

16. Any symptoms you had today that we did not ask you about?

a. Please describe in detail - the symptom and the duration

Remember to fill out your Supplement Compliance and Physical Activity form!

Day 1 *DATE (MM/DD/YYYY)* ____/__ /_____	***AM DOSE TIME*** ☐☐☐☐ ***AM*** ***PM***	***PM DOSE TIME*** ☐☐☐☐ ***AM*** ***PM***	***If a dose was missed, use key above to complete this section*** ***Reason*** ☐ ***AM*** ***PM*** ☐☐
Day 1 **Physical Activity**	**Did you exercise today?** **Yes** **No** ☐☐ ***If you check No, please disregard the next 3 exercise questions.***	**How many hours did you exercise?** <1 hr. ○ 1-2 hrs. ○ 2-3 hrs. ○ >3 hrs. **What type of exercise?** ○ Cycle ○ Run ○ Weight training ○ Climb ○ Hike ○ Walk ○ Other If other, please list _____	**Please use the RPE scale above to describe the intensity of your exercise.** ☐

Appendix D: 24-Hour Dietary Recall Form

24-Hour Dietary Recall Form			Participant ID:		
			Investigator name:		
			Date:		
Individual:					
Time	Serving size	Meals :	How prepared:	Where	Note :
		Breakfast			
		Snack			
		Lunch			
		Snack			
		Dinner			

Appendix E: Recruitment Flyer

IS CYCLING YOUR MAIN SPORT?

The Effect of Quercetin and Citrulline on Nitric Oxide Metabolite Production and Cycling Performance

A research team invites you to participate in a study that aims to investigate the effect of supplementing with quercetin + citrulline on the nitric oxide metabolite production (blood vessel expansion) and 20-km cycling performance outcomes (oxygen consumption, time to completion, average cadence, speed, mean power, heart rate, rating of perceived exertion, and breath-by-breath analysis).

Testing will involve:

- Body composition testing (DEXA)
- 20-kilometer time-trial performance test– **3 visits**
- **Visit 1** – familiarization of 20-km TT
- **Visit 2** – baseline (pre-supplementation) 20-km test
  - Blood draw pre-and post-exercise
  - **Randomized supplement of** 1) quercetin + citrulline, 2) quercetin, 3) citrulline, **or** 4) non-supplement powder (placebo)
- **Visit 3** – post- 20-km test
  - Blood draw pre-and post-exercise

- Supplementation 2 x/day for 4 weeks dissolved in a zero-calorie orange flavored crystal light package and water after meals
- This research will be used to provide nutritional recommendations of consuming quercetin and citrulline to improve blood vessel expansion, cycling performance, and gastrointestinal distress

What's in it for you?

- You will be provided with 28 days of supplementation, 16 oz water bottles, all performance and body composition (DEXA) data

Georgia State University

Time & Location

- Three short visits (~1-2 hours each) to Georgia State University (Atlanta, GA)

Are you eligible?

- A trained developed cyclist
  - **Age for Men: 18-55**
  - **Age for Women: 18-45**
- Training with a purpose to compete
- Have ridden (stationary bike, bike trainer, outdoor (road, mountain, gravel) 3-5 hours per week, at least 3 years prior to enrollment
- Must have your own cycling bike
  - **NOT** currently consuming sport supplements, pain, or blood pressure medications
  - **NOT** currently on medicines that cause blood vessels to dilate, blood pressure or inflammatory medicines, or certain birth control pills called "triphasic"

If you are interested or have any questions text, call, email, or scan the QR code:

SCAN ME

Jennifer.kurtz06@gmail.com; 720.436.2084

Appendix F: Supplement Compliance Dosing Diary

This diary was provided for each week of the supplementation period. As an example, we have provided just day 1 of the week 1 diary. Questions repeat for each day for the 4 weeks and complete diaries are available upon request.

<table>
<tr><td><u>Week 1</u></td><td>Subject ID #
_____</td><td colspan="2">1=Forgot
2= Physically Unable
3=Fell asleep
4= Took more
5= Other</td></tr>
<tr><td></td><td>RPE SCALE</td><td colspan="2">The Borg Rating of Perceived Exertion
RPE
INTENSITY SCALE
20 Maximal Exertion
19 Extremely hard
18
17 Very hard
16
15 Hard (heavy)
14
13 Somewhat hard
12
11 Light
10
9 Very light
8
7 Extremely light
6 No exertion at all</td></tr>
<tr><td>Day 1
DATE (MM/DD/YYYY)
_____/___ /_____</td><td>PM DOSE TIME
☐☐☐☐
PM PM</td><td>PM DOSE TIME
☐☐☐☐
PM PM</td><td>If a dose was missed, use key above to complete this section
Reason
☐
PM PM
☐☐</td></tr>
<tr><td>Day 1 Physical Activity</td><td>Did you exercise today?
Yes No
☐ ☐
If you check No, please disregard the next 3 exercise questions.</td><td>How many hours did you exercise?
<1 hr.
○ 1-2 hrs.
○ 2-3 hrs.
○ >3 hrs.
What type of exercise?
○ Cycle
○ Run
○ Weight training
○ Climb
○ Hike
○ Walk
○ Other
If other, please list
_____</td><td>Please use the RPE scale above to describe the intensity of your exercise.
☐</td></tr>
</table>

Appendix G: Sleep Quality Questionnaire

1. During the past week, what time have you usually gone to bed at night?

Bedtime _____

2. During the past week, how long (in minutes) has it taken you to fall asleep each night?

Number of Minutes _____

3. During the past week, what time have you usually gotten up in the morning?

Getting up time _____

4. During the past week, how many hours of actual sleep did you get at night? (*This may be different than the number of hours you spent in bed.)*

Hours of sleep per night _____

5. During the past week, how often have you had trouble sleeping because you…
    - Cannot get to sleep within 30 minutes
        - Less than once a week
        - Once or twice a week
        - Three or more times a week
    - Feel too hot or cold
    - Had bad dreams
    - Had pain

Other reason(s), please describe

_____

_____

Appendix H: RPE Scale

The Borg Rating of Perceived Exertion

RPE

INTENSITY SCALE

20	Maximal Exertion
19	Extremely hard
18	
17	Very hard
16	
15	Hard (heavy)
14	
13	Somewhat hard
12	
11	Light
10	
9	Very light
8	
7	Extremely light
6	No exertion at all