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L-CITRULLINE SUPPLEMENTATION, EXERCISE CAPACITY, AND RELATED PSYCHOPHYSIOLOGICAL RESPONSES DURING EXERCISE IN HEALTHY ADULTS

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Symbols and Abbreviations

NO	Nitric oxide
NOS	Nitric oxide synthase
ADMA	Asymmetric dimethylarginine
$\dot{V}O_2$	Oxygen consumption
LT	Lactate threshold
MMSS	Maximal metabolic steady state
OCT	Ornithine carbamoyltransferase
ASS	Argininosuccinate synthetase
CIT_{max}	L-citrulline peak plasma concentration
nNOS	Neuronal nitric oxide synthase
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
OAT	Ornithine aminotransferase
CPS-I	Carbamoyl phosphate synthetase-I
ASL	Argininosuccinate lyase
NADPH	Nicotinamide adenine dinucleotide phosphate
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide

sGC	Guanylate cyclase
CPS	Carbamoyl phosphate synthetase
OTC	Ornithine transcarbamylase
ROS	Reactive oxygen species
GTP	Guanosine triphosphate
cGMP	Cyclic guanosine monophosphate
PDE-5	Phosphodiesterase type 5
RNS	Reactive nitrogen species
BTB	Blood-brain tumour barrier
NIRS	Near-infrared spectroscopy
TR	Treadmill running
CE	Cycle ergometer
TT	Time trial
TTE	Time to exhaustion
Pla	Placebo
PO peak	Peak power output
GET	Gas exchange threshold
RCP	Respiratory compensation point
Cit6	6 g of L-citrulline
Cit12	12 g of L-citrulline
BP	Blood pressure
SBP	Systolic blood pressure

DBP	Diastolic blood pressure
MAP	Mean arterial pressure
[Arg]	L-arginine concentration
[Orn]	L-ornithine concentration
[Lys]	L-lysine concentration
MRT	Mean response time
Hb	Haemoglobin
Mb	Myoglobin
Oxy(Hb+Mb)	Oxygenated haemoglobin and myoglobin
Deoxy(Hb+Mb)	Deoxygenated haemoglobin and myoglobin
$[La^-]$	Blood lactate concentration
RPE	Rating of perceived exertion
AAI	Arginine availability index
$\dot{V}_E$	Pulmonary ventilation
$\dot{V}CO_2$	Carbon dioxide production
RER	Respiratory exchange ratio
PO_2	End-tidal partial pressure of O_2
PCO_2	End-tidal partial pressure of CO_2
RSS	Residual sum of squares
HPLC	High-performance liquid chromatography

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Abstract

L-citrulline is a naturally occurring non-essential amino acid that is both endogenously synthesised and obtained from dietary sources. Although it plays a critical role in the urea cycle, L-citrulline is primarily recognised for its contribution to nitric oxide (NO) synthesis, a gaseous signalling molecule generated as a by-product of the conversion of L-arginine to NO. Dietary supplementation with NO precursor L-arginine has shown improved exercise economy and tolerance in healthy adults. However, findings have been inconsistent, likely due to the limited bioavailability of the L-arginine resulting from extensive pre-systemic and systemic metabolism. In contrast, dietary supplementation with L-citrulline can bypass these metabolic barriers, thereby enhancing plasma L-arginine availability and stimulating NO production effectively. As a result, L-citrulline has emerged as a promising nutritional strategy to improve exercise "performance" by increasing NO bioavailability. The scientific literature on the ergogenicity of L-citrulline, however, remains equivocal. Inconsistencies among studies may arise, in part, from differences in exercise protocols and/or variations in supplementation protocols, including dosage. These factors, however, have not been systematically examined, and the occurrence of a dose-response relationship remains still uncertain. To address this knowledge gap, the present thesis investigated the effects of different doses of pure L-citrulline on exercise tolerance and associated physiological responses during ramp-incremental (***Study 1***) and constant work-rate cycling tests (***Study 2***). A group of moderately trained adults visited the laboratory on

separate occasions and completed a ramp-incremental cycling test and/or moderate- and severe-intensity step transitions following supplementation of either L-citrulline or energy-matched placebo. Plasma concentrations of L-lysine ([Lys]), L-ornithine ([Orn]), L-arginine ([Arg]), and the arginine availability index (AAI), along with blood pressure and blood lactate, were assessed before and after exercise. Pulmonary gas exchange and ventilation and NIRS-derived muscle oxygenation were continuously measured throughout the exercise tests. Plasma [Orn], [Arg], as well as AAI were significantly elevated ($P < .05$) following L-citrulline supplementation, while blood pressure remained unaltered ($P > .05$). No differences were found between conditions ($P > .05$) for pulmonary gas exchange, ventilation, or muscle oxygenation during either ramp-incremental or constant work rate cycling. Time to task failure (TTF) and peak power output (PO peak) were also similar between conditions ($P > .05$). Collectively, the current findings do not support the ergogenicity of L-citrulline under the studied conditions. This result provides valuable evidence concerning the safety and practical applicability of L-citrulline supplementation in healthy, trained adults.

1 | Introduction

L-citrulline is a naturally occurring non-essential amino acid that is both endogenously synthesized and obtained through dietary sources. It is found primarily in watermelon (*citrullus vulgaris*) and, to a lesser extent, in cucumbers, muskmelons, bitter melons, pumpkins, squashes, and gourds (Wu et al., 1998). L-citrulline plays a key role in the urea cycle, an essential metabolic pathway for nitrogen disposal (Rabier et al., 1995). Within this cycle, L-citrulline is formed indirectly from L-arginine via L-ornithine as part of ammonia sequestration (Wu et al., 1998). However, L-citrulline is also involved in NO synthesis, where it is produced as a by-product of the conversion of L-arginine to NO, a reaction catalysed by nitric oxide synthase (NOS) enzymes (Wu et al., 1998; Förstermann et al., 2012).

NO is a gaseous signalling molecule pivotal in the human body (Stamler et al., 2001). Indeed, NO plays an important role in a plethora of physiological processes, including the vasodilation, neurotransmission, mitochondrial respiration, and skeletal muscle contractile function (Lundberg and Weitzberg, 2009). The multifaceted physiological signalling molecule NO, however, might also possess ergogenic potential. Previous studies (Vanhatalo et al., 2010; Bailey et al., 2010; Buford et al., 2004) have reported enhanced exercise economy and exercise tolerance following dietary supplementation with the NO precursor L-arginine. Other investigations, however, have less optimistic

findings, reporting minimal or no ergogenic benefit (Stevens et al., 2000; Bescós et al., 2009; Koppo et al., 2009). These inconsistent results cast doubt on the ergogenicity of L-arginine. The reasons for such discrepancies remain uncertain, but may relate, at least in part, to variations in dosage, timing, and duration of supplementation protocols (Vanhatalo et al., 2013). Limited bioavailability due to a myriad of pre-systemic and systemic elimination processes may also contribute to these inconsistencies. Indeed, a considerable portion of orally ingested L-arginine is catabolised by intestinal bacteria and arginase enzymes on the first pass (Castillo et al., 1993), while additional systemic L-arginine is extracted and metabolised by the liver (O'Sullivan et al., 1988; van de Poll et al., 2007). Consequently, the effectiveness of oral L-arginine supplementation as a valid intervention to stimulate NO synthesis and improve exercise economy and exercise tolerance remains questionable.

More recently, L-citrulline has emerged as a more effective endogenous precursor for L-arginine synthesis. Unlike L-arginine, orally ingested L-citrulline is not metabolized in the intestine or liver and does not stimulate arginase enzymes (Morris, 2016). Upon absorption, L-citrulline is transported into the kidney and other tissues via facilitated mechanisms, since it, also in contrast to L-arginine, does not compete with asymmetric dimethylarginine (ADMA) and other L-arginine analogues for cellular uptake via the y^+ transporter system, including the hCAT-2B carrier (Closs et al., 1997). Once inside the cell, L-citrulline can be readily converted to L-arginine through the citrulline-NO cycle, thereby elevating plasma (Kuhn et al., 2002; Kuhn et al., 2011; Osowska et al., 2004) and tissue (Wijnands et al., 2012) L-arginine concentrations, and increasing NO

synthesis more effectively than an equivalent dose of L-arginine. Collectively, these findings suggest that L-citrulline may serve as a superior precursor for NO production, with potential implications for exercise performance enhancement.

L-citrulline, similar to other dietary supplements that enhance NO production, has attracted growing interest in recent years as a nutritional aid for improving both aerobic and anaerobic exercise performance (for an in-depth review, see Gonzalez et al., 2020). The primary mechanism proposed to underlie the ergogenic effects of L-citrulline is its ability to increase NO production, leading to endothelium-mediated vasodilation and improved blood flow and oxygen delivery to the active skeletal muscles (Allerton et al., 2018). A direct consequence of improved oxygen transport is increased oxygen consumption ($\dot{V}O_2$) by the working muscles, which may ultimately enhance exercise efficiency and exercise tolerance in humans (Bassett, 2000; Burtscher, 2013). Recent studies indicates that L-citrulline supplementation not only augments NO synthase (Wijnands et al., 2012) and increases NO biomarkers (Schwedhelm et al., 2008), but also improves skeletal muscle oxygenation and accelerates $\dot{V}O_2$ kinetics (Bailey et al., 2015). L-citrulline supplementation has also been shown to improve skeletal muscle metabolism and contractile efficiency (Bendahan et al., 2002; Giannesini et al., 2009), potentially enhancing fatigue resistance (Bailey et al., 2015). Another proposed mechanism concerning linking L-citrulline to enhanced exercise performance involves buffering ammonia through the urea cycle. By reducing circulating ammonia levels, L-citrulline may promote the aerobic utilisation of pyruvate, thereby diminishing reliance on anaerobic metabolism and lactate accumulation, and, ultimately, sustaining

muscle contraction and delaying the onset of fatigue (Mutch et al., 1983). However, the current literature remains inconclusive regarding the effects of supplementation with L-citrulline on anaerobic, primarily lactate, metabolism and the consequences on athletic performance (Martínez-Sánchez et al., 2017). Despite such inconsistencies, the collective evidence suggests that short-term L-citrulline supplementation might represent a promising nutritional approach to enhance exercise efficiency and exercise tolerance in humans (Martínez-Sánchez et al., 2017; Stanelle et al., 2020; Suzuki et al., 2016).

Although some evidence suggests that supplementation with L-citrulline may enhance NO production and improve physiological responses in conjunction with optimised exercise performance, the scientific literature about the ergogenic potential remains inconclusive, with findings to date being mixed. The reasons for such discrepancies are not fully understood, but may be attributed, at least in part, to variations in exercise protocols, which largely differ in terms of exercise intensity (Martínez-Sánchez et al., 2017). In fact, exercise intensity is typically categorised into three distinct domains, defined by two genuine metabolic boundaries: the moderate-intensity domain (below the lactate threshold, LT), the heavy-intensity domain (between LT and maximal metabolic steady state, MMSS), and the severe-intensity domain (above the MMSS; Poole and Jones, 2012). Because the degree of intramuscular and systemic homeostasis disruption during exercise is linked to the intensity domain in which the exercise is performed (Burnley et al., 2012; Vanhatalo et al., 2016; Black et al., 2017), it is plausible that the effectiveness of L-citrulline supplementation on exercise efficiency

and/or tolerance would exhibit domain-specificity. Varied results between studies may also arise from differences in dosage used in L-citrulline supplementation protocols. This hypothesis, however, is speculative and remains to be studied. Since all exercise-performance studies completed to date with L-citrulline have employed dosages of 6 g or lower (Suzuki et al., 2016), further research is warranted to determine whether a dose-response relationship exists between L-citrulline intake, physiological responses to exercise, and exercise performance. Establishing the optimal dosage for enhancing exercise efficiency and tolerance is of importance, given the increasing popularity of L-citrulline supplementation in both basic research and applied exercise settings.

The present thesis, therefore, aims to address the aforementioned gap in the literature by investigating the effects of different doses of acute L-citrulline supplementation on exercise tolerance and related physiological responses during both ramp-incremental and constant-power output cycling in humans. Specifically, the thesis is structured into six chapters: *Chapter 1* presents an introduction to the topic; *Chapter 2* provides the state-of-the-art, summarizes current findings, and identifies research gaps; *Chapter 3* outlines the methodology, including experimental design, participant characteristics, supplementation protocols, and study procedures; *Chapter 4* displays the main results, while *Chapter 5* discusses the findings based upon the existing literature, addresses limitations, and proposes future research directions. Lastly, the *Chapter 6* summarizes the main findings and offers a comprehensive perspective on the effects of L-citrulline supplementation on exercise "performance" in humans.

2 | Topic and Purpose of the Study

2.1 | Physical and Chemical Properties of L-Citrulline

Citrulline (CAS Registry: 372-75-8) is a colourless solid compound under ambient temperature and pressure, with a recorded melting point of 222°C. As an α-amino acid with an asymmetric carbon, it exists in two enantiomeric forms. Its naturally occurring form is L-citrulline (dextrorotatory, $[\alpha_D^{20}] = 3.7^o$, with an absolute S configuration), like many other amino acids. Citrulline can also exist as salt in its cationic form, such as citrulline hydrochloride, another dextrorotatory compound ($[\alpha_D^{20}] = 17.9^o$) (Curis et al., 2005). It exhibits a crystalline structure with complex chemical properties. With a molar mass of 175 $g \cdot mol^{-1}$, chemical properties of citrulline are influenced by amino acid function and a ureido group in its aliphatic chain, which replaces the α-carbon. Due to its polar side chain, citrulline exhibits relatively high hydrophilicity but low solubility in methanol and ethanol. The partition coefficient between octanol and water is reported as log P = -3.19 ± 0.11 (Pliška et al., 1981). In the context of Brønsted-Lowry acid-base equilibrium, Citrulline exhibits two pKa values: 2.4 for the carboxyl group and 9.4 for the amino group, both characteristic of amino acids. However,

depending on experimental conditions, these pKa values can vary between 2.1–2.4 for pKa_1 and 8.6–9.7 for pKa_2 (Steglish et al., 2000). Notably, citrulline is a diacid, and considering its micro-acidities, its predominant neutral form is zwitterionic (>99.9%; Noszál and Kassai-Tánczos, 1991). Under alkaline pH conditions, both the carboxyl and amino groups can act as Lewis acids, forming coordination complexes with metal cations, such as the ternary complex [CuII (His)(Cit)] (Yamauchi et al., 1980). The biological significance of these complexes, however, remains a topic of intense debate in the scientific literature (Curis et al., 2005).

Citrulline shares the typical reactivity of α-amino acids, enabling it to form peptide bonds and become incorporated into proteins, thus generating “citrullinated proteins”. However, its reactivity is primarily influenced by its terminal ureido group, which closely resembles the functional group of arginine (Toffoli et al., 1987). Interestingly, the electrophilic carbon of the ureido group is highly reactive, probably due to electron withdrawal by the adjacent nitrogen and oxygen atoms, contributing to the distinct biochemical properties of the Citrulline. Therefore, R, a relatively weak nucleophilic compound reacts with the carbon atom to form a stable intermediate, $N_2C(R)O^-$, which is subsequently stabilized by the expulsion of one of its four ligands: R (no reaction),

NH_3, R-NH_2 (forming ornithine), or water. These reactions occur with proton donor and acceptor ions (Curis et al., 2005).

From a biological perspective, the most relevant reaction involves aspartate as the nucleophilic compound R, leading to the formation of argininosuccinate in the urea cycle (Curis et al., 2005). However, these reactions can also proceed in the reverse direction, yielding citrulline as a final product. This mechanism underlies the industrial production of citrulline from arginine, where water can serve as the nucleophile and ammonia as the leaving group. In most biological systems, the leaving group may be phosphate – within the urea cycle, following ornithine condensation via carbamoyl phosphate synthetase – or NO (Morris, 2000).

The quantification of citrulline in biological tissues is currently achieved through various analytical techniques, broadly classified into two categories: generic amino acid quantification methods and citrulline-specific assays (Neveux et al., 2004). Generic methods rely on detecting amino acid functional groups and, being non-specific, are typically integrated with separation techniques such as high-performance liquid chromatography (Martens-Lobenhoffer and Bode-Bøger, 2003). In constrast,

citrulline-specific methods are based on urea quantification, as citrulline is structurally related to substituted ureas. These approaches can even detect citrulline residues in proteins (Sugawara et al., 1998). However, their apparent technical advantage can be mitigated by their inability to distinguish between free citrulline and protein-bound citrulline residues, potentially leading to overestimating intracellular and tissue citrulline concentrations. The aforementioned methods, whether generic or specific, also have the disadvantage of providing a static view of citrulline levels in a sample, thereby overlooking the dynamic flux of citrulline and the rate of synthesis of its metabolites. These factors can be better assessed using isotopic labelling techniques (Keilhoff and Wolf, 2003; Castillo et al., 1993).

Regardless of the quantification method employed, it is now well-established that citrulline – due to its strong association with arginine – is present in nearly all living organisms, with variations in concentration and function (Wu, 1998; Wu et al., 2007). In microorganisms, such as bacteria and parasitic nematodes, citrulline is a carbon and energy source. Like mammals, two key enzymes – ornithine carbamoyltransferase and argininosuccinate synthetase – regulate citrulline metabolic pathways. The next section of this thesis will provide a more detailed exploration of these enzymes. It is

also worth noting that citrulline is found in the plant kingdom, with high concentrations in cucurbitaceous plants, such as watermelon (*citrullus vulgaris*—after which it is named), and certain algae, such as Grateloupia vulgaris. In plants, citrulline is involved in nitrogen transport (Ludwig, 1993) and protects against oxidative stress (Akashi et al., 2001).

2.2 | L-Citrulline Biosynthesis and Metabolism

L-citrulline metabolism follows three distinct biological pathways. The first pathway is L-arginine biosynthesis, in which L-citrulline is exchanged and utilized throughout the body to support systemic amino acid metabolism. The second is the NO cycle, which involves local recycling of L-citrulline sustaining NO synthesis, a key regulator of vascular function and cellular signalling. The third pathway, the urea cycle, occurs in the liver and is essential for ammonia detoxification and nitrogen balance. These pathways are tightly regulated by three key enzymes: nitric oxide synthase (NOS; EC 1.14.13.39) and ornithine carbamoyltransferase (OCT; EC 2.1.3.3), both of which are involved in CIT synthesis, and argininosuccinate synthetase (ASS; EC 6.3.4.5), also referred to as citrulline-aspartate ligase, which plays a crucial role in the catabolism of

CIT into argininosuccinate (Morris, 2006). An in-depth analysis of these enzymes and their specific roles in CIT metabolism is provided in subsequent sections.

2.2.1 | Ornithine Carbamoyltransferase (OCT)

Ornithine carbamoyltransferase (OCT), also called ornithine transcarbamylase, is an essential enzyme in various biological kingdoms. It plays a pivotal role in both the biosynthesis and catabolism of L-arginine. OCT is a key component of the urea cycle, catalysing the conversion of L-ornithine into L-citrulline via the following reaction (Wu and Morris, 1998):

$$\text{L-ornitina} + \text{carbamoil fosfato} \rightarrow \text{L-citrulina} + \text{fosfato}$$

$$R - NH2 + H2N - CO - Pi \rightarrow R - NH - CO - NH2 + Pi$$

where Pi represents phosphate, and R is the shared radical structure among L-ornithine, L-citrulline, and L-arginine. The anabolic OCT isoform is involved in L-citrulline biosynthesis via the L-arginine synthesis pathway, where L-citrulline could serve as the reaction product. From a thermodynamic perspective, OCT strongly favours L-

citrulline formation, as indicated by its standard Gibbs free energy change (ΔrGm = −29.8 kJ·mol^{-1}) under physiological conditions (pH 6.7–7.4, ionic strength I = 0.1 M, temperature T = 38°C), resulting in an equilibrium constant of K' = 10^5 (Reicherd, 1957). In prokaryotes and some eukaryotes, OCT is typically found in the cytoplasm. However, in higher eukaryotes, including mammals, the enzyme is localized within the mitochondrial matrix (Takiguchi et al., 1989; Wu and Morris, 1998). In humans, OCT is predominantly expressed in the liver and intestinal mucosa (Takiguchi and Mori, 1995). Structurally, OCT is a crystallized enzyme that has directly evolved from aspartate carbamoyltransferase (ATC; EC 2.1.3.2), thus highlighting its evolutionary significance in nitrogen metabolism (Houghton et al., 1984).

2.2.2 | Nitric Oxide Synthase (NOS)

NOS is a family of enzymes that catalyse the production of NO from L-arginine. NOS enzymes play a fundamental role in various physiological processes, such as vascular homeostasis, immune defence, and neurotransmission. This enzyme family comprises three distinct isoforms, which differ in their expression patterns, cellular localization,

and physiological functions: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (Morris, 2016).

nNOS, predominantly expressed in specific central nervous system neurons, plays a critical role on regulating synaptic plasticity, a mechanism essential for learning and memory formation (Förstermann & Sessa, 2012). NO produced by nNOS can regulate blood pressure (Moncada and Higgs, 2006), modulate neurotransmission, and affect physiological processes such as peristalsis, vasodilation, erectile function, among others (Burnett, 1995).

iNOS is expressed in various cell types, remarkably immune and inflammatory cells, and is essential for host defence against intracellular pathogens, such as *Leishmania parasites* (Wei et al., 1995). Unlike nNOS and eNOS, both constitutively expressed, iNOS is inducible in response to inflammatory stimuli. NO synthesized by iNOS exerts pro-inflammatory and antimicrobial effects, playing a key role in the immune response (Rafiee et al., 2002). Moreover, iNOS-derived NO influences vasodilation and blood pressure regulation in pathological conditions, such as sepsis (Wong and Billiar, 1995).

eNOS is predominantly expressed in vascular endothelial cells and is fundamental for vascular homeostasis. The NO produced by eNOS induces vasodilation, inhibits platelet aggregation and adhesion, and exerts anti-thrombotic effects, thereby reducing the risk of vascular occlusion and thrombosis (Li and Förstermann, 2000). Moreover, eNOS-derived NO regulates gene expression in atherosclerosis prevention, thereby highlighting its importance as an anti-atherogenic factor (Vanhoutte et al., 2016). For an in-depth analysis of NOS enzyme expression and function in humans, please refer to Förstermann and Sessa (2012).

2.2.3 | Argininosuccinate Synthetase (ASS)

Like OCT, ASS is widely distributed across biological kingdoms, playing a crucial role in L-citrulline catabolism. This cytosolic enzyme catalyses the following reaction (Wu and Morris Jr, 1998):

L-citrulina + Aspartato + ATP → Argininosuccinato + pirofosfato + AMP

where ATP stands for adenosine triphosphate and AMP for adenosine monophosphate. In a thermodynamic perspective, ASS favours L-citrulline degradation, with a ΔrGm of -2.0 kJ·mol^{-1} at pH 6.91, ionic strength (I) = 0.01 M, and temperature (T) = 25°C, yielding an equilibrium constant K' = 2.14 (Schuegraf et al., 1960). Notably, in higher eukaryotes, including mammals, ASS is widely expressed, with the highest levels found in the liver and kidneys and lower concentrations in the intestine (Husson et al., 2003). In humans, the expression of the ASS gene varies across different tissues and appears to be regulated by hormonal signals and nutritional status, highlighting its role in metabolic adaptability (Curis et al., 2005). Structurally, ASS is likely to be encoded on chromosome 9 and features a considerably well-defined crystalline structure with specific binding sites for ATP, L-citrulline, and aspartate (Lemke and Howell, 2002). Kinetic studies suggest that ASS operates through a sequential binding mechanism, where ATP is the first to bind, followed by L-citrulline and aspartate (Rauschel and Seigle, 1983). The binding of aspartate induces the formation of a citrulline-adenylate intermediate, which is processed to release argininosuccinate, pyrophosphate (PPi), and AMP in a stepwise manner (Rauschel and Seigle, 1983; Ghose and Rauschel, 1985). Magnesium ions (Mg^{2+}) are also involved in the reaction, although their exact role remains unclear.

2.3 Endogenous Synthesis of L-Citrulline

L-citrulline is synthesized primarily in the small intestine by metabolizing various amino acid substrates, including glutamine, proline, and L-arginine. Within intestinal enterocytes, glutamine is partially catabolized into L-citrulline through a series of enzymatic reactions, which involves glutaminase, pyrroline-5-carboxylate synthase, ornithine aminotransferase (OAT), OCT, ornithine transcarbamylase, and carbamoyl phosphate synthetase-I (CPS-I) (Aguayo et al., 2021). Traditionally, glutamine has been considered the key precursor of intestinal L-citrulline synthesis. Prior evidence, however, suggests that L-arginine plays a predominant role, as nearly 40% of dietary arginine is converted into ornithine through arginase II activity in enterocytes, which is then further metabolized into L-citrulline by OCT (Marini et al., 2004). Proline also contributes to L-citrulline synthesis, albeit to a lesser extent, being converted into Δ^1-l-pyrroline-5-carboxylate by proline oxidase, follow its transformation into ornithine via OAT. Notably, ornithine from portal circulation can serve as a notable alternative L-citrulline precursor, particularly under conditions of low L-arginine availability. Since OCT catalyses the conversion of ornithine into L-citrulline, it plays a dual role,

contributing to L-citrulline biosynthesis and urea cycle function. Of note, the systemic concentration of citrulline reflects both intestinal absorption from dietary sources and *de novo* synthesis, making it a valuable biomarker of functional enterocyte mass and small intestinal health (Curis et al., 2005).

2.3.1 | Nitric oxide cycle

2.3.1.1 | L-Arginine Biosynthesis

In mammals, L-arginine is classified as a semi-essential or conditionally essential amino acid, as its requirement could vary based on health status and developmental stage (Luiking et al., 2012). L-arginine is obtained from endogenous synthesis, protein breakdown or dietary intake. Among these recognized sources, L-citrulline conversion to L-arginine accounts for approximately 60% of the *de novo* L-arginine synthesis in the human body, yet only 5–15% of this pool circulates in plasma (Morris et al., 2020). Enterocytes in the small intestine release L-citrulline into the portal circulation, which is transported to the kidneys. Within proximal tubule cells, approximately 83% of L-citrulline is converted to L-arginine, a process mediated by ASS, which catalyses the

formation of argininosuccinate from L-citrulline, aspartate, and ATP. Thereafter, the enzyme Argininosuccinate is then hydrolysed by argininosuccinate lyase (ASL) to yield fumarate and L-arginine (van de Poll, et al., 2004).

The intestinal-renal axis is the principal site of L-arginine synthesis, with roles played by intestinal epithelial cells and renal proximal tubule cells (Bescós, et al., 2012). While other tissues also express ASS and ASL, allowing for localized L-arginine production, the liver and kidneys remain the primary sites of biosynthesis (Mori, et al., 2004). Moreover, plasma L-citrulline concentrations are considered kidney function biomarkers, as L-citrulline degradation in the kidney is a key step in renal L-arginine synthesis. Notably, L-arginine undergoes extensive hepatic catabolism, whereas L-citrulline bypasses hepatic metabolism, making L-citrulline supplementation a valid strategy to enhance L-arginine production and bioavailability while avoiding hepatic degradation (Windmueller, et al., 1981).

2.3.1.2 | L-Arginine Catabolism

L-arginine metabolism is highly compartmentalized and primarily regulated by the enzyme arginase, a manganese-dependent metalloenzyme that hydrolyses L-arginine into L-ornithine and urea. There are two isoforms of arginase, each with distinct tissue distribution, subcellular localization, and physiological function. In particular, the cytosolic enzyme arginase I is predominantly expressed in the liver, where it plays a fundamental role in the urea cycle, facilitating nitrogen excretion from amino acid metabolism. However, low levels of arginase I have also been detected in extrahepatic tissues, including endothelial cells and vascular smooth muscle cells (Durante et al., 2007).

In contrast, arginase II is a mitochondrial enzyme widely distributed in extrahepatic tissues, including the kidney, small intestine, brain, red blood cells, and immune cells (Pernow et al., 2013). The intestinal mucosa exhibits high arginase II activity, with the splanchnic region extracting ~40% of dietary L-arginine, contributing to low plasma L-arginine levels. Constitutive arginase activity in endothelial cells reduces L-arginine bioavailability for NO synthesis by NOS, thereby affecting vascular tone and

endothelial function (Vanhoutte et al., 2017). Given that arginase and NOS compete for the same substrate, the relative activities are determined by tissue type and external stimuli (Wu et al., 1998).

2.3.1.3 | NO Biosynthesis

NO synthesis occurs via two primary pathways – the reduction of inorganic nitrate and the enzymatic conversion of L-arginine to L-citrulline by NOS with NO as a byproduct (Bode-Böger et al., 1998). This reaction uses O_2, nicotinamide adenine dinucleotide phosphate (NADPH), and multiple cofactors, including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin, and heme. The recycling of L-citrulline to L-arginine, mediated by ASS, sustains NO production (Husson et al., 2003).

NO induces vasodilation by activating soluble guanylate cyclase (sGC) in vascular smooth muscle, leading to increased intracellular cyclic guanosine monophosphate (cGMP) levels and further relaxation (Suzuki, et al., 2016). Beyond the vasodilation, NO inhibits platelet aggregation, prevents leukocyte adhesion to the endothelium, and

modulates mitochondrial O_2 consumption by inhibiting cytochrome oxidase (Luiking et al., 2010; Morita et al., 2014). Notably, impaired NO production is implicated in a great number of cardiovascular diseases, including hypertension, atherosclerosis, and angiogenesis-related disorders (Luiking et al., 2012).

Given that NO synthesis is directly influenced by L-arginine availability, a myriad of factors regulates its production:

- Intracellular L-arginine synthesis from L-citrulline is particularly dependent on L-citrulline availability.
- Extracellular L-arginine transport, which affects substrate availability.
- Arginase activity, which competes with NOS for L-arginine utilization.

2.3.2 | Urea cycle

The urea cycle, which takes place primarily in the liver, is a fundamental metabolic pathway responsible for detoxifying ammonia derived from dietary protein and muscle metabolism by converting it into urea for excretion (Windmueller et al., 1981). Within the liver, L-citrulline metabolism is highly compartmentalized and remains

separate from other L-citrulline-related pathways (Curis et al., 2005). Of considerable interest, hepatocytes involved in the urea cycle cannot uptake L-citrulline from the portal circulation (Bescós et al., 2012). The cycle is initiated by the carbamoyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC) enzymes, which are also expressed in the small intestine. OTC facilitates carbamoyl phosphate and L-ornithine reaction, leading to L-citrulline synthesis (Kaore et al., 2013). A deficiency in OTC can result in hypocitrullinemia, hyperammonemia, and elevated plasma levels of glutamine, alanine, and lysine, potentially leading to severe metabolic disorders, coma, or even death (Breuillard, et al., 2015). The L-citrulline, however, produced in the urea cycle is highly labile, as argininosuccinate synthetase rapidly can convert it into argininosuccinate, preventing its release into circulation (Bescós et al., 2012; Windmueller et al., 1981). Argininosuccinate is then metabolized by argininosuccinate lyase into arginine and fumarate, with L-arginine subsequently being hydrolysed by arginase into L-ornithine and urea. While L-ornithine is largely recycled within the cycle, urea is excreted by the kidneys, ensuring the efficient removal of nitrogenous waste. This process highlights the crucial interplay between the liver and kidneys in nitrogen homeostasis and ammonia detoxification.

2.4 | L-Citrulline Pharmacokinetics and Pharmacodynamics

2.4.1 | Transport, Absorption, and Distribution

No specific L-citrulline transporter has been identified (Lee and Kang, 2017). Instead, L-citrulline is transported into cells via general amino acid transporters, and in some instances, some cells can uptake or release L-citrulline without the involvement of transport proteins (Hilderman et al., 2000). Notably, all cells in the nervous system, such as microglia, macroglia, and neurons, possess a L-citrulline transport mechanism, although the precise pathways remain uncertain (Devés and Boyd, 1998). It has been suggested that this process may involve L-type long-chain amino acid carriers, which function independently of sodium (Schmidlin et al., 2000).

In aortic endothelial cells, L-citrulline uptake is sodium-independent and occurs via a poorly characterised transport system distinct from the L-arginine transport pathway. This is clear from the inability of the y^+ transport system, which facilitates L-arginine transport, to carry L-citrulline (Hilderman et al., 2000). In contrast, L-citrulline transport in aortic smooth muscle cells are sodium-dependent and pH-insensitive.

Likewise, enterocytes also can require sodium-dependent transport mechanisms for L-citrulline uptake (Vadgama and Evered, 1992). In the kidneys, no specific L-citrulline transporter has been identified (Lee and Kang, 2017). However, competition of L-citrulline with dibasic amino acids for transport suggests such a mechanism. This is supported by findings that show increased L-citrulline excretion in urine following L-lysine use, indicating competitive renal transport (Oyanagi et al., 1981).

L-citrulline is synthesized by OCT in the mitochondria but catabolized by ASS in the cytosol (Morris, 2006). This could suggest the existence of an intracellular transport mechanism in cells expressing both OCT and ASS, which are capable of partial or full urea cycle activity (Porter, 2000). This intracellular transport system may involve a citrulline-ornithine exchanger located in the inner mitochondrial membrane, along with proton (H^+) and calcium (Ca^{2+}) ion transport (Poolman et al., 1987).

L-citrulline is absorbed in the small intestine, predominantly in the mid to distal ileum. L-citrulline accumulation in enterocytes suggests the presence of specific transport mechanisms (Vadgama and Evered, 1992). However, dietary intake is not the primary source of circulating L-citrulline. Instead, most free L-citrulline is synthesized from

glutamine within enterocytes (Wu and Morris Jr., 1998). Other amino acids, including glutamate, proline, and L-arginine also contribute to L-citrulline synthesis (Wu, 1998). Glutamine, in particular, is obtained from diet and systemic circulation (Plauth et al., 1999). Enterocytes could metabolize 25–33% of arterial glutamine, 66% of luminal glutamine, and 96% of luminal glutamate (Wu, 1998).

The intestinal production of L-citrulline plays a crucial physiological role. Disruptions in this process have been linked to intestinal motility disorders (Crenn et al., 2003) and growth retardation in animals (Hoogenraad et al., 1985) and humans (Casanello and Sobrevia, 2002). If not utilized in NO synthesis, L-citrulline undergoes renal metabolism, converting it into L-arginine (Levillain et al., 1990). This occurs through a partial urea cycle involving ASS and ASL. The L-arginine synthesised from L-citrulline is then released into the bloodstream in sufficient amounts to meet daily L-arginine requirements in adults, with about 60% of *de novo* L-arginine synthesis being attributed to the renal conversion of L-citrulline (Morris, 2016).

It is known that ~83% of the L-citrulline released by the intestine is metabolized in the kidneys, representing 35% of circulating L-citrulline (Morris, 2006). The intestinal

renal metabolic axis allows L-citrulline to bypass hepatic metabolism and ensure L-arginine availability for peripheral tissues. Differently from dietary L-arginine, which is extracted mainly by the hepatic portal circulation, L-citrulline avoids hepatic uptake due to the limited ability of the liver to transport L-citrulline (Windmueller and Spaeth, 1981). Once L-citrulline reaches the kidneys, it is efficiently converted into L-arginine and released into circulation, making it an important precursor for systemic L-arginine supply (Morris, 2016).

L-citrulline metabolism differs from related amino acids such as L-arginine and L-ornithine. Notably, peak plasma concentrations (CIT_{max}) of L-citrulline are higher than those of L-arginine or L-ornithine following oral administration (Moinard et al., 2008; Schwedhelm et al., 2008). This is due to ability of the L-citrulline to bypass splanchnic extraction, as intestine or liver does not metabolize it, not being a substrate for arginase enzymes (Moinard et al., 2008). Notably, L-citrulline also inhibits arginase activity, which may contribute to its superior ability to increase plasma L-arginine levels when supplemented alongside L-arginine (Schwedhelm et al., 2008).

Prior studies have indicated that oral L-citrulline supplementation effectively increase plasma citrulline and arginine concentrations dose-dependently. In fact, Schwedhelm et al. (2008) reported that 1.5 g twice-daily supplementation for one-week elevated plasma L-citrulline and L-arginine levels more effectively than an equivalent dose of L-arginine supplementation. Similarly, Moinard et al. (2008a) noted that in elderly individuals, a single 10 g dose of L-citrulline increased plasma concentrations of L-arginine compared to an equivalent dose of L-arginine (9.94 g). In a subsequent study, Moinard et al. (2008b) investigated oral L-citrulline loads (2, 5, 10, and 15 g) and revealed a rapid and large increase in plasma L-citrulline concentrations – ranging from 10-fold (2 g) to 100-fold (15 g) – with levels returning to baseline within 5-8 hours post-ingestion. Notably, even at high doses, L-citrulline was well tolerated and did not cause gastrointestinal disturbances, suggesting intestinal absorption is not a limiting factor in its bioavailability.

The transport mechanisms of L-citrulline also differ from those of L-arginine. While L-arginine is primarily absorbed via Na+-independent cationic amino acid transporters (CAT-1, 2, and 3), L-citrulline is transported across enterocytes using Na+-dependent neutral amino acid transporters, such as ASC or B0+ transporters (Schwedhelm et al.,

2007). These differences in transport and metabolism favour L-citrulline as a superior strategy for increasing plasma L-arginine concentrations, particularly for applications targeting NO production and vascular function.

2.4.2 | Safety of Oral Citrulline Supplementation

L-citrulline is well tolerated, even at high doses, and has not been associated with significant gastrointestinal side effects. Unlike L-arginine and L-ornithine, which at doses exceeding 9 g per day (L-arginine) or 10 g (L-ornithine) can induce nausea, vomiting, and diarrhea due to intestinal absorption limitations, L-citrulline does not lead to osmotic diarrhea or gastrointestinal distress (Schwedhelm et al., 2007; Moinard et al., 2008). This makes L-citrulline supplementation more practical and effective for increasing systemic L-arginine availability. The highest documented safe doses of L-citrulline include 0.18 g/kg/day (~12.6 g/day for a 70 kg individual) over seven days and a single dose of 15 g, both of which were well tolerated with no adverse effects (Moinard et al., 2008).

2.5 | L-Citrulline as a Therapeutic Agent

Over recent decades, growing scientific and clinical interest has focused on the therapeutic potential of L-citrulline, mainly due to its role as a precursor to L-arginine through its ability to enhance NO bioavailability. Cells that synthesize NO from L-arginine can also utilize circulating L-citrulline, thereby indirectly supporting NO-dependent physiological processes such as vascular relaxation and the modulation of vascular tone (Vanhoutte et al., 2016). These attributes seem to position L-citrulline as a potential natural agent for treating and preventing pathophysiological conditions such as cardiovascular diseases, diabetes, erectile dysfunction, and cancer (Bahri et al., 2013; Romero et al., 2006).

2.5.1 | L-Citrulline and Cardiovascular Diseases

Endothelial dysfunction – impaired endothelium-dependent vasodilation resulting from reduced NO bioavailability – represents a fundamental pathological mechanism underlying the increased morbidity and mortality in cardiovascular diseases (Romero et al., 2006; Sharifi-Rad et al., 2020; Versari et al., 2009). A substantial body of

evidence links endothelial NO deficiency to the development and progression of a number of cardiovascular conditions, including arterial hypertension, atherosclerosis, and diabetic vascular complications (Khalaf et al., 2019; Le Roux-Mallouf et al., 2019; Romero et al., 2006). In vascular endothelial cells, NO is synthesized by eNOS, where it plays a fundamental role in regulating vascular tone, maintaining blood pressure homeostasis, and exerting vasoprotective, anti-inflammatory, and anti-atherosclerotic effects. Therefore, enhancing NO production may represent a promising therapeutic strategy to slow or mitigate the progression of these cardiovascular disorders.

Growing evidence supports the efficacy of L-citrulline supplementation in modulating blood pressure (Figueroa et al., 2017). For instance, Wong et al. (2016) reported significant reductions in both brachial systolic and diastolic blood pressure (~7/3 mmHg), as well as aortic pressure (~9/3 mmHg) following eight weeks of L-citrulline supplementation (6 g/day) in obese, prehypertensive, and hypertensive women. In a similar manner, Orozco-Gutierrez et al. (2010) observed comparable reductions in patients with coronary artery disease after eight weeks of L-citrulline supplementation. Although these antihypertensive effects are consistently observed in populations with elevated cardiovascular risk, findings in normotensive individuals are still uncertain.

Alsop and Hauton (2016) reported reductions of approximately 7/11 mmHg in brachial systolic and diastolic pressure in healthy adults after seven days of L-citrulline intake (3 g/day). In contrast, Ochiai et al. (2012) found no significant blood pressure changes following a similar supplementation regimen (5.8 g/day for seven days) in middle-aged normotensive men. These mixed outcomes, confirmed by other studies (Figueroa et al., 2013; Figueroa et al., 2017), suggest that the primary utility of L-citrulline lies in therapeutic applications for individuals with impaired vascular function rather than as a preventive strategy in normotensive populations (Figueroa et al., 2017; Vanhoutte et al., 2016).

2.5.2 | L-Citrulline and Diabetes

As previously discussed, endothelial dysfunction is a premature and critical event in the pathogenesis of cardiovascular diseases such as hypertension and diabetes mellitus. In diabetes, vascular complications primarily stem from both structural and functional impairments of blood vessels, emphasizing the necessity of prophylactic strategies. A primary mechanism underlying diabetic vascular dysfunction involves the diminished availability of arginine for eNOS, leading to reduce NO production (Romero et al.,

2008). Hyperglycaemia can exacerbate this process by promoting the production of reactive oxygen species (ROS), also diminishing NO bioavailability, and contributing to the development of diabetic atherosclerosis. Under high-glucose conditions, NOS substrate supplementation has been shown to attenuate endothelial senescence, likely through modulation of the redox balance in endothelial cells (Hayashi et al., 2005). L-arginine serves as a substrate for both eNOS and arginase, and enhanced arginase activity in diabetes competes for L-arginine, depleting its availability, impairing NO synthesis, and worsening endothelial dysfunction (Romero et al., 2008). Experimental studies have demonstrated that administering L-citrulline, norvaline, and L-ornithine alleviates diabetes-related hypertension in animal models (El-Bassossy et al., 2012). Furthermore, L-citrulline alone or combined with L-arginine supplementation delayed high-glucose-induced endothelial senescence (Tsuboi et al., 2018). Similarly, four weeks of dietary supplementation with L-arginine (0.2%) or watermelon extract rich in L-citrulline (63%) improved endothelium-dependent relaxation in diabetic mouse models (Wu et al., 2007).

Clinical investigations support the vascular benefits of arginase inhibition. In diabetic patients with microvascular complications, pharmacological inhibition of arginase can

improve endothelial function in both resistance and conduit arteries. The upregulation of arginase reduces L-arginine availability for NO production. It is associated with increased ROS generation, a phenomenon partly driven by "eNOS uncoupling", where eNOS produces superoxide instead of NO under conditions of L-arginine deficiency (Förstermann et al., 2012; Kövamees et al., 2016). Arginase inhibition may also offer vascular protection through direct mechanisms – preserving endothelial-dependent relaxation and enhancing NO production – and indirect mechanisms, including improving insulin sensitivity and reducing hypertriglyceridemia (El-Bassossy et al., 2013). Further, in vivo studies have shown that supplementation with L-arginine and L-citrulline enhances microcirculatory function by increasing L-arginine availability and NO production, particularly under conditions of L-arginine depletion induced by arginase activity (Wijnands et al., 2015). However, not all findings are consistent. For instance, You and colleagues (2014) reported that supplementation with L-arginine or L-citrulline over nine weeks failed to prevent or mitigate renal injury in a mouse model of type 1 diabetes, despite increases in kidney and plasma L-arginine concentrations. Lastly, a recent systematic review concluded that L-citrulline and watermelon extract positively affect glycaemic control and inflammation in diabetes mellitus. However,

evidence regarding their antioxidant and lipid-lowering effects remains limited (Azizi et al., 2020).

2.5.3 | L-Citrulline and Erectile Dysfunction

Erectile dysfunction is defined as the persistent inability to achieve or maintain an erection sufficient for sexual activity. NO plays a fundamental role in penile erection (Barassi et al., 2017) functioning both as a neurotransmitter in the penile non-adrenergic, non-cholinergic nerve fibers and as a vasodilator of the smooth muscle cells within the penile arteries, sinusoids, and trabeculae (Barassi et al., 2017). During sexual stimulation, NO is released into the smooth muscle tissue of the penis, where it activates soluble guanylate cyclase, catalysing the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The increase in cGMP activates protein kinases, leading to the phosphorylation of proteins and ion channels, which reduces intracellular calcium concentrations and promotes smooth muscle relaxation, allowing penile erection to occur. The process is further regulated by phosphodiesterase type 5 (PDE-5) enzymes, which hydrolyse cGMP into inactive GMP, resulting in the cessation of the erection (Cormio et al., 2011). PDE-5 inhibitors,

which enhance NO signalling by preventing cGMP degradation, are currently the most effective oral pharmacological treatments for male erectile dysfunction.

Emerging evidence suggests that alterations in L-arginine metabolism may contribute to erectile dysfunction pathophysiology. Lacchini and Tanus-Santos (2014) indicated elevated plasma levels of arginase type II in patients with vasculogenic erectile dysfunction, indicating that strategies to increase L-arginine availability are beneficial. In agreement with those findings, Shiota et al. (2013) demonstrated in a rat model of acute arteriogenic erectile dysfunction that oral L-citrulline supplementation improved erectile function, likely through increased plasma NO concentrations. Furthermore, Shirai et al. (2018) evaluated a combination therapy consisting of L-citrulline and trans-resveratrol with PDE-5 inhibitors in men with ED, finding that this adjunctive treatment improved erectile function compared to placebo, suggesting that L-citrulline supplementation may enhance the efficacy of standard PDE-5 inhibitor therapy.

2.5.4 | L-Citrulline and Cancer

Dietary antioxidants play a crucial role in preventing several human diseases, including cancer, atherosclerosis, stroke, neurodegenerative disorders, and diabetes, as oxidative stress and redox imbalance are strongly implicated in the pathogenesis of these conditions (Salehi et al., 2020). In this context, iNOS – one of the three isoforms of NOS – is a potent producer of NO and contributes to immune responses, including in certain types of cancer (Förstermann et al., 2012). However, the effects of NO on biological tumor are complex and depend on a plethora of factors, including local concentration, exposure duration, the tumor microenvironment, and the surrounding redox status (Burke et al., 2013).

At low concentrations (nanomolar range), NO promotes tumor growth by enhancing angiogenesis and inhibiting apoptosis (Fahey et al., 2015). In contrast, at superior concentrations (micromolar to millimolar range), NO can increase oxidative stress, leading to apoptotic cell death (Carpenter et al., 2012; Seabra et al., 2017). Increased arginase activity reduces NO bioavailability by competing for the standard substrate arginine, leading to uncoupled NOS activity and the generation of ROS and reactive

nitrogen species (RNS) instead of NO, thus promoting carcinogenesis (Pernow et al., 2013). Arginase can contribute to tumor progression through the polyamine pathway or reduce NO production (Timosenko et al., 2017). Myeloid-derived suppressor cells (MDSCs) express both arginase I and NOS, producing urea, L-ornithine, NO, and L-citrulline, respectively, to suppress tumor-specific T cell proliferation and cytotoxic activity (Bronte et al., 2005). Since L-arginine is essential for T cell function, its depletion can impair immune surveillance and enhance tumor progression (Munder, 2009). Therapeutic strategies targeting arginase activity have shown to be promising. For example, arginase inhibitors, either alone or in combination with other treatments, have effectively reduced and controlled tumor growth in murine models of Lewis lung carcinoma by improving immune responses (Timosenko, 2017). A small-molecule arginase inhibitor, CB-1158 (2-amino-6-borono-2-(1-(2,4-dichlorobenzyl) piperidin-4-yl) hexanoic acid), has entered phase I clinical trials for patients with advanced or metastatic solid tumors (Pham et al., 2018). However, the relationship between NO and cancer is complex. Overexpression of iNOS and elevated NO production have been associated with the initiation and progression of oral precancerous lesions and gastric carcinoma (Ambe et al., 2016; Rajendran et al., 2007; Yamaguchi et al., 2005). Interestingly, deletion of arginase II expression in a murine prostate cancer model

resulted in more aggressive tumor growth, suggesting that alternative polyamine biosynthesis pathways may become upregulated without arginase II (Mumenthaler et al., 2008).

NO donors have also been proposed as a valid strategy to enhance the delivery of chemotherapeutics to malignant brain tumors. The blood-brain tumor barrier (BTB) limits effective drug delivery. However, administration of L-arginine, or hydroxyurea, has been shown to increase BTB permeability by elevating NO, L-citrulline and cGMP levels within tumor tissues (Xyin et al., 2008). Elevated levels of cGMP can activate cGMP-dependent protein kinase, stimulating calcium-activated potassium (KCa) channels (Onoue et al., 1997), which are highly expressed in tumor microvessels. Activating these channels facilitates enhanced vesicular transport of therapeutic agents from the bloodstream into brain tumor tissues (Ningaraj et al., 2002).

2.6 | Proposed Ergogenic Mechanisms of L-Citrulline

Ergogenic aids are substances or strategies that enhance metabolic energy production, regulation, and efficiency to improve exercise performance (Suzuki et al., 2016). L-

citrulline has emerged as a promising nutritional supplement with potential ergogenic properties mediated by multiple physiological mechanisms. As indicated earlier, the supplementation with L-citrulline serves as a precursor for the renal synthesis of L-arginine, and oral administration of the substance can significantly increase plasma L-arginine concentrations, thus enhancing L-arginine bioavailability for NO production (Schwedhelm et al., 2008). Interestingly, L-citrulline supplementation can effectively restore NO synthesis under limited L-arginine availability (Kaore et al., 2013).

It is known that arginase converts L-arginine, synthesized from L-citrulline, into L-ornithine and urea within the urea cycle. Through this pathway, supplementation with L-citrulline contributes to improved ammonia homeostasis by facilitating the removal of excess ammonia (Breuillard et al., 2015). This is particularly crucial during exercise, as elevated ammonia levels are associated with muscle fatigue, partly due to their role in promoting anaerobic glycolysis and lactic acid accumulation during high-intensity efforts (Cutrufello et al., 2015).

2.6.1 | NO Production and Vasodilation

NO is pivotal in several physiological processes and is increasingly acknowledged as a potential ergogenic aid. As a critical regulator of vascular tone and mitochondrial function, NO affects fundamental physiological processes such as blood flow, oxygen delivery, and energy metabolism during exercise. Emerging research suggests that increasing NO bioavailability may positively impact muscle function and improve exercise performance (Petroczi et al., 2010). Enhancing NO availability may improve O_2 and nutrient delivery to active muscles by improving vascular dilation and blood flow, lowering the ATP cost of active muscle contraction, and supporting endurance performance (Bescos et al., 2012).

L-citrulline has received considerable scientific interest due to its potential to improve NO bioavailability and exercise performance. Recent investigations suggest that L-citrulline supplementation can enhance skeletal muscle metabolism and contractile efficiency (Bailey et al., 2015; Giannesini et al., 2009; Schwedhelm et al., 2008), promote greater tissue oxygenation and, ultimately, improve $\dot{V}O_2$ (Bendahan et al., 2002; Wijnands et al., 2012). This improved peripheral vasodilation in active muscles

(Gonzales et al., 2017), could ultimately represent enhanced fatigue resistance during exercise (Bendahan et al., 2002; Gonzalez et al., 2020).

A number of studies further support these findings, demonstrating that short-term L-citrulline supplementation can increase NO biomarkers (Wijnands et al., 2012) and NO production, improving vascular function. In a group of 10 healthy, young men, Bailey et al. (2015) found significant increases in plasma L-arginine, L-citrulline, and NO metabolites following seven days of supplementation with 6 g/day of L-citrulline. Supporting these findings, a subsequent study by the same group reported similar elevations in NO-related biomarkers levels after 16 days of supplementation with 3.4 g/day of L-citrulline provided as watermelon juice in eight recreationally active men (Bailey et al., 2016). Consistent results have also been documented in a study involving 20 trained male cyclists, who reported enhanced NO-related biomarkers following a 14-day supplementation of 1.47 g/day of L-citrulline via watermelon juice before completing a 75-km cycling time trial (Shanely et al., 2016).

Despite these promising findings, evidence linking elevated plasma L-arginine levels to enhanced flow-mediated vasodilation in healthy individuals remains inconclusive.

Cutrufello et al. (2015) observed no improvements in resting brachial artery flow-mediated vasodilation within 60–120 minutes after ingesting either 6 g of L-citrulline or 710 mL of watermelon juice. Near-infrared spectroscopy (NIRS) investigations, however, indicated enhanced muscle oxygenation after L-citrulline supplementation in recreationally active young men (Bailey et al., 2015; Bailey et al., 2016). These discrepancies may be attributed to variations in supplement dosage and timing, and training status of the participants (Gills et al., 2020; Gonzalez et al., 2020). It has been suggested that well-trained individuals typically exhibit higher baseline NO levels and may experience diminished responsiveness to NO-boosting interventions compared to less-trained counterparts (Bloomer et al., 2010).

2.6.2 | $\dot{V}O_2$ *kinetics*

A number of dietary supplements, including L-citrulline, beetroot juice, and inorganic nitrates, have been investigated for their potential to enhance $\dot{V}O_2$ kinetics – the rate at which $\dot{V}O_2$ adjusts to meet the energetic demands of exercise. Accelerated $\dot{V}O_2$ kinetics can facilitate a faster transition to oxidative metabolism, thereby reducing reliance on anaerobic pathways and improving exercise performance, particularly

during the onset of moderate - to high-intensity activity. In particular, L-citrulline has shown promising effects on muscle oxygenation and $\dot{V}O_2$ kinetics during exercise. The changes in such dynamic responses are likely to be linked to its role in enhancing NO bioavailability, which promotes vasodilation, improved blood flow, and more efficient oxygen delivery to active skeletal muscles. In a preliminary study, Bailey et al. (2015) demonstrated that a short-term L-citrulline supplementation enhanced NIRS-derived estimates of oxygen delivery to the muscle microvasculature, leading to a faster $\dot{V}O_2$ dynamics response during the initial phase of a severe-intensity cycling exercise. This suggests a greater reliance on oxidative metabolism early in during vigorous exercise. Suzuki et al. (2016) corroborated those findings and reported a significant association between plasma NO levels and the power output/$\dot{V}O_2$ ratio, an indicator of mechanical efficiency (Peyré-Tartaruga et al., 2018), following L-citrulline supplementation. This remarkable finding could indicate enhanced muscular efficiency at a given $\dot{V}O_2$. It should be noted, however, that not all studies have confirmed these preliminary effects. Indeed, a later study by Bailey et al. (2016) failed to detect changes in $\dot{V}O_2$ kinetics, possibly due to the use of a reduced L-citrulline dose. Moreover, existing evidence does not support a significant impact of L-citrulline on $\dot{V}O_2$ max. Further research is

warranted to focus on refining supplementation approaches in terms of dosage and timing to clarify the role of L-citrulline supplementation on exercise performance.

2.6.3 | Buffering of ammonia

The involvement of L-citrulline in the urea cycle has been proposed as a mechanism underlying its potential ergogenic effects, particularly through its capacity to buffer ammonia, enhance aerobic pyruvate utilization, and reduce lactate production through anaerobic pathways. This mechanism is theorized to delay lactate accumulation and the associated muscular fatigue during intense exercise. However, evidence supporting this pathway as the primary responsible for any benefits of citrulline supplementation remains inconclusive. While some studies have shown reduced post-exercise blood lactate concentrations following L-citrulline supplementation (Farney et al., 2019; Wax et al., 2016), these changes have not consistently translated into improved exercise performance. For example, Martínez-Sánchez et al. (2017a) reported lower blood lactate concentrations after ingesting L-citrulline-enriched watermelon juice before a half marathon, even without any improvement in race time. Interpreting the lactate responses during exercise, however, is complex, since enhanced performance

– indicated by either increased exercise intensity or duration – could elevate lactate production and potentially offset any lactate-lowering effects of supplementation. Furthermore, because high-intensity strength and power exercises rely more heavily on anaerobic glycolysis than endurance activities, the benefits of ammonia buffering might be more pronounced in these contexts.

2.7 | Effects of L-Citrulline Supplementation on Endurance Performance

Based on the proposed ergogenic mechanisms, several studies have proposed that L-citrulline supplementation may enhance exercise performance by improving skeletal muscle oxygen transport and utilization and/or reducing lactic acid production during exercise (Bailey et al., 2015; Martínez-Sánchez et al., 2017a; Martínez-Sánchez et al., 2017b). As such, L-citrulline represents a potentially valuable, non-pharmacological strategy to support athletic performance. As illustrated in **Table 1**, the current body of evidence presents a mixed yet promising perspective on the ergogenic potential of L-citrulline supplementation. A substantial number of studies indicates improvements in exercise efficiency and tolerance and related physiological responses, particularly in high-intensity and endurance-based exercise contexts. In fact, Bailey et al. (2015)

demonstrated that a 7-day supplementation with 6 g/day of L-citrulline significantly improved pulmonary $\dot{V}O_2$ kinetics, peak power output (+9%), and exercise tolerance (+12%) during severe-intensity cycling in recreationally active young men. These enhancements occurred without concomitant changes in muscle oxygenation or lactate levels, suggesting that the ergogenic benefits of L-citrulline may be mediated through improved efficiency on oxygen utilisation rather than altered metabolic by-products. A study by the same group (Bailey et al., 2016), using 3.4 g/day of L-citrulline delivered via watermelon juice for 16 days, revealed increased plasma L-citrulline, L-arginine, and plasma nitrite. While no significant improvements in exercise tolerance were observed, the supplementation led to greater total work completed during the first 10 seconds of maximal effort, thereby suggesting potential benefits for explosive or short-duration tasks that relies upon anaerobic metabolism. Complementary findings from Stanelle et al. (2020) revealed a 5.2% reduction in 40-km time trial completion time and a 5.4% increase in average power output in trained male cyclists after 7-day supplementation with 6 g/day of L-citrulline. Similarly, Suzuki et al. (2016) found that 7 days of 2.4 g/day L-citrulline supplementation reduced time trial completion time by 1.5% and increased power output by 2%, while improving perceived measures of fatigue and concentration. Collectively, these findings suggest that moderate doses of

L-citrulline confer tangible ergogenicity, especially in endurance-based disciplines. Martínez-Sánchez et al. (2017) offers further support to the applicability of L-citrulline supplementation. Those authors showed that L-citrulline-enriched watermelon juice significantly improved post-exercise recovery in amateur runners by reducing muscle soreness and increasing post-race plasma L-arginine levels. This aligns with earlier evidence suggesting that L-citrulline enhances NO production, a mechanism thought to promote vasodilation and nutrient delivery to muscles during recovery.

It should be noted, however, that not all studies support the plausible ergogenic effects of L-citrulline. For example, Cutrufello et al. (2014) and Gills et al. (2021) found no improvements in exercise performance or $\dot{V}O_2$ max following supplementation with either L-citrulline or citrulline malate, respectively. In a preliminary study, Hickner et al. (2005) found that oral L-citrulline supplementation reduced time to exhaustion and increased perceived exertion, potentially indicating a negative impact under certain conditions. These discrepancies may arise from variations in supplement dosage, timing, delivery (e.g., pure citrulline *vs.* citrulline malate *vs.* watermelon juice), or even training status of the participants. As previously mentioned, well-trained individuals tend to exhibit a blunted response due to already elevated baseline NO levels. Although

numerous studies have demonstrated increases in plasma L-arginine and L-citrulline concentrations following L-citrulline supplementation, the magnitude of such changes that could be translated to improvements in health status or exercise performance remains elusive.

In summary, improved exercise efficiency and/or tolerance have not been consistently demonstrated following L-citrulline supplementation in healthy adults. In particular, acute L-citrulline administration have generally failed to enhance time-to-exhaustion or time-trial outcomes. The reviewed studies, however, employed a wide range of supplementation protocols, with dosages varying from as low as 2.4 g/day to 8 g/day, administered in different forms, including pure L-citrulline, citrulline malate, and watermelon juice (for a review, see **Table 2**). Such heterogeneity in dosage, timing, and form likely contributes to the inconsistent findings across studies. Whether a more sustained or greater dosing might produce more pronounced ergogenic effects is still uncertain, particularly in individuals with reduced baseline NO bioavailability or under prolonged, high-intensity exercise conditions. Future studies are therefore needed to investigate how differences in L-citrulline regimen could affect the ergogenicity of the supplement.

Table 1. Exercise protocols employed in L-citrulline supplementation studies.

Reference (First author, year)	**Subjects, *n*** age in years (mean ± SD)	**Training Status**	**Exercise Modality**	**Performance Task**	**Exercise Protocol**	**Main Findings**
Bailey et al (2015)	10 ♂ 19 ± 1	Recreationally active	CE	TTE	TTE at severe intensity	↑ Time to exhaustion
Bailey et al (2016)	8 ♂ 22 ± 2	Recreationally active	CE	TTE	TTE at moderate and severe intensity	↔ Time to exhaustion
Cutrufello et al (2014)	11 ♂| 11♀ 20.6 ± 1.2 | 21.0 ± 1.3	Recreationally trained	TR	TTE	Graded exercise test (Modified Bruce protocol)	↔ Time to exhaustion
Gills et al (2021)	28 ♂ 20.9 ± 2.8	Recreationally active	CE	TTE	TTE at 90% of $\dot{V}O_2$peak	↔ Time to exhaustion
Hickner et al. (2005)	17 ♀and♂ 18 - 40	Physical active	TR	TTE	Graded Exercise Test	↓ Time to exhaustion

Martínez-Sánchez et al. (2017)	21 ♂ 35.3 ± 11.4	Amateur runners	Running	TT	Half marathon time trial	↔ Time-to-completion
Shanely et al. (2016)	20 ♂ 48.5 ± 2.3 years	Trained cyclists	CE	TT	75-km time trial	↔ Time-to-completion
Stanelle et al (2019)	9 ♂ 24 ± 3	Trained triathletes or cyclists	CE	TT	40-km time trial	↔ Time-to-completion
Suzuki et al (2016)	22 ♂ 29 ± 8.4	Trained	CE	TT	4-km cycling time trial	↓ Time-to-completion

Note. Exercise modality: TR = treadmill running; CE = cycle ergometer; Performance task: TT time trial, TTE time to exhaustion; ↔: no significant difference ($P > .05$) compared to placebo, ↑: significantly greater ($P < .05$) than placebo, ↓: significantly less ($P < .05$) than placebo.

Table 2. Supplement protocols employed in L-citrulline supplementation studies.

Reference (First author)	**Placebo Substance**	**Supplement Intervention**				**Washout Period**
		Type of Supplement	**L-citrulline dose** (g per day)	**Protocol Administration** time before trial (min)	**Days of Supplementation**	
Bailey et al (2015)	Maltodextrin Beverage	L-citrulline powder + maltodextrin mixed with 500 ml of water	6 g	90	7 days	7-10 days
Bailey et al (2016)	Apple juice	Watermelon juice concentrate 300 mL	~ 3.4 g	75	16 days	7-10 days
Cutrufello et al (2014)	Sucrose Beverage	710 ml watermelon juice L-citrulline powder	1 g 6 g	60 or 120	Single dose	7 days
Gills et al (2021)	Dextrose beverage	Citrulline-malate powder + 12 g dextrose	8 g	60	Single dose	7 days

Hickner et al (2005)	NM	L-citrulline	3 g	180	Single dose	1 day
			9 g	24 hours	2 days	2 days
Martínez-Sánchez et al. (2017)	Beverage without L-citrulline	500 ml Watermelon juice enriched with L-citrulline	3.45 g	120	Single dose	NM
Shanely et al. (2016)	6% Carbohydrate beverage	980 ml Watermelon juice	1.47 g	During	14 days	2 weeks
Stanelle et al (2019)	Maltodextrin capsules	L-citrulline capsules	6 g	120	7 days	7 days
Suzuki et al (2019)	Cornstarch capsules	L-citrulline capsules	2.4 g	120	8 days	3 weeks

2.8 | Aims and Hypotheses

The primary aim of the present work was to determine the effects of different doses of L-citrulline supplementation on exercise performance in healthy adults. In particular, the specific aims and hypothesis of this study included:

Study 1. To determine the effects of different doses of orally ingested L-citrulline on exercise tolerance and associated physiological responses to ramp-incremental test in healthy adults. It was hypothesized that, relative to energy-matched placebo, different doses of L-citrulline supplementation would increase plasma L-arginine concentration and availability, alter physiological responses, and enhance exercise capacity in a dose-dependent manner.

Study 2. To determine the effects of different doses of orally ingested L-citrulline on exercise tolerance and associated physiological responses to moderate- and severe-intensity constant power output exercise tests in healthy adults. We hypothesized that, relative to energy-matched placebo, different doses of L-citrulline supplementation

would dose-dependently alter physiological responses to both moderate- and severe-intensity exercise or improve exercise tolerance in healthy adults.

2.9 | Scientific Rationale

In recent years, a number of studies have established that L-citrulline supplementation can increase the activity of NOS enzymes (Wijnands et al., 2012) and augment NO biomarkers (Schwedhelm et al., 2008). There is also mounting evidence that short-term supplementation with L-citrulline can improve skeletal muscle metabolism and contractile efficiency (Bailey et al., 2015; Bendahan et al., 2002; Giannesini et al., 2009) and enhance fatigue resistance (Bailey et al., 2015). Accordingly, exogenous L-citrulline administration may serve as a valid alternative to increase the amount of L-arginine provided to NOS and to improve exercise efficiency and exercise tolerance in healthy adults. However, as the majority of studies to date have employed L-citrulline doses of 6 g or less, it is unclear whether greater doses may elicit further improvements in exercise tolerance and associated physiological responses. Establishing the optimal dose of L-citrulline for enhancing exercise performance is essential, particularly in light of the growing interest in its use within the athletic performance context.

Whether the ergogenicity of L-citrulline is exacerbated or nullified according to the exercise protocol is also uncertain. Early L-citrulline studies used incremental tests, which, while valuable scientifically, are tests of exercise tolerance rather than exercise performance (Jeukendrup et al., 1996). Other L-citrulline studies, in turn, have adopted constant power-to-fatigue tests to evaluate volitional exhaustion, which have been extensively used in exercise physiology to evaluate exercise tolerance (Koppo et al., 2009; Vanhatalo et al., 2010). Constant power-to-fatigue tests are similarly criticized for lacking ecological validity and having poor test-retest reliability (Jeukendrup et al., 1996). When pre-exercise conditions are adequately standardized and familiarized, however, such tests may be more consistent in performance than previously thought (Amman et al., 2008).

2.10 | Relevance and Contribution

Few studies have examined the dose-response effects of pure L-citrulline on tolerance and associated physiological responses during exercise in young adults. Moreover, the extent to which specific exercise protocols influence this relationship remains unclear.

The present thesis aims to address this gap in the literature by providing data on the ergogenicity of L-citrulline supplementation. From a practical perspective, the current findings may offer valuable insights for researchers, sport nutritionists, and sport and exercise professionals on the potential efficacy and optimal application of L-citrulline as a non-pharmacological intervention to enhance exercise performance in young, healthy individuals.

3 | General Methods

3.1 | Participants

In ***Study 1***, nine young, healthy men (mean ± SD: age 26 ± 5 yr, body mass 80.9 ± 7.8 kg, height 178 ± 6.2 cm) were recruited and volunteered to participate. In ***Study 2***, ten participants with comparable characteristics (mean ± SD: age 23 ± 4 yr, body mass 75.5 ± 10.9 kg, height 174 ± 5.5 cm) were recruited. All participants were moderately trained, based on their peak O_2 uptake ($\dot{V}O_2$ peak) values (see *Results* for details), and reported engaging in regular physical activity more than four times per week. They were familiar with the laboratory testing procedures and accustomed to high-intensity cycling exercise, thereby minimizing any possible learning effects during the study. Written informed consent was obtained from all participants prior to the start of the study, after the experimental procedures, associated risks, and benefits of participation had been explained. The present study was conducted in accordance with the latest version of the Declaration of Helsinki.

3.1.1 | Inclusion Criteria

Participants were eligible for inclusion if they self-declared to be in good health and reported no history of medical conditions that could contraindicate their participation in the study. Eligibility also required that participants were non-smokers and did not use any medications or dietary supplements known to affect somatic and/or cognitive functions.

3.1.2 | General Instructions and Standardized Laboratory Conditions

All participants were instructed to arrive at the laboratory rested and well-hydrated, having fasted for at least three hours. They were also required to abstain from alcohol, caffeine, chewing gum, and the use of antibacterial mouthwash for at least 24 hours prior to each experimental session, and to avoid strenuous exercise during the same period. Participants were encouraged to maintain their usual daily routines, including habitual food and fluid intake, throughout the study. However, to minimise potential confounding factors, they were provided with a list of foods rich in nitrate, L-arginine,

and L-citrulline, and were instructed to avoid these items for at least 24 hours prior to each trial (see *Nutrient Intake* for details). All experimental trials were separated by a minimum of 72 hours and conducted at the same time of the day (±2 hours) in an environmentally controlled room (temperature: 21-23ºC; humidity: 50-60%).

3.1.3 | Nutrient Intake

All participants were instructed to record their food and fluid intake for 24 hours before the first experimental trial and were asked to replicate the same diet prior to subsequent trials. Under the supervision of a nutritionist, participants were advised to adhere to a diet rich in carbohydrates (> 4 $g \cdot kg^{-1}$ body weight), which, in combination with rest or light training, would ensure adequate glycogen stores at the start of each experimental trial (Pfeiffer et al., 2010). Dietary intake was assessed using 24-hour dietary recalls on the days before each experimental trials, and carbohydrate intake for all participants was confirmed to exceed 4 $g \cdot kg^{-1}$ body weight. Participants were instructed to report any adverse events related to supplementation with *Cit* or *Pla* in their dietary records. Of note, participants fasted overnight before each trial and consumed a standardised

breakfast rich in carbohydrates (324 kcal; carbohydrate 87%, protein 7%, and fat 6%) following ingestion of *Cit* or *Pla*.

3.2 | Experimental Design

All volunteers were required to attend the laboratory on seven (***Study 1***) or eight (***Study 2***) separate occasions over a 3- to 4-wk period, which included one or two preliminary visits, respectively, followed by six experimental visits (see **Fig. 1** and **2**). During the first visit, participants were further familiarised with the testing procedures utilised in the study (***Studies 1*** and ***2***) and completed a ramp-incremental cycling test (***Study 2***) to determine $\dot{V}O_2$ peak (Poole and Jones, 2017), peak power output (PO peak; Zhang et al., 2021), gas exchange threshold (GET; the respiratory surrogate of lactate threshold, LT; Beaver et al., 1986), and respiratory compensation point (RCP; the respiratory surrogate of maximal metabolic steady state, MMSS; Whipp et al., 1989). After these preliminary visits, participants returned to the laboratory for additional visits, during which they were randomly assigned, in a double-blind, counterbalanced manner, to ingest either 6 g (*Cit6*) or 12 g (*Cit12*) of L-citrulline, or energy-matched

placebo (*Pla*), ~90 minutes prior to performing either a ramp-incremental cycling test (***Study 1***) or a constant-power output cycling test (***Study 2***) (see *Supplementation Procedures* for further details).

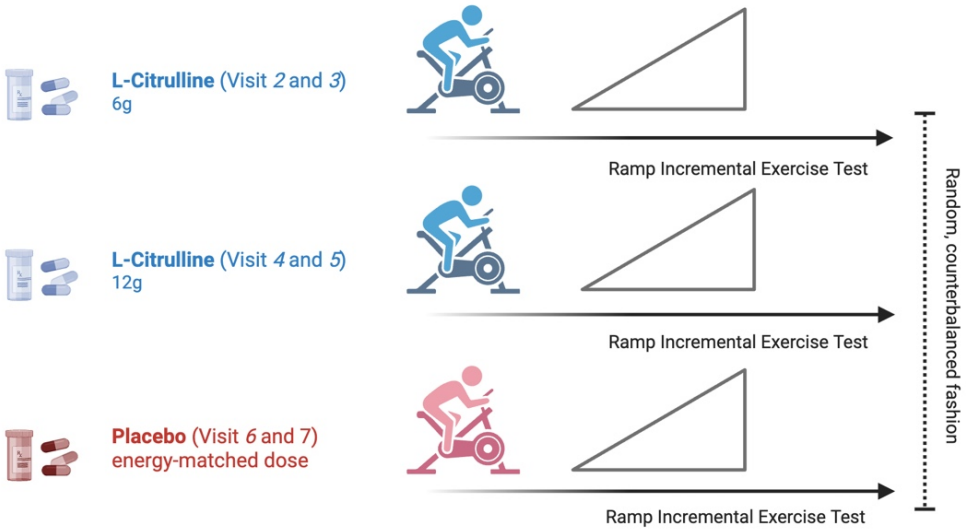


Fig. 1. Schematic illustration of the experimental design for ***Study 1***. Following one preliminary visit, participants performed six ramp-incremental cycling tests to task to determine PO peak, $\dot{V}O_2$ peak, GET, and RCP after ingestion of either 6 g (*Cit6*) or 12 g of pure L-citrulline (*Cit12*), or energy-matched placebo (*Pla*) gelatinous capsules. Figure created with BioRender.com and published with permission.

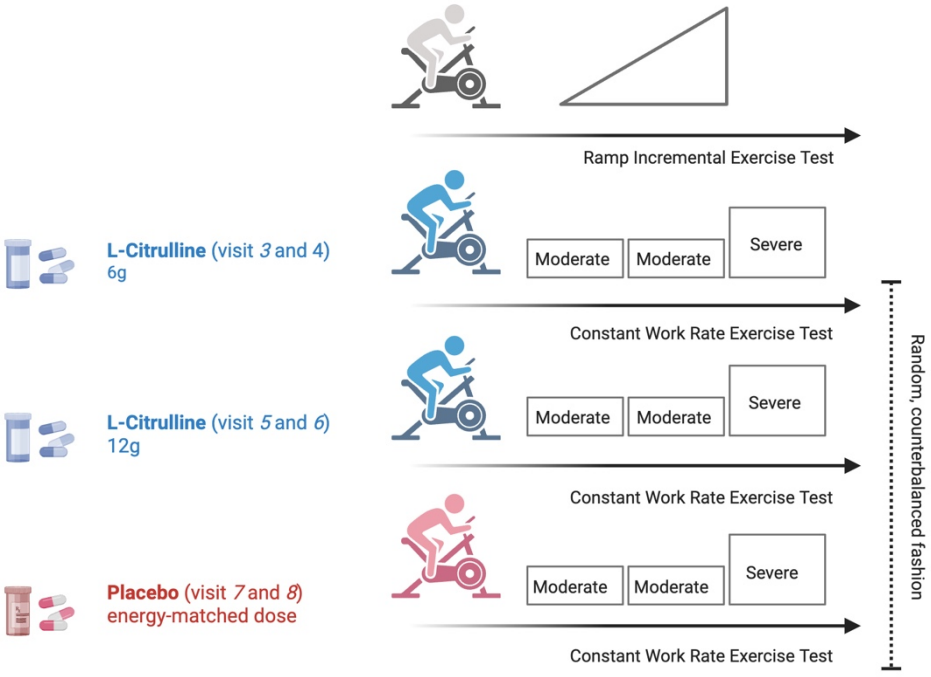


Fig. 2. Schematic illustration of the experimental design for ***Study 2***. Following a preliminary visit, participants completed a ramp-incremental cycling test to task failure to assess PO peak, $\dot{V}O_2$ peak, GET, and RCP, and six constant-power output cycling trials after ingestion of either 6 g (*Cit6*) or 12 g of pure L-citrulline (*Cit12*), or energy-matched placebo (*Pla*) gelatinous capsules. Each constant-power output cycling trial comprised two moderate-intensity "step" tests and one severe-intensity exercise bout. Figure created with BioRender.com and published with permission.

3.3 | Experimental tests

3.3.1 | Preliminary Visit

An anthropometrical assessment was conducted during the initial laboratory visit for all participants. Height was measured to the nearest 0.1 cm using a Seca stadiometer (model 264, Seca Ltd., Birmingham, UK), and body weight was recorded to the nearest 0.05 kg using a portable, digital electronic scale (model 813, Seca Ltd., Birmingham, UK). Subsequently, all participants underwent further familiarisation with the testing procedures used in the study to minimize the risk of order effect on exercise tolerance results attributable to learning effects. In particular, participants completed a constant-power output cycling trial within the severe-intensity domain to task failure.

3.3.2 | Experimental Visits

Upon arrival at the laboratory, participants rested for 10 minutes in a seated position in an isolated room. While seated, brachial artery blood pressure (BP) was measured using an automated sphygmomanometer (Dinamap Pro, GE Medical Systems, Tampa,

FL). Four measurements were recorded, and the mean of the final three was used for later analysis of systolic blood pressure (SBP) and diastolic blood pressure (DBP). Mean arterial pressure (MAP) was calculated according to the following formula:

MAP = (1/3 × SBP) + (2/3 × DBP) (***Studies 1*** and ***2***)

A venous blood sample was then drawn into lithium-heparin tubes and immediately stored for subsequent analysis of plasma L-arginine ([Arg]), L-ornithine ([Orn]), and L-lysine ([Lys]) concentrations (see *Measurement* for further details).

Following these initial procedures, participants were randomly assigned, in a double-blind, counterbalanced manner, to receive either L-citrulline (*Cit6* or *Cit12)* or placebo (*Pla*) ~90 minutes prior to performing either a ramp-incremental cycling test (***Study 1***) or a constant-power output cycling test (***Study 2***) (see *Supplementation Procedures* for further details). After ingestion of *Cit* or *Pla*, participants were given a standardised breakfast and then rested within an isolated room. During this period, participants were required to fast, although water intake was permitted *ad libitum*. Then, ~90 minutes

following ingestion of the supplement, a BP measurement was replicated, and a venous blood sample was drawn, after which the exercise test commenced (**Fig. 3 and 4**).

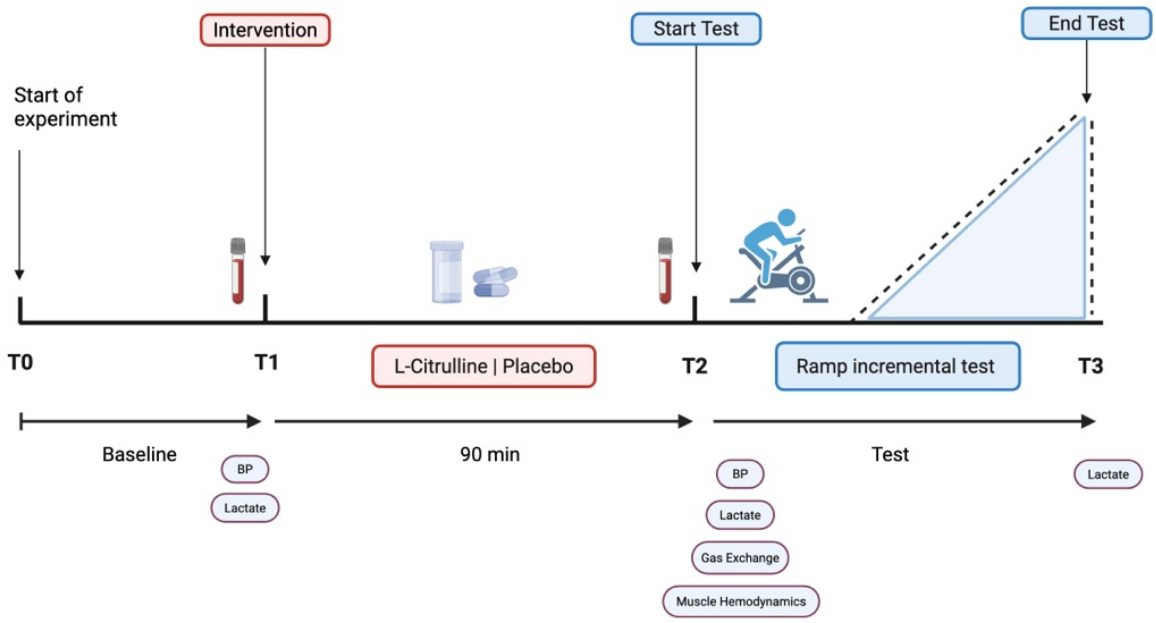


Fig. 3. Schematic illustration of the experimental procedures (***Study 1***). Figure created with BioRender.com and published with permission.

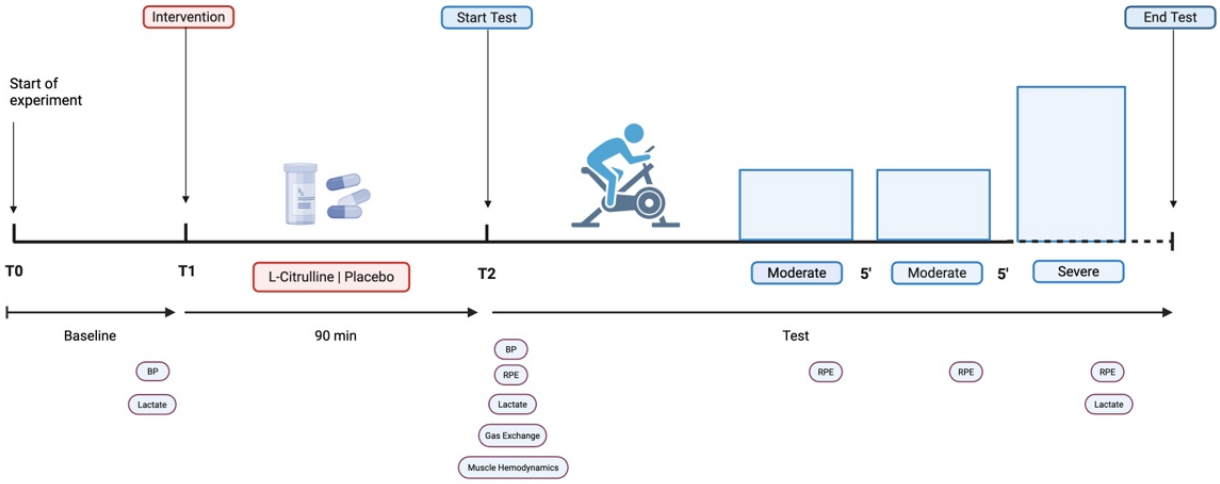


Fig. 4. Schematic illustration of the experimental procedures (***Study 2***). Figure created with BioRender.com and published with permission.

*3.3.2.1 | Ramp-Incremental Cycling Test (for **Studies 1** and 2)*

At 90 minutes after ingestion of *Cit* or *Pla*, participants performed a ramp-incremental test to task failure on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The test began with 3 minutes of baseline cycling at 20

W, followed by 2 minutes at 40 W, after which the work rate was increased linearly at a rate of 20 W·min^{-1} (or 1 W every 3 seconds). The baseline value of 40 W was selected to ensure that any potential changes in mechanical efficiency at low work rates would not interfere with the progressive and linear increase in $\dot{V}O_2$ (Boone et al., 2008). The test was terminated when participants voluntarily disengaged or failed to maintain the required cadence despite verbal encouragement (i.e., a drop of > 10 rpm for more than 5 seconds) (Vanhatalo et al., 2010). Cadence was self-selected during familiarisation within a range of 75–95 rpm and was recorded along with saddle and handlebar height configuration, which were then replicated in all subsequent tests. The instantaneous pedal rate was continuously displayed on the cycle ergometer to ensure participants remained informed of their cadence during the test. However, investigators covered all timing devices, and no feedback was provided regarding elapsed time or work rate during the test (**Fig. 3**).

3.3.2.2 | Constant-Power Output Cycling Test (for ***Study 2***)

Following completion of the ramp-incremental cycling test to task failure, participants performed constant-power output cycling tests on separate occasions (Poole and Jones,

2012). At ~90 minutes after ingestion of *Cit* or *Pla*, participants completed a constant-power output cycling tests consisting of two moderate-intensity step tests followed by one severe-intensity step test. The moderate-intensity step tests were designed to assess pulmonary $\dot{V}O_2$ kinetics and cycling economy in absence of the $\dot{V}O_2$ slow component, whereas the severe-intensity test was intended to evaluate $\dot{V}O_2$ kinetics in the presence of a slow component, during which $\dot{V}O_2$ peak is attained and the tolerable duration of exercise is less than 20 minutes (Poole et al., 1988).

Each step test began with 3 minutes of baseline cycling at 20 W, followed by an abrupt transition to the target work rate. For the moderate-intensity step tests, the target work rate corresponded to 80% of the work rate at the GET. For the severe-intensity step test, the target was set at 75% of the difference between GET and PO peak (i.e., 75%Δ), with adjustment made for the mean response time (MRT) of $\dot{V}O_2$ observed during the ramp-incremental test (see *Data Analysis* for further details). Each moderate-intensity step test lasted 6 minutes and was separated by a 6-minute passive recovery period. The severe-intensity step test lasted 6 minutes and continued until task failure, which was used as an indicator of exercise tolerance (Bailey et al., 2015). Time-to-task failure was defined as the point at which participants either voluntarily disengaged from the

task or failed to maintain the cadence within the target range, defined as a reduction of > 15 rpm for more than 10 seconds (Iannetta et al., 2022), despite considerable verbal encouragement. Prior to each test, participants cycled at their self-selected cadence (±5 rpm) within a range of 75–95 rpm and were reminded of the task failure criteria. Cadence, but not work rate and time elapsed, were displayed to the participants throughout the tests. Participants were not informed of the work rates and the time-to-task failure in any of the tests until the entire study had been completed (**Fig. 4**).

3.4 | Supplementation Procedures

Participants were randomly allocated in a balanced, randomised crossover design to receive either 6 g (*Cit6*) or 12 g (*Cit12*) of pure L-citrulline (Arboretum, Brazil) or an energy-matched placebo (*Pla*) of similar appearance, odour, and texture. *Cit6, Cit12,* and *Pla* were administered with 0.5 L of water ~90 minutes prior to performing either a ramp-incremental cycling test (***Study 1***) or a constant-power output cycling test (***Study 2***). This supplementation protocol was based on the findings of Moinard et al. (2007), who demonstrated that oral ingestion of pure L-citrulline in doses ranging from

2 to 15 g resulted in dose-dependent increases in plasma concentrations of L-arginine ([Arg]), with peak levels observed between ~70- and ~140-minutes post-ingestion. A washout period of 72 hours was maintained between each supplement condition (*Pla*, *Cit6*, and *Cit12*), which is considered sufficient given the reported half-life of ~6.4 hours for a single 15 g oral dose of L-citrulline (Moinard et al., 2007).

3.5 | Measurement

3.5.1 | Plasma Amino Acid Concentrations

Venous blood samples were collected into lithium-heparin tubes and stored on ice prior to processing. Samples were subsequently centrifuged at 4000 rpm for 10 minutes at 4 °C. The resulting plasma was extracted and immediately stored at −80 °C for further analysis of plasma concentrations of L-arginine ([Arg]), L-ornithine ([Orn]), and L-lysine ([Lys]) using high-performance liquid chromatography (HPLC) (Van Eijk et al., 1993).

3.5.2 | Pulmonary Gas Exchange and Ventilation

During the ramp-incremental cycling test (***Studies 1*** and ***2***) and the constant-power output cycling trials (***Study 2***), pulmonary gas exchange and ventilation were measured breath-by-breath using an automated open-circuit gas analysis system ($K4B^2$, Cosmed, Rome, Italy) (Duffield et al., 2004). A turbine digital transducer measured inspired and expired airflow, while an electrochemical cell oxygen (O_2) analyser and an infrared carbon dioxide (CO_2) analyser simultaneously measured expired gases. Participants wore a facemask, which was held in place by a head cap and fastened to the turbine. The inspired and expired gas volume and gas concentration signals were continuously sampled via a capillary line connected to the turbine. Both O_2 and CO_2 gas analysers were calibrated before each test with a gas mixture of known concentration (16%O_2, 5%CO_2, balance N_2), and the turbine volume transducer was calibrated using a 3-L syringe (Hans Rudolph, Kansas City), according to the manufacturer's instructions. HR was recorded by means of the $K4B^2$, system from a chest belt transmission (Polar Electro, Kempele, Finland).

3.5.3 | NIRS-Derived Indices of Muscle Oxygenation

Muscle oxygenation changes in the *vastus lateralis* of the right leg were assessed using near-infrared spectroscopy (NIRS) (Pilotto et al., 2022). The reliability of NIRS-derived tissue oxygenation indices has been reported as very high, with intraclass correlation coefficients indicating excellent consistency across repeated measurements on the same individual across different days (Subudhi et al., 2007). Furthermore, NIRS measurements in skeletal muscle have shown a strong correlation with local venous O_2 saturation (Wilson et al., 1989). A continuous-wave NIR photometer (PortaMon, Artinis Medical Systems, Elst, The Netherlands) was used for data acquisition. This device emits light at wavelengths of 760 and 850 nm through three channels, enabling tissue penetration depths of 15, 17.5, and 20 mm (Van Beekvelt et al., 2001). This device provides measurements of concentration changes in oxygenated haemoglobin (Hb) and myoglobin (Mb) {Δ[oxy(Hb+Mb)]} and deoxygenated Hb and Mb {Δ[deoxy (Hb+Mb)]} relative to a baseline value arbitrarily set to zero and expressed in arbitrary units (a.u.). The sum of these two parameters {Δ[oxy(Hb+Mb) + deoxy(Hb+Mb)]} reflects changes in total haemoglobin volume within the monitored muscle region of

interest (Shiga et al., 1997). In accordance with previous literature (e.g., Grassi et al., 2003; Ferreira et al., 2005), Δ[deoxy (Hb+Mb)] was considered a valid surrogate for fractional O_2 extraction in skeletal muscle, due to its relative insensitivity to blood volume fluctuations compared to Δ(oxyHb+Mb).

The NIRS apparatus was securely affixed to the skin overlying the distal third of the *vastus lateralis* muscle (~10–12 cm proximal to the patella), aligned parallel to the longitudinal axis of the thigh. Apparatus placement was standardised using a surgical skin marker to ensure reproducibility across experimental trials. To eliminate ambient light interference, the apparatus was covered with black opaque tape, then enclosed in a sealed transparent polyethylene bag for waterproofing. It was then firmly secured to the limb to prevent displacement and to minimise water ingress between the skin and the sensor (Van Beekvelt et al., 2001).

Prior to the first experimental trial, skinfold thickness at the site of application of the NIRS apparatus was assessed using a Harpenden skinfold caliper (British Indicators, Burgess Hill, UK). The combined thickness of adipose tissue and skin was calculated and confirmed to be less than half the interoptode distance, that is, the distance between the light-emitting optode and the detector (Ferrari et al., 2004), ensuring that NIRS

signals primarily originated from the underlying muscle tissue. The NIRS system was connected to a personal computer by Bluetooth for data acquisition (10 Hz), analog-to-digital conversion, and subsequent analysis.

3.5.4 | Blood Lactate Concentration

Capillary blood samples (20 μL) were collected from a fingertip using heparinised capillary tubes and immediately analysed for blood lactate concentration ($[La^{-}]$) using an automated lactate analyser (YSI1500, Yellow Springs Instruments, Yellow Springs, USA). In ***Study 1***, samples were obtained 30 seconds prior to ingestion of either *Cit* or *Pla*, 20 seconds before the onset of the incremental exercise test, and immediately upon exhaustion. In ***Study 2***, samples were collected 30 seconds prior to ingestion of either *Cit* or *Pla*, 20 seconds before the onset of the first moderate-intensity step test, and within the final 20 seconds of both the first moderate-intensity step exercise and subsequent severe-intensity step exercise.

3.5.5 | Rate of Perceived Exertion

Rating of perceived exertion (RPE) for the overall body was assessed using a 15-point category scale (Borg, 1982). Participants were previously anchored to the scale using memory-anchoring procedures (Noble and Robertson, 1996). Standard definitions of perceived exertion, also known as perception of effort, along with a set of instructions on using the scale, were read to the participants before each test (DaSilva et al., 2011). All participants were asked to rate their conscious sensation of how hard, heavy, and strenuous the physical task was (Marcora et al., 2010). RPE was assessed in the final 20 seconds of both the first moderate-intensity step exercise and subsequent severe-intensity step exercise (***Study 2***).

3.6 | Data Analysis

3.6.1 | Plasma Amino Acid Concentrations

Plasma amino acid concentrations were determined as described by Wijnands et al. (2012). The arginine availability index (AAI) was subsequently calculated using the

equation [Arg] / ([Orn] + [Lys]) (Wu, 1995; Wu, 1998). This index accounts for the plasma concentrations of [Arg], [Orn], and [Lys], which are competitively transported via the shared y^+ cationic amino acid transporter system (for review, Closs et al., 1997)

3.6.2 | Pulmonary Gas Exchange and Ventilation

Breath-by-breath gas exchange and ventilation data from the ramp-incremental test (***Studies 1*** and *2*) were first examined to identify and remove errant breaths, defined as those positioned more than four standard deviations from the local mean. The breath-by-breath data were then linearly interpolated on a second-by-second basis and averaged into 10-seconds bins (Origin Pro, Origin Lab, Northampton, MA) (Lamarra et al., 1987; Iannetta et al., 2022). The profiles of ventilation ($\dot{V}_E$), carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (RER) and end-tidal partial pressure of O_2 (PO_2) and CO_2 (PCO_2), along with the ventilatory equivalents for both O_2 ($\dot{V}_E/\dot{V}O_2$) and CO_2 ($\dot{V}_E/\dot{V}CO_2$), were plotted against $\dot{V}O_2$ for each participant and examined to estimate the $\dot{V}O_2$ corresponding to the GET and the RCP (Keir et al., 2022). Both the estimated GET and RCP were determined by two trained researchers independently,

and in cases of disagreement of more than 200 mL·min^{-1}, a third researcher reevaluated the profiles until consensus was reached (Keir et al., 2016). The GET was estimated as the $\dot{V}O_2$ at which $\dot{V}CO_2$ and $\dot{V}_E$ began to increase disproportionately in relation to $\dot{V}O_2$, with a systematic rise in PO_2, while PCO_2 and $\dot{V}_E/\dot{V}CO_2$ remained stable (Beaver et al., 1986). The RCP was determined as the $\dot{V}O_2$ at which PCO_2 began to fall after a period of isocapnia (Whipp et al., 1987), coinciding with a second and first breakpoint in the $\dot{V}_E$-to-$\dot{V}O_2$ and $\dot{V}_E$-to-$\dot{V}CO_2$ relationships, respectively (Whipp et al., 1989). Linear regression was used to determine the slope of the relationship between the change in $\dot{V}O_2$ and the change in work rate ($\Delta\dot{V}O_2/\Delta PO$) during the ramp-incremental test, accounting for the MRT (Whipp et al., 1981). The MRT denotes the delay in $\dot{V}O_2$ increase during ramp-incremental exercise and was calculated by deducting two-thirds of the ramp rate (i.e., 20 W) from the work rate at GET, as described elsewhere (Jones et al., 2004; Bailey et al., 2009; Vanhatalo et al., 2016). The left-shifted $\dot{V}O_2$ data were also used to identify the $\dot{V}O_2$ and PO corresponding to the RCP (Iannetta et al., 2018). The work rates for both moderate and severe-intensity step transitions were normalised using the left-shifted $\dot{V}O_2$ data. $\dot{V}O_2$ peak was computed as the highest $\dot{V}O_2$ from a 30-s rolling average during the ramp-incremental test, while the highest PO recorded at the end of the test was considered PO peak (Zhang et al., 2021).

Breath-by-breath $\dot{V}O_2$ data from each constant-power output cycling trial were also cleaned, interpolated to 1-second intervals, and subsequently time-aligned to the onset of exercise (***Study 2***). For each participant, identical repetitions of both moderate- and severe-intensity step transitions were ensemble-averaged to improve signal-to-noise ratio (Lamarra et al., 1987). A nonlinear least-squares fitting algorithm was applied to the averaged data, with the initial cardiogenic phase (i.e., phase I response) excluded by omitting the first 20 seconds of data, in line with established recommendations (Hughson, 2009; Murias et al., 2011). A mono-exponential model was employed to characterise the $\dot{V}O_2$ response to moderate-intensity exercise, whereas a bi-exponential model was applied for severe-intensity transitions, as described by the following equations:

$$\dot{V}O_2(t) = \dot{V}O_{2\,\text{baseline}} + Ap \times (1 - e^{-(t - TDp\,/\,\tau p)}) \qquad (\textit{moderate})$$

$$\dot{V}O_2(t) = \dot{V}O_{2\,\text{baseline}} + Ap \times (1 - e^{-(t - TDp\,/\,\tau p)}) + As \times (1 - e^{-(t - TDs\,/\,\tau s)}) \qquad (\textit{severe})$$

where $\dot{V}O_2$ (t) represents the absolute $\dot{V}O_2$ at a given time point t, and $\dot{V}O_{2\,\text{baseline}}$ denotes the mean $\dot{V}O_2$ in the baseline period. The parameters Ap, TDp, and τp

represents the amplitude, time delay, and time constant, respectively, characterising the primary (phase II) component of the $\dot{V}O_2$ response. Specifically, Ap represents the increase in $\dot{V}O_2$ from baseline to the steady-state level (i.e., steady-state $\dot{V}O_2$ minus $\dot{V}O_{2\ baseline}$); TDp denotes the time delay prior to the onset of the exponential response (i.e., the point at which the exponential rise in $\dot{V}O_2$ begins, allowing the model not to be constrained to the origin); and τp reflects the time constant of the primary response, defined as the time required to achieve 63% of the amplitude of this component (Poole and Jones, 2012). Similarly, As, TDs, and τs represent the amplitude, time delay, and time constant, respectively, of the $\dot{V}O_2$ slow component. As quantifies the magnitude of the slow component rise above the steady-state level; TDs indicates the delay before its onset; and τs characterises the rate at which the slow component develops over time (Jones et al., 2011).

An iterative fitting procedure was employed to minimise the sum of squared errors between the fitted function and the observed data. The fitting window was constrained to the time point at which a deviation from fundamental monoexponential behaviour was observed, as determined by visual inspection of the residuals plot. $\dot{V}O_{2\ baseline}$ was defined as the mean $\dot{V}O_2$ during the final 90 seconds of the resting period. The $\dot{V}O_2$ at 360 seconds was calculated as the mean value between 330 and 360 seconds, while

the $\dot{V}O_2$ at task failure was defined as the mean $\dot{V}O_2$ during the final 30 seconds of the exhaustive exercise bout. Given that the As of the exponential term describing the $\dot{V}O_2$ slow component may exceed the actual value attained at the cessation of exercise, the realised amplitude of the $\dot{V}O_2$ slow component at end-exercise was denoted As′. This As′ parameter was compared across all *Cit* and *Pla* conditions at the same isotime (360 seconds). The amplitude of the slow component was additionally expressed relative to the entire $\dot{V}O_2$ response (Vanhatalo et al., 2010; Bailey et al., 2015).

The functional gain of the primary $\dot{V}O_2$ response was calculated by dividing the amplitude of the primary response (Ap) by the change in work rate (Δ work rate). To characterise the overall kinetics of the $\dot{V}O_2$ response to moderate- and severe-intensity step transitions, a monoexponential model without time delay was fitted to the data from exercise onset (0 seconds) to task termination. The resulting MRT was ultimately used to estimate the O_2 deficit using the following equation:

O_2 deficit = MRT (min) × $\Delta\dot{V}O_2$ (L)

Where $\Delta\dot{V}O_2$ is the difference in $\dot{V}O_2$ between 360 seconds and baseline.

Baseline values for $\dot{V}_E$, $\dot{V}CO_2$, RER, and HR were computed as the mean over the final 60 seconds preceding exercise onset. End-exercise values were determined as the mean values obtained during the final 30 seconds of both the moderate- and severe-intensity step transitions (Vanhatalo et al., 2010; Bailey et al., 2015).

3.6.3 | NIRS-Derived Indices of Muscle Oxygenation

To provide information on NIRS-derived muscle oxygenation changes in the *vastus lateralis* during ramp-incremental exercise test (***Study 1***), a "double-linear" piecewise equation was used and normalised based on work rate (0 to 100%) (Boone et al., 2008).

3.6.4 | Blood Lactate Concentration

Blood lactate accumulation ($\Delta[La^-]_{acc}$) was calculated as the difference between blood $[La^-]$ measured at end exercise and baseline values ($[La^-]_b$) (McDonagh et al., 2016).

The change in blood [La^-] attributable to supplementation ($\Delta[La^-]$) was determined as the difference between [La^-] measured ~90 minutes after ingestion of the supplement (*Cit* or *Pla*) and $[La^-]_b$.

3.7 | Statistical Analysis

Data are presented as means ± SD, unless stated otherwise. Assumptions of normality and sphericity were tested with Shapiro–Wilk and Mauchly tests, respectively. Where the assumption of sphericity was violated, the Greenhouse-Geisser correction factor was applied to adjust the degrees of freedom. Two-way repeated-measures ANOVA were used to assess differences in physiological responses to ramp-incremental cycling test (***Study 1***) and constant work-rate cycling test (***Study 2***) at moderate- and severe-intensity step transitions across supplement conditions (*Pla*, *Cit6*, and *Cit12*) and over time. Significant condition × time interactions were followed up with changes relative to baseline within the supplement conditions, assessed using one-way repeated-measures ANOVA with Bonferroni-corrected pairwise comparisons as appropriate. Bonferroni-adjusted paired-samples *t*-tests (with a 95% confidence interval, 95% CI)

were used to identify differences between conditions at specific time points. One-way repeated-measures ANOVA was also used to assess between-condition differences in blood pressure, plasma concentrations of [Lys], [Orn], [Arg], and AAI, parameters of pulmonary gas exchange and ventilation and NIRS-derived muscle oxygenation, and exercise tolerance (PO peak and time to task failure). Significant effects were further explored using simple contrasts, with the alpha level adjusted through a Fisher's LSD correction. Statistical procedures were performed using a commercially available statistical package (SPSS; IBM, Armonk, NY), with statistical significance accepted at $P < .05$. Based on previous work using a similar experimental design to the present thesis (Bailey et al., 2015) and using an $\alpha = 0.05$, $\beta = 0.80$, Cohen's effect size = 0.9, and a correlation of 0.5 for repeated measures (Quinn et al., 2024), a minimum sample size of 9 participants was determined *a priori* to detect an effect (G*power software, Heinrich Heine-Universitat, Dusseldorf, Germany; Faul et al., 2007).

4 | Results

4.1 | Main Findings from *Study 1*

The *Pla*, *Cit6,* and *Cit12* supplementation protocols used in ***Study 1*** were well tolerated and no harmful side effects were reported. These results are consistent with findings from previous research (Wijnands et al., 2012; Bailey et al., 2015; Bailey et al., 2016).

4.1.1 | Plasma Amino Acid Concentrations

Plasma concentrations of [Lys], [Orn], [Arg], as well as the AAI, for the *Pla*, *Cit6,* and *Cit12* conditions are summarised in **Table 3** and **Fig. 5**. A one-way ANOVA revealed a significant effect of condition on plasma [Orn] ($F_{2,24} = 3.61$; $\eta^2_p = 0.232$; $P < .05$), [Arg] ($F_{2,24} = 49.30$; $\eta^2_p = 0.804$; $P < .01$), and AAI ($F_{2,24} = 25.15$; $\eta^2_p = 0.677$; $P < .01$). *Post hoc* comparisons found that, relative to *Pla*, plasma [Orn] was significantly elevated (~100%) after supplementation with *Cit12* (95% CI, -278.2 to -9.95; $P < .01$), but not following supplementation with *Cit6* (95% CI, -215.3 to -53.04; $P > .05$), when compared with pre-supplementation baseline values. *Post hoc* analysis also revealed

significant increases in plasma [Arg] relative to *Pla* following both *Cit6* (95% CI, -100.4 to -46.09; $P < .01$) and *Cit12* (95% CI, -132.5 to -78.17; $P < .01$) supplementation. Notably, plasma [Arg] increases were dose-dependent, with the higher dose (*Cit12*) inducing a more pronounced elevation than the smaller dose (*Cit6*) (115% *vs*. 183%, respectively; 95% CI, -59.23 to -4.92; $P < .05$). The AAI was significantly elevated from presupplementation baseline values following both *Cit6* (95% CI: -0.26 to -0.07; $P < .01$) and *Cit12* (95% CI: -0.36 to -0.17; $P < .01$) supplementation, as indicated by the observed responses differing from *Pla*. Consistent with the pattern observed for plasma [Arg], increases in AAI were also dose-dependent, with *Cit12* eliciting a more pronounced effect than *Cit6* (81% *vs*. 148%, respectively; 95% CI, -0.19 to -0.00; $P < .05$).

4.1.2 | Blood Pressure

Resting blood pressure data for the *Pla*, *Cit6,* and *Cit12* conditions are summarised in **Table 4** and **Fig. 6** A one-way ANOVA revealed no significant effect of condition on SBP ($F_{2,24} = 0.55$; $P = .57$), DBP ($F_{2,24} = 1.70$; $P = .20$), and MAP ($F_{2,24} = 0.30$; $P = .74$).

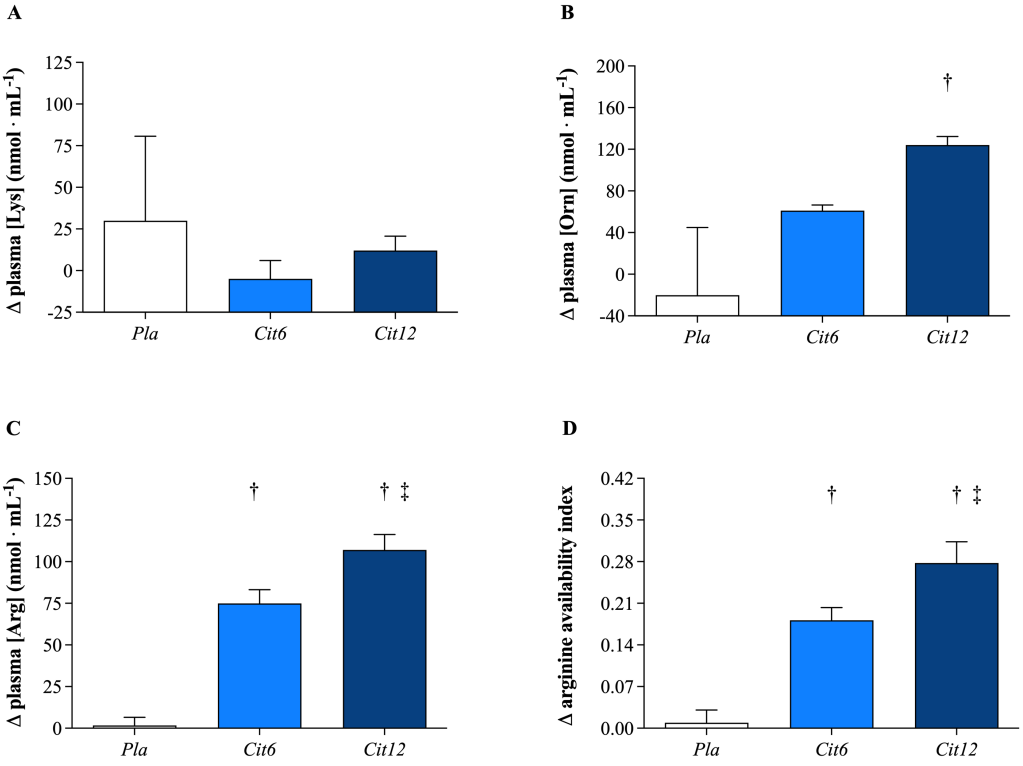


Fig. 5. Changes (Δ) from presupplementation baseline in plasma concentrations of (**A**) L-lysine ([Lys]), (**B**) L-ornithine ([Orn]), (**C**) L-arginine ([Arg]), and the (**D**) arginine availability index (AAI) following supplementation with *Pla*, *Cit6,* and *Cit12*. Venous blood samples for plasma amino acid analysis were drawn before and 90 minutes after supplement ingestion (see *Measurement* for details). Data are means ± SEM. † denotes a significant difference from *Pla* ($P < 0.05$); ‡ denotes a significant difference from *Cit6* ($P < 0.05$).

Table 3. Plasma [Lys], [Orn], [Arg], and the AAI following supplementation with *Pla*, *Cit6,* and *Cit12*.

	Pla	*Cit6*	*Cit12*	*Pla vs. Cit6*	*Pla vs. Cit12*
				Mean difference (95% CI)	**Mean difference (95% CI)**
Before supplementation					
[Lys]	239.0 ± 86.36	192.0 ± 36.72	203.8 ± 21.92	- 47.00 (- 37.71, 131.7)	35.22 (- 47.65, 118.0)
[Orn]	287.9 ± 172.5	99.89 ± 18.92 †	123.2 ± 23.32 †	188.0 (23.55, 352.4)	164.6 (0.120, 329.2)
[Arg]	60.81 ± 17.14	64.69 ± 19.33 †	58.37 ± 24.20 †	- 3.878 (- 27.93, 20.18)	2.444 (- 21.61, 26.50)
AAI	0.129 ± 0.057	0.223 ± 0.069 †	0.186 ± 0.101	- 0.094 (- 0.173, - 0.016)	- 0.057 (- 0.161, 0.046)
After supplementation					
[Lys]	267.7 ± 115.2	187.0 ± 29.40	215.8 ± 35.32	80.66 (- 3.641, 164.9)	51.88 (- 32.41, 136.1)
[Orn]	267.8 ± 161.2	160.9 ± 21.70	247.2 ± 33.69	106.88 (- 5.981, 219.7)	20.55 (- 92.31, 133.4)
[Arg]	62.53 ± 20.90	139.7 ± 37.10	165.4 ± 48.37	- 77.12 (- 120.9, 33.32)	- 102.8 (- 146.6, 59.08)
AAI	0.138 ± 0.060	0.405 ± 0.113 †	0.464 ± 0.189 †	- 0.266 (- 0.381, - 0.152)	- 0.325 (- 0.508, - 0.143)
Changes (Δ) from presupplementation baseline					
[Lys]	29.89 ± 152.5	-5.000 ± 33.05	12.00 ± 26.05	34.89 (- 72.64, 142.4)	17.89 (- 89.64, 125.4)
[Orn]	- 20.11 ± 195.1	61.00 ± 16.67	124.0 ± 24.90 †	- 81.11 (- 215.2, 53.04)	- 144.1 (- 278.2, - 9.952)
[Arg]	1.722 ± 14.51	74.97 ± 24.54 †	107.0 ± 27.99 †	- 73.24 (- 100.4, - 46.09)	- 105.3 (- 132.5, - 78.17)
AAI	0.009 ± 0.063	0.181 ± 0.064 †	0.277 ± 0.107 †	- 0.172 (- 0.268, - 0.076)	- 0.268 (- 0.364, - 0.173)

Data are presented as means ± SD. 95% CI = 95% confidence interval. † denotes a significant difference from *Pla* ($P < 0.05$).

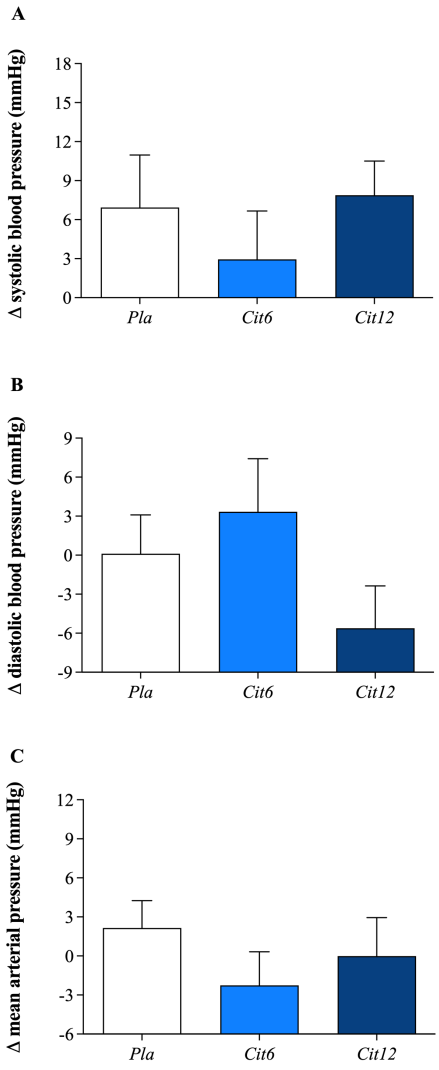


Fig. 6. Changes (Δ) from presupplementation baseline in (**A**) systolic blood pressure (SBP), (**B**) diastolic blood pressure (DBP), and (**C**) mean arterial pressure (MAP) following supplementation with *Pla*, *Cit6,* and *Cit12*. Brachial artery blood pressure was measured before and 90 minutes after supplement ingestion (see *Measurement* for details). Data are means ± SEM.

Table 4. Resting BP following supplementation with *Pla*, *Cit6,* and *Cit12*.

	Pla	*Cit6*	*Cit12*	*Pla vs. Cit6*	*Pla vs. Cit12*
				Mean difference (95% CI)	**Mean difference (95% CI)**
Before supplementation					
SBP	121.0 ± 5.55	121.5 ± 7.962	112.8 ± 21.49	- 0.444 (- 16.47, 15.58)	8.222 (- 7.803, 24.25)
DBP	68.97 ± 3.805	67.86 ± 6.062	70.17 ± 5.178	1.111 (- 4.893, 7.115)	- 1.194 (- 7.199, 4.810)
MAP	86.32 ± 3.215	85.73 ± 5.912	84.38 ± 7.181	0.592 (- 6.096, 7.281)	1.944 (- 4.744, 8.633)
After supplementation					
SBP	127.8 ± 12.84	115.1 ± 23.57	120.9 ± 10.20	12.69 (- 6.823, 32.21)	6.833 (- 12.68, 26.35)
DBP	68.83 ± 5.912	67.67 ± 7.336	66.08 ± 6.911	1.167 (- 6.775, 9.108)	2.750 (- 5.192, 10.69)
MAP	88.48 ± 7.368	83.47 ± 10.55	84.37 ± 6.633	5.009 (- 4.832, 14.85)	4.111 (- 5.730, 13.95)
Changes (Δ) from presupplementation baseline					
SBP	6.944 ± 12.09	2.944 ± 11.18	7.889 ± 7.837	4.000 (- 8.394, 16.39)	- 0.944 (- 13.34, 11.45)
DBP	0.111 ± 8.964	3.333 ± 12.27	- 5.611 ± 9.752	- 3.222 (- 15.49, 9.049)	5.722 (- 6.549, 17.99)
MAP	2.157 ± 6.285	-2.259 ± 7.756	-0.009 ± 8.892	4.417 (- 4.670, 13.50)	2.167 (- 6.920, 11.25)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

4.1.3 | Time to Task Failure and Peak Power Output

TTF and PO peak during the ramp-incremental cycling test for the *Pla*, *Cit6,* and *Cit12* conditions are summarised in **Table 5** and **Fig. 7**. A one-way ANOVA revealed no significant effect of condition on TTF ($F_{2,24} = 0.02$; $P = .97$) and PO peak ($F_{2,24} = 0.03$; $P = .96$).

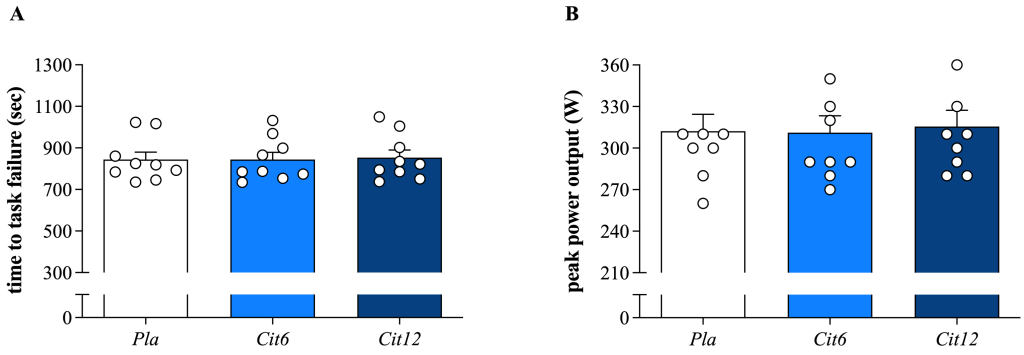


Fig. 7. Time to task failure (TTF) (**A**) and peak power output (PO peak) (**B**) in the ramp-incremental cycling test following supplementation with *Pla*, *Cit6,* and *Cit12*. Data are means ± SEM. White circles represent individual responses to supplements.

4.1.4 | Pulmonary Gas Exchange and Ventilation

Pulmonary gas exchange and ventilation responses to the incremental cycling test for the *Pla*, *Cit6,* and *Cit12* conditions are summarised in **Table 5** and **Fig. 8** and **9**. A one-way ANOVA revealed no significant effect of condition on $\dot{V}O_2$ peak ($F_{2,24} = 0.04$; $P = .96$), RCP ($F_{2,24} = 0.00$; $P = .99$), and GET ($F_{2,24} = 0.00$; $P = .99$). No significant between-condition differences were also observed for $\dot{V}_E$ max ($F_{2,24} = 2.36$; $P = .11$), $\dot{V}CO_2$ max ($F_{2,24} = 0.96$; $P = .39$), RER max ($F_{2,24} = 1.93$; $P = .16$), and HR max ($F_{2,24} = 0.88$; $P = .42$). A two-way ANOVA revealed no significant main effects of time or condition for any pulmonary gas exchange and ventilatory response during moderate- or severe-intensity step exercise ($P > .05$ for all comparisons; **Fig. 10**).

4.1.5 | NIRS-Derived Indices of Muscle Oxygenation

NIRS-derived muscle oxygenation responses during the ramp-incremental cycling test for the *Pla*, *Cit6,* and *Cit12* were analysed using a two-way ANOVA. There were no significant main effects of time or condition for any parameter of muscle oxygenation during moderate- or severe-intensity step exercise ($P > .05$ for all comparisons).

Table 5. TTF, PO peak, and pulmonary gas exchange and ventilation during the ramp-incremental cycling test following supplementation with *Pla*, *Cit6,* and *Cit12*.

	Pla	*Cit6*	*Cit12*	*Pla vs. Cit6*	*Pla vs. Cit12*
				Mean difference (95% CI)	**Mean difference (95% CI)**
TTF	844.6 ± 106.8	844.4 ± 103.9	853.5 ± 110.3	- 0.111 (- 125.9, 126.1)	-8.944 (- 134.9, 117.0)
PO peak	312.2 ± 36.67	311.1 ± 36.55	315.6 ± 35.04	1.111 (- 41.38, 43.60)	- 3.333 (- 45.82, 39.16)
$\dot{V}_E$ max ($L.min^{-1}$)	146.4 ± 23.33	162.3 ± 20.64	168.7 ± 23.11	- 15.91 (- 42.27, 10.45)	- 22.26 (- 48.62, 4.094)
$\dot{V}O_2$ max ($L.min^{-1}$)	3556 ± 379.4	3511 ± 309.6	3541 ± 315.7	44.11 (- 351.9, 440.1)	14.44 (- 381.6, 410.4)
$\dot{V}CO_2$ max ($L.min^{-1}$)	4340 ± 420.1	4538 ± 285.1	4570 ± 418.3	- 197.4 (- 644.5, 249.8)	- 229.3 (- 676.4, 217.8)
RER max	1.252 ± 0.146	1.369 ± 0.122	1.324 ± 0.110	- 0.116 (- 0.266, 0.032)	- 0.072 (- 0.221, 0.077)
HR max (bpm)	180.2 ± 12.21	191.0 ± 25.48	182.3 ± 14.35	- 10.82 (- 32.35, 10.72)	- 2.083 (-23.62, 19.45)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

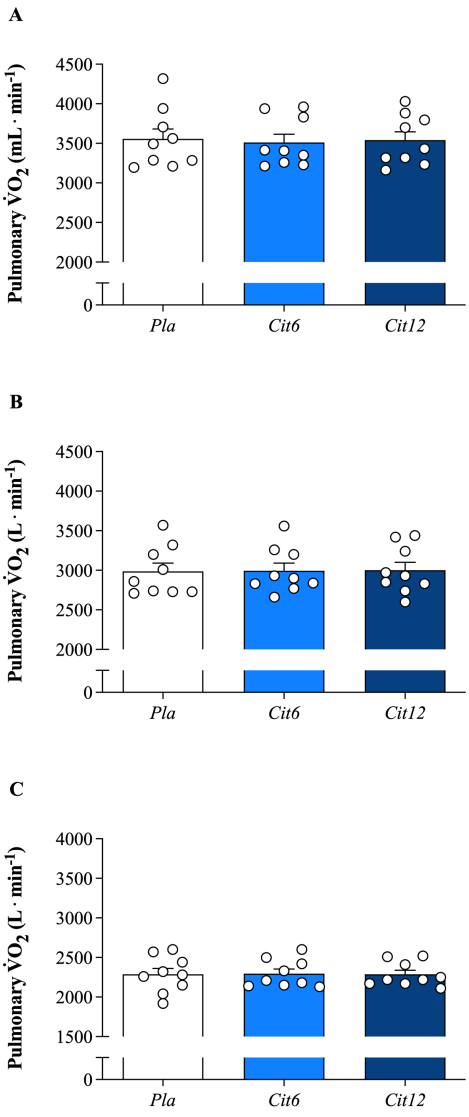


Fig. 8. Pulmonary $\dot{V}O_2$ responses during the ramp-incremental cycling test following supplementation with *Pla*, *Cit6,* and *Cit12*. (**A**) peak oxygen uptake ($\dot{V}O_2$ peak), (**B**) respiratory compensation point (RCP), and (**C**) gas exchange threshold (GET). Data are means ± SEM.

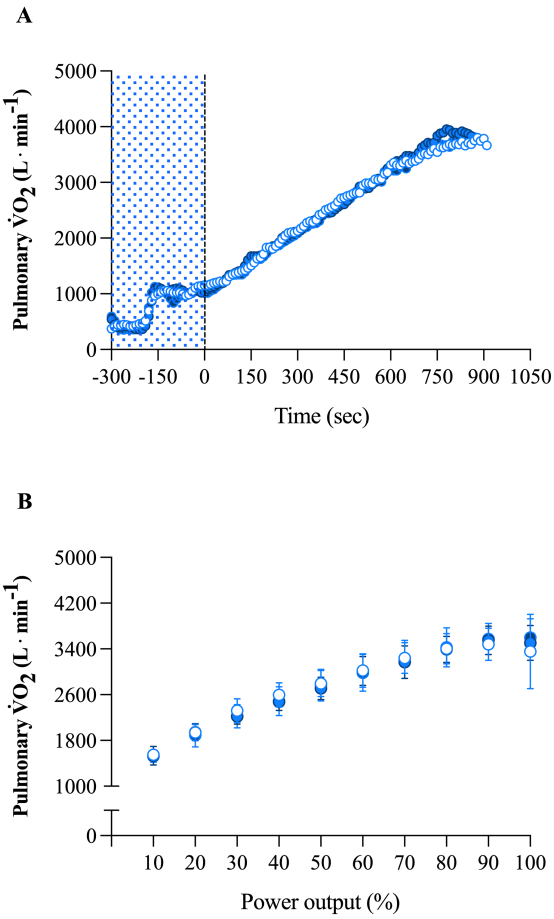
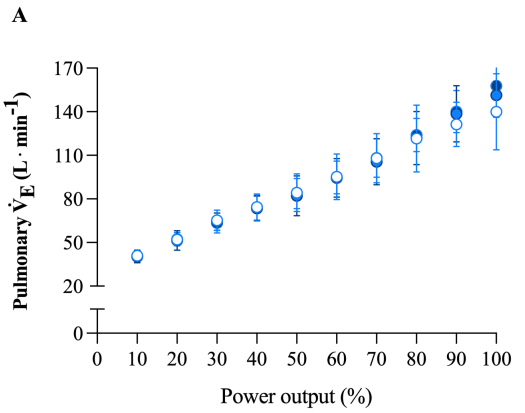
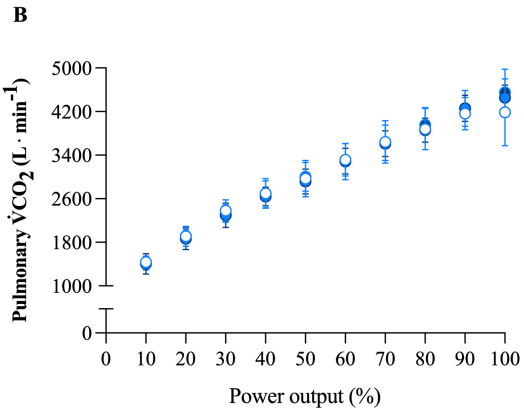


Fig. 9. Pulmonary $\dot{V}O_2$ responses during the ramp-incremental cycling test following supplementation with *Pla*, *Cit6,* and *Cit12*. Responses following *Cit6* (royal blue) and *Cit12* (navy blue) are shown as solid circles, while the *Pla* responses are shown as open circles. The dotted vertical line indicates the onset of the incremental exercise from a baseline of unloaded cycling. (**A**) $\dot{V}O_2$ responses of a representative individual. (**B**) Group mean (± SD) $\dot{V}O_2$ responses throughout the ramp-incremental cycling test plotted against %PO peak.


A
Pulmonary $\dot{V}_E$ (L · min^{-1})
170
140
110
80
50
20
0
0 10 20 30 40 50 60 70 80 90 100
Power output (%)


B
Pulmonary $\dot{V}CO_2$ (L · min^{-1})
5000
4200
3400
2600
1800
1000
0
0 10 20 30 40 50 60 70 80 90 100
Power output (%)

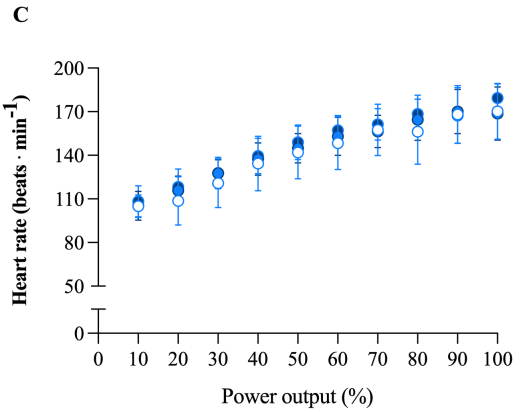

C
Heart rate (beats · min^{-1})
200
170
140
110
80
50
0
0 10 20 30 40 50 60 70 80 90 100
Power output (%)

Fig. 10. Pulmonary gas exchange and ventilation responses in the ramp-incremental cycling test following supplementation with *Pla*, *Cit6,* and *Cit12*. Responses following *Cit6* (royal blue) and *Cit12* (navy blue) are shown as solid circles and the *Pla* responses are shown as open circles. The dotted vertical line indicates the onset of the ramp incremental exercise from a baseline of unloaded cycling. Group mean (± SD) (**A**) $\dot{V}_E$, (**B**) $\dot{V}CO_2$, and (**C**) HR responses throughout the ramp-incremental cycling test plotted against %PO peak.

4.1.6 | Blood Lactate Concentration

Blood [La^-] responses following supplementation with *Pla*, *Cit6,* and *Cit12* are shown in **Table 6** and **Fig. 11**. A one-way ANOVA revealed no significant effect of condition on the Δ[La^-] from baseline to ~90 minutes post-supplementation ($F_{2,24}$ = 0.32; P = .72), nor on $\Delta[La^-]_{acc}$ during the ramp-incremental cycling test ($F_{2,24}$ = 0.47; P = .62).

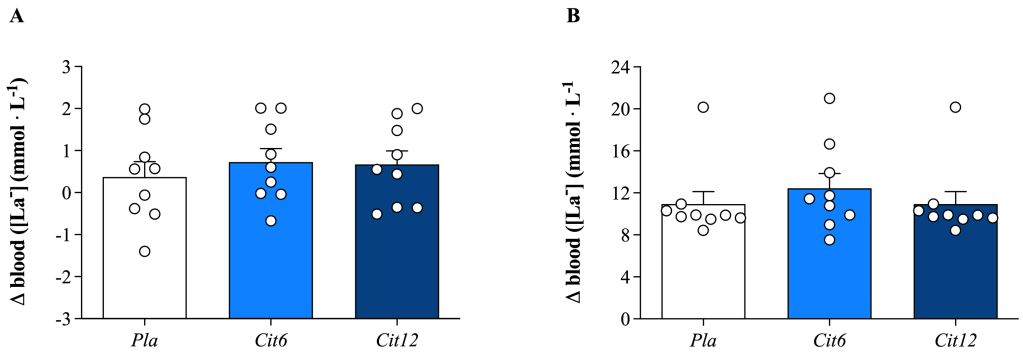


Fig. 11. Blood lactate ([La^-]) responses following supplementation with *Pla*, *Cit6,* and *Cit12*. Changes in [La^-] from baseline to ~90 minutes post-supplementation ($\Delta[La^-]$) (**A**) and blood lactate accumulation during the ramp-incremental test ($\Delta[La^-]_{acc}$) (**B**). Data are means ± SEM. White circles represent individual responses to supplements.

Table 6. Blood [La^-] responses following supplementation with *Pla*, *Cit6,* and *Cit12*.

	Pla	*Cit6*	*Cit12*	*Pla vs. Cit6*	*Pla vs. Cit12*
				Mean difference (95% CI)	**Mean difference (95% CI)**
Before supplementation					
[La^-]	1.763 ± 0.586	1.561 ± 0.746	1.521 ± 0.356	0.202 (- 0.487, 0.891)	0.242 (- 0.447, 0.931)
After supplementation					
[La^-]	2.137 ± 0.772	2.290 ± 0.544	2.248 ± 0.616	- 0.153 (- 0.920, 0.613)	- 0.111 (- 0.877, 0.655)
End exercise					
[La^-]	11.59 ± 4.782	14.00 ± 3.984	11.42 ± 1.324	- 2.408 (- 6.733, 1.917)	0.176 (- 4.148, 4.501)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

4.2 | Main Findings from *Study 2*

4.2.1 | Blood Pressure

Resting blood pressure data for the *Pla*, *Cit6,* and *Cit12* conditions are shown in **Table 7** and **Fig. 12**. A one-way ANOVA revealed no significant effect of condition on SBP ($F_{2,27} = 0.08$; $P = .91$), DBP ($F_{2,27} = 0.33$; $P = .71$), and MAP ($F_{2,27} = 0.48$; $P = .73$).

4.2.2 | Ramp-Incremental Exercise Test

The $\dot{V}O_2$ peak attained in the ramp-incremental cycling test was 3571 ± 398 $L \cdot min^{-1}$, which were not significantly different from the $\dot{V}O_2$ at task failure measured during the severe-intensity exercise trial following *Pla* supplementation ($P > .05$). The $\dot{V}O_2$ corresponding to the estimated GET and RCP averaged 2336 ± 316 $L \cdot min^{-1}$ and 3066 ± 372 $L \cdot min^{-1}$, respectively (see **Fig. 13** for further details).

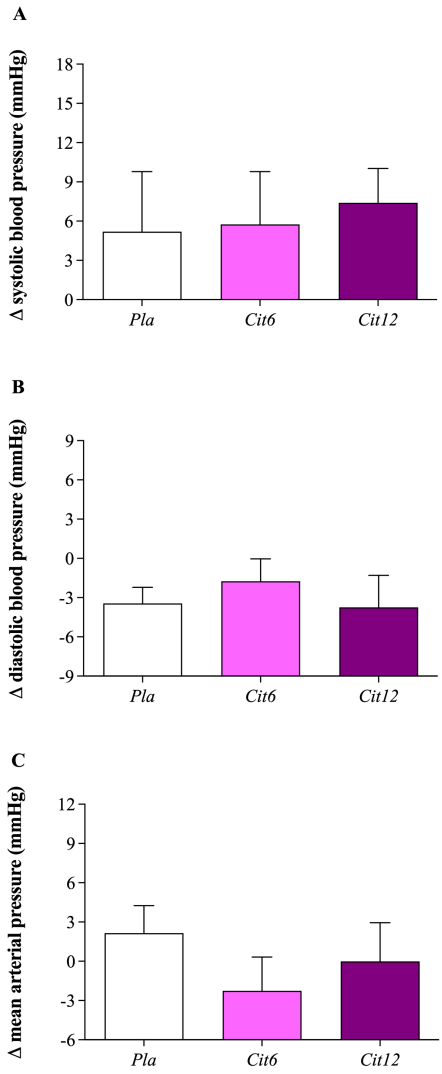


Fig. 12. Changes (Δ) from presupplementation baseline in (**A**) systolic blood pressure (SBP), (**B**) diastolic blood pressure (DBP), and (**C**) mean arterial pressure (MAP) following supplementation with *Pla*, *Cit6,* and *Cit12*. Brachial artery blood pressure was measured before and 90 minutes after supplement ingestion (see *Measurement* for details). Data are means ± SEM.

Table 7. Resting BP following supplementation with *Pla*, *Cit6,* and *Cit12*.

	Pla	*Cit6*	*Cit12*	*Pla vs. Cit6*	*Pla vs. Cit12*
				Mean difference (95% CI)	**Mean difference (95% CI)**
Before supplementation					
SBP	126.6 ± 15.39	121.4 ± 11.51	114.2 ± 8.879	5.200 (- 8,353, 18.75)	12.40 (- 1.153, 25.95)
DBP	61.80 ± 7.029	68.20 ± 6.771	64.70 ± 8.077	- 6.400 (- 14.51, 1.710)	- 2.900 (- 11.01, 5.210)
MAP	83.40 ± 7.046	85.93 ± 6.258	81.20 ± 6.953	- 2.533 (- 10.03, 4.964)	2.200 (- 5.298, 9.698)
After supplementation					
SBP	126.6 ± 15.39	127.2 ± 14.95	121.6 ± 9.966	- 0.550 (- 15.69, 14.59)	5.000 (- 10.14, 20.14)
DBP	61.80 ± 7.029	66.45 ± 7.418	60.95 ± 4.907	- 4.650 (- 11.91, 2.607)	- 0.850 (- 6.407, 8.107)
MAP	83.40 ± 7.046	86.68 ± 7.055	81.17 ± 5.120	- 3.283 (- 10.46, 3.892)	2.233 (- 4.942, 9.409)
Changes (Δ) from presupplementation baseline					
SBP	5.200 ± 14.52	5.750 ± 12.76	7.400 ± 8.296	- 0.550 (- 14.02, 12.92)	- 2.200 (- 15.67, 11.27)
DBP	- 3.450 ± 3.891	- 1.750 ± 5.402	- 3.750 ± 7.736	- 1.700 (- 8.234, 4.834)	0.300 (- 6.234, 6.834)
MAP	2.157 ± 6.285	-2.259 ± 7.756	-0.009 ± 8.892	4.417 (- 4.670, 13.50)	2.167 (- 6.920, 11.25)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

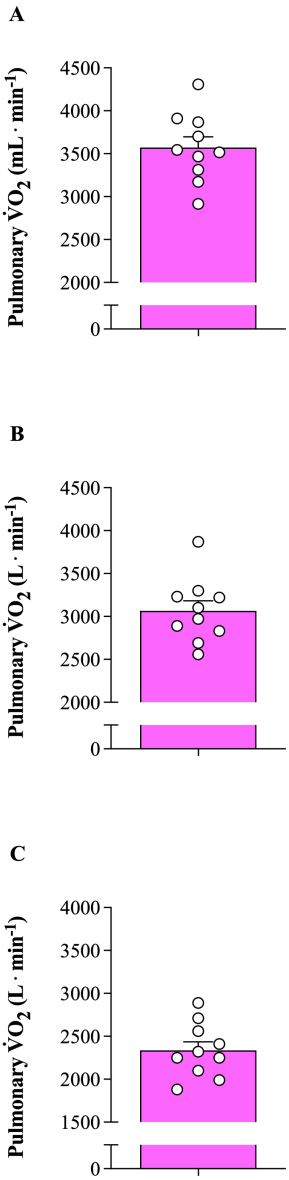


Fig. 13. Pulmonary $\dot{V}O_2$ responses during the ramp-incremental cycling test following supplementation with *Pla*, *Cit6,* and *Cit12*. (**A**) peak oxygen uptake ($\dot{V}O_2$ peak), (**B**) respiratory compensation point (RCP), and (**C**) gas exchange threshold (GET). Data are means ± SEM.

4.2.3 | Constant-Power Output Cycling Test

Pulmonary $\dot{V}O_2$ responses to the constant work rate cycling test for the *Pla*, *Cit6,* and *Cit12* conditions are summarised in **Table 8** and **9**. In the moderate-intensity step test, both $\dot{V}O_2$ baseline and $\dot{V}O_2$ end-exercise were not affected by the supplement ($P >$ .05). Accordingly, all parameters of pulmonary $\dot{V}O_2$ kinetics during the exercise test, including mean response time, primary amplitude, and phase II constant time, did not differ between *Pla*, *Cit6,* and *Cit12* conditions ($P >$ .05 for all comparisons). Similarly, both $\dot{V}O_2$ baseline and $\dot{V}O_2$ end-exercise were not affected by the supplement ($P >$ .05). Additionally, no between-condition differences were observed for any studied parameter of pulmonary $\dot{V}O_2$ kinetics, including slow component amplitude and overall mean response time ($P >$ .05 for all comparisons). No significant between-condition differences were also observed for $\dot{V}_E$, $\dot{V}CO_2$, RER, and HR across all times ($P >$ .05).

Table 8. Pulmonary $\dot{V}O_2$ responses during the moderate-intensity step test for the *Pla*, *Cit6,* and *Cit12* conditions.

	Pla	***Cit6***	***Cit12***	***Pla vs. Cit6***	***Pla vs. Cit12***
				Mean difference (95% CI)	**Mean difference (95% CI)**
$\dot{V}O_2$					
Phase II constant time, s	36.94 ± 10.49	33.25 ± 7.72	30.34 ± 11.91	3.684 (- 7.616, 14.98)	6.598 (-4.702, 17.90)
Mean response time, s	48.70 ± 7.385	45.97 ± 7.888	43.81 ± 10.79	2.728 (- 7.048, 12.50)	4.890 (-4,886, 14.67)
Primary amplitude, $L.min^{-1}$	1279 ± 150.1	1276 ± 196.7	1266 ± 173.9	3.400 (- 190.2, 197,0)	12.70 (-180.9, 206.3)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

Table 8. Pulmonary $\dot{V}O_2$ responses during the moderate-intensity step test for the *Pla*, *Cit6,* and *Cit12* conditions.

	Pla	***Cit6***	***Cit12***	***Pla vs. Cit6***	***Pla vs. Cit12***
				Mean difference (95% CI)	**Mean difference (95% CI)**
$\dot{V}O_2$					
Phase II constant time, s	36.94 ± 10.49	33.25 ± 7.72	30.34 ± 11.91	3.684 (- 7.616, 14.98)	6.598 (-4.702, 17.90)
Mean response time, s	48.70 ± 7.385	45.97 ± 7.888	43.81 ± 10.79	2.728 (- 7.048, 12.50)	4.890 (-4,886, 14.67)
Primary amplitude, $L.min^{-1}$	1279 ± 150.1	1276 ± 196.7	1266 ± 173.9	3.400 (- 190.2, 197,0)	12.70 (-180.9, 206.3)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

4.2.4 | Time to Task Failure

TTF during the severe intensity cycling exercise test under the *Pla*, *Cit6,* and *Cit12* conditions is summarised in Fig. 14. A one-way ANOVA revealed no significant effect of condition on TTF on during either the first ($F_{2,27}$ = 0.15; P = .85) or second ($F_{2,27}$ = 0.01; P = .98) severe-intensity step transition.

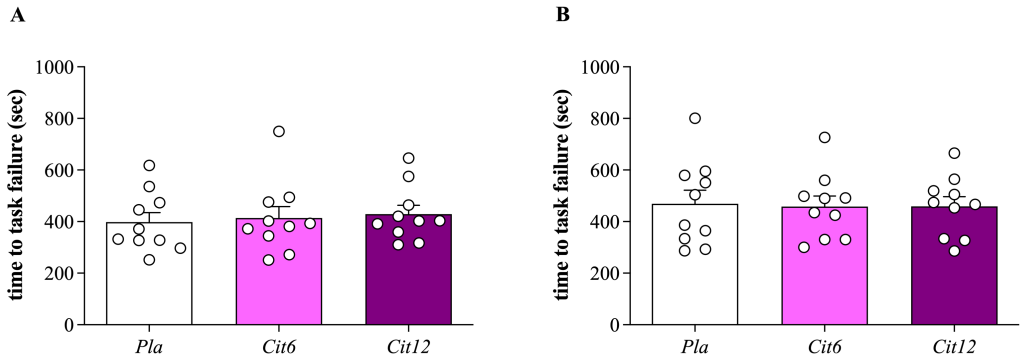


Fig. 14. Time to task failure (TTF) during the constant work-rate cycling test under the *Pla*, *Cit6,* and *Cit12* conditions. (**A**) Severe-intensity step transition on *Day 1*, and (**B**) Severe-intensity step transition on *Day 2*. Notice that the exercise transitions were performed on separate days. Data are means ± SEM. White circles represent individual responses to supplements.

4.2.5 | Blood Lactate Concentration

Blood [La^-] responses following supplementation with *Pla*, *Cit6,* and *Cit12* are shown in **Table 10** and **Fig. 15**. A one-way ANOVA revealed no significant effect of condition on the Δ[La^-] from baseline to ~90 minutes post-supplementation ($F_{2,27}$ = 0.82; P = .44), nor on $\Delta[La^-]_{acc}$ during the severe-intensity step transition ($F_{2,27}$ = 0.16; P = .84).

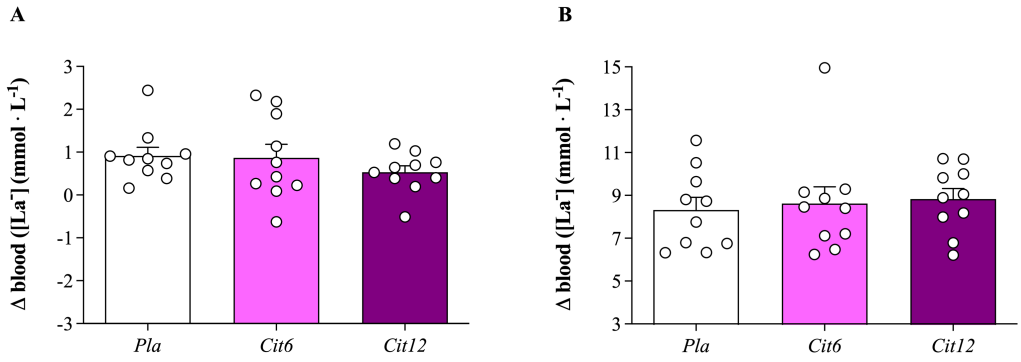


Fig. 15. Blood lactate ([La^-]) responses following supplementation with *Pla*, *Cit6,* and *Cit12*. Changes in [La^-] from baseline to ~90 minutes post-supplementation (Δ[La^-]) (**A**) and blood lactate accumulation during the severe-intensity step test ($\Delta[La^-]_{acc}$) (**B**). Data are means ± SEM. White circles represent individual responses to supplement.

Table 10. Blood [La^-] responses following supplementation with *Pla*, *Cit6,* and *Cit12*.

	Pla	***Cit6***	***Cit12***	***Pla vs. Cit6***	***Pla vs. Cit12***
				Mean difference (95% CI)	**Mean difference (95% CI)**
Before supplementation					
[La^-]	1.274 ± 0.403	1.503 ± 0.481	1.595 ± 0.42	- 0.229 (- 0.714, 0.256)	- 0.321 (- 0.806, 0.163)
After supplementation					
[La^-]	2.272 ± 0.549	2.169 ± 0.712	2.602 ± 0.414	- 0.103 (- 0.530, 0.739)	- 0.330 (- 0.963, 0.303)
Severe-intensity step exercise					
[La^-]	10.40 ± 1.993	12.08 ± 2.617	11.56 ± 1.550	- 1.676 (- 4.004, 0.651)	- 1.153 (- 3.481, 1.175)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

4.2.6 | Rate of Perceived Exertion

Perceived exertion responses during the constant work-rate cycling test under the *Pla*, *Cit6,* and *Cit12* conditions are presented in **Table 11** and **Fig. 16**. A one-way ANOVA demonstrated no significant effect of condition on perceived exertion during either the moderate-intensity ($F_{2,27} = 0.09$; $P = .91$) or severe-intensity ($F_{2,27} = 0.73$; $P = .48$) step transitions.

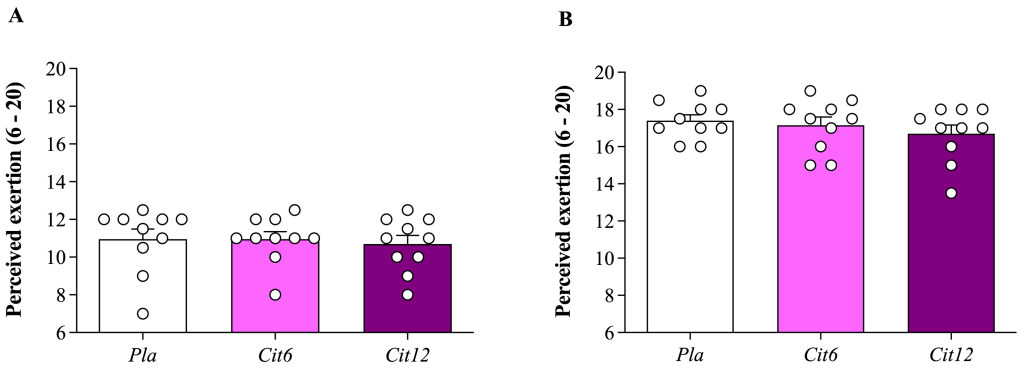


Fig. 16. Perceived exertion (RPE) responses during the constant work-rate cycling test following *Pla*, *Cit6,* and *Cit12* supplementation. (**A**) RPE responses during moderate-intensity step transition, and (**B**) RPE responses during severe-intensity step transition on *Day 2*. Data are means ± SEM. White circles represent individual responses to supplements.

Table 11. RPE responses following supplementation with *Pla*, *Cit6,* and *Cit12*.

	Pla	*Cit6*	*Cit12*	*Pla vs. Cit6*	*Pla vs. Cit12*
				Mean difference (95% CI)	**Mean difference (95% CI)**
Moderate-intensity step exercise					
RPE	10.95 ± 1.723	10.95 ± 1.257	10.70 ± 1.438	0.000 (- 1.647, 1.647)	0.250 (- 1.397, 1.897)
Severe-intensity step exercise					
RPE	17.40 ± 0.994	17.15 ± 1.395	16.70 ± 1.476	0.250 (- 1.198, 1.698)	0.700 (- 0.747, 2.148)

Data are presented as means ± SD. 95% CI = 95% confidence interval.

5 | Discussion

It is well established that both elite and recreational athletes frequently utilise dietary supplements in combination with a balanced diet, with the aim of maintaining general health and enhancing performance during competition. One such supplement of great interest is inorganic nitrate (Jones, 2014), a naturally occurring compound found in various foods, especially green leafy vegetables, and commonly ingested by athletes in the form of beetroot juice (McMahon et al., 2017). Dietary nitrate supplementation can enhance muscle efficiency by reducing the O_2 cost of submaximal exercise, thus improving exercise performance (Vanhatalo et al., 2011; Jones et al., 2018). It has also been shown to augment skeletal muscle contractile function (Bailey et al., 2009), an effect that could result in increased muscle power and improved performance in sprint-based exercise (Jones, 2014). The proposed mechanisms underpinning the ergogenic effects of the dietary nitrate involve the nitrate–nitrite–nitric oxide (NO_3^-–NO_2^-–NO) pathway (Lundberg et al., 2008; Kapil et al., 2010), which facilitates the stepwise reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and subsequently to NO and other reactive nitrogen species. NO is a gaseous signalling molecule that plays a fundamental role in

numerous human physiological processes, including vasodilation, neurotransmission, mitochondrial respiration, and skeletal muscle contractility (Lundberg and Weitzberg, 2009). Accordingly, NO may possess considerable ergogenic potential. Notably, while previous studies have confirmed that dietary nitrate supplementation can elevate NO biomarkers, reduce blood pressure, and improve exercise efficiency and tolerance in healthy adults (Jones, 2014), recent investigations into alternative NO precursors have yielded less optimistic findings (see Harnden et al., 2023 for review). In recent years, L-arginine supplementation has gained popularity due to its capacity to enhance NO synthesis and availability, thereby influencing exercise performance and associated physiological responses. Preliminary studies (Buford et al., 2004; Bailey et al., 2010; Vanhatalo et al., 2010) have demonstrated improvements in exercise efficiency and/or tolerance following supplementation with L-arginine. Other investigations, however, have found conflicting findings, indicating minimal or no ergogenic effect (Stevens et al., 2000; Bescós et al., 2009; Koppo et al., 2009a). These inconsistent results cast doubt on the efficacy of L-arginine as a performance-enhancing supplement and highlight the need for further research into nutritional strategies capable of modulating NO responsiveness and enhancing exercise performance in humans.

L-citrulline, a naturally occurring non-essential amino acid that is both endogenously synthesized and obtained through dietary sources, emerged as a promising approach for stimulating NO production and enhancing exercise performance in healthy adults. L-citrulline plays a key role in the NO synthesis, where it is generated as a by-product during the conversion of L-arginine to NO, a reaction catalysed by NOS enzymes (Wu et al., 1998). Differently from L-arginine, orally ingested L-citrulline is not subject to extensive metabolism in the liver or intestine and does not stimulate arginase enzymes (Morris, 2016), thereby allowing for more efficient systemic availability of L-arginine. Consequently, L-citrulline may serve as a more effective precursor for NO production, with plausible ergogenic benefits (for an in-depth review, see Gonzalez et al., 2020). Recent studies confirmed that oral L-citrulline supplementation not only augments NO synthase (Wijnands et al., 2012) and increases NO biomarkers (Schwedhelm et al., 2008) but also improves skeletal muscle oxygenation and accelerates $\dot{V}O_2$ kinetics (Bailey et al., 2015). Oral L-citrulline supplementation has also been shown to improve skeletal muscle metabolism and contractile efficiency (Bendahan et al., 2002; Giannesini et al., 2009), potentially enhancing fatigue resistance (Bailey et al., 2015). In addition, L-citrulline supplementation might also enhance exercise tolerance v ia buffering ammonia through the urea cycle. By reducing circulating ammonia levels,

L-citrulline promotes the aerobic utilisation of pyruvate, thereby diminishing reliance on anaerobic metabolism and lactate accumulation, and, ultimately, sustaining muscle contraction and delaying the onset of fatigue (Martínez-Sánchez et al., 2017a). The current literature, however, remains elusive regarding the effects of supplementation with L-citrulline on both aerobic and anaerobic performance in humans (Suzuki et al., 2016; Stanelle et al., 2020).

The reasons for such discrepancies between studies on the ergogenicity of L-citrulline are not fully understood, but may be attributed, at least in part, to variations in exercise protocols, which largely differ in terms of exercise intensity (Martínez-Sánchez et al., 2017b). Because the degree of intramuscular and systemic homeostasis perturbations during exercise is linked to the intensity domain in which the exercise is performed (Burnley et al., 2012; Vanhatalo et al., 2016; Black et al., 2017), it is plausible that the effectiveness of L-citrulline supplementation on exercise efficiency and/or exercise tolerance would exhibit domain-specificity. Varied results between studies may also arise from differences in dosage used in L-citrulline supplementation protocols. Since exercise-performance studies completed to date with L-citrulline have employed dosages of 6 g or lower (Suzuki et al., 2016), it is uncertain whether a dose-response

relationship exists between L-citrulline intake, physiological responses to exercise, and exercise performance. Establishing the optimal dosage for enhancing exercise efficiency and/or tolerance, therefore, is of importance, given the increasing popularity of L-citrulline supplementation in both basic research and applied exercise settings. Hence, the present thesis addressed the aforementioned gap in the existing literature by investigating the effects of different doses of oral L-citrulline supplementation on exercise tolerance and related physiological responses during both ramp-incremental and constant-power output cycling in humans.

The principal original finding of this investigation was that plasma [Arg], relative to *Pla*, significant increased following both *Cit6* and *Cit12* supplementation (see **Table 3** and **Fig. 5** for further details). This plasma [Arg] increases was dose-dependent, with larger doses inducing a more pronounced elevation than the smaller doses. The AAI, a well-established indicator of L-arginine availability (Wu, 1995; Wu, 1998), was also elevated from presupplementation baseline in both *Cit6* and *Cit12* supplementation, as indicated by the observed responses differing from *Pla*. Consistent with the pattern observed for plasma [Arg], increases in AAI were also dose-dependent, with *Cit12* eliciting a more pronounced effect than *Cit6*. These preliminary findings do support

our preliminary hypothesis and are in line with previous studies (Wijnands et al., 2012; Van Wijck et al., 2014). Orally ingested L-citrulline is not metabolized in the intestine or liver and does not stimulate arginase enzymes (Morris, 2016). Upon absorption, L-citrulline is transported into the kidney and other tissues via facilitated mechanisms and not compete with ADMA and other L-arginine analogues for cellular uptake via the y^+ transporter system, including the hCAT-2B carrier (Closs et al., 1997). Once inside the cell, L-citrulline can be readily converted to L-arginine via the citrulline-NO cycle, thereby elevating plasma (Kuhn et al., 2002; Kuhn et al., 2011; Osowska et al., 2004) and tissue (Wijnands et al., 2012) L-arginine concentrations, and increasing NO synthesis more effectively than an equivalent dose of L-arginine. The significant increase (~100%) of plasma [Orn], when compared with presupplementation baseline values, following *Cit12* supplementation, but not following *Cit6* supplementation, is a somewhat surprising finding. The conversion of orally ingested L-citrulline in the liver primarily occurs via the urea cycle (Morris, 2016), where both amino acids, L-arginine and L-ornithine, are components. L-citrulline and L-ornithine play a crucial role in the urea production and ammonia detoxification in the liver (Morris, 2016). Therefore, an increased plasma [Orn] following supplementation of large doses of L-citrulline could be possible.

Contrary to our preliminary hypothesis and inconsistent findings from previous studies (e.g., Bailey et al., 2015), orally ingested L-citrulline did not significantly affect blood pressure (see **Tables 4** and 7). Although plasma [Arg] and AAI increased significantly, with larger doses eliciting a more pronounced elevation, no significant reductions were observed in resting mean, systolic, or diastolic blood pressure. These findings suggest that the increase in L-arginine bioavailability following L-citrulline supplementation may not have been sufficient to elicit reductions in resting blood pressure among young, normotensive adults. This finding is somewhat unexpected, as a reduction in blood pressure is typically considered a hallmark of enhanced NO synthesis, mediated in part by NO-induced vascular smooth muscle relaxation (Gruetter et al., 1979), and has been observed in response to dietary supplementation with nitrate (Bailey et al., 2009), L-arginine (Bailey et al., 2010), and L-citrulline (Bailey et al., 2015). Future research is, therefore, warranted to examine whether L-citrulline supplementation may influence vascular function and other physiological responses associated with blood pressure regulation in both normotensive individuals and clinical populations.

We hypothesised that short-term L-citrulline supplementation would alter pulmonary gas exchange or ventilatory responses during ramp-incremental exercise and constant

work rate cycling test. However, contrary to our preliminary hypothesis, L-citrulline supplementation did not significantly affect pulmonary or ventilatory responses during exercise, irrespective of the administered dose. In particular, no significant differences between *Pla* and *Cit* conditions were observed for $\dot{V}O_2$ peak, a recognised indicator of cardiorespiratory fitness with established relevance to both health (Harber et al., 2017) and athletic performance (Jones et al., 2024). Similarly, no between-group differences were observed for the GET – a respiratory surrogate of the LT (Beaver et al., 1986) – or for the RCP – a surrogate of the MMSS (Whipp et al., 1989). Likewise, $\dot{V}_E$ max, $\dot{V}CO_2$ max, RER max, and HRmax did not differ between the *Cit* and *Pla* conditions (see **Table 5** for details). These findings diverge somewhat from those of Bailey et al. (2015), who reported discrete differences in pulmonary gas exchange and ventilatory responses during constant work rate cycling following seven days of supplementation with L-citrulline (6 g per day), despite improved tolerance to severe-intensity exercise and increased total work completed. In contrast, our results align with those of Hickner et al. (2006), who observed no significant effect of supplementation with L-citrulline on pulmonary gas exchange or ventilatory variables, including $\dot{V}O_2$ max, during ramp-incremental exercise test in healthy adults. Our findings also contrast the findings of Giannessini et al. (2011), who found that L-citrulline malate supplementation reduced

both phosphocreatine (PCr) and oxidative cost during skeletal muscle contraction *in situ* in the rat gastrocnemius muscle. The differences between studies may be attributed to the experimental model (human skeletal muscle contraction *in vivo vs.* isolated rat skeletal muscle *in situ*) and the supplementation protocol (L-citrulline *vs.* L -citrulline combined with malate). The NIRS-derived muscle oxygenation responses to the ramp-incremental cycling test, however, further support the absence of significant between-condition differences in pulmonary gas exchange and ventilatory responses (see **Fig. 11**). During ramp-incremental cycling, neither [oxy(Hb+Mb)] nor [deoxy(Hb+Mb)] differed between the *Cit* and *Pla* conditions. In particular, Δ[deoxy (Hb+Mb)] – a valid proxy for fractional O_2 extraction in skeletal muscle (Shiga et al., 1997) – supports our notion that muscle microvascular O_2 delivery, which could permit a greater $\dot{V}O_2$ during exercise, remains unaffected by L-citrulline supplementation. It is important to note, however, that NIRS-derived muscle oxygenation responses during exercise are subject to considerable spatial heterogeneities across the contracting quadriceps (Koga et al., 2007). Therefore, our data may reflect changes only within a discrete site of the vastus lateralis. Nonetheless, the absence of any differences in pulmonary gas exchange or ventilatory dynamics between supplement conditions (see **Tables 8 – 11** for further

details) reinforces the idea that L-citrulline had no discernible effect on systematic or local muscle oxygenation during exercise.

A proposed mechanism by which L-citrulline supplementation may influence exercise performance involves the buffering of ammonia via urea cycle. By reducing circulating ammonia levels, L-citrulline is thought to facilitate the aerobic utilisation of pyruvate, diminishing reliance on anaerobic metabolism and attenuating lactate accumulation. This metabolic shift may help to sustain muscle contraction and delay the onset of fatigue (Mutch et al., 1983). Supporting this notion, early work in humans observed that supplementation with L-citrulline malate decreased ammonia levels and mitigated metabolic acidosis, an effect potentially associated with muscle fatigue (Callis et al., 1991). However, the literature remains inconclusive concerning the extent to which L-citrulline supplementation alters anaerobic metabolism, primarily lactate dynamics, and its consequent impact on exercise performance (Martínez-Sánchez et al., 2017a). In the current study, no significant differences in blood [La^-] were demonstrated during either the ramp-incremental cycling test (**Table 6**) or the constant work rate cycling test (**Table 12**) following supplementation with *Pla* or *Cit12*, thus suggesting a limited effect of L-citrulline on lactate metabolism under these exercise conditions. Given the

apparent absence of changes in indicators of both aerobic and anaerobic metabolism, it is unsurprising that no improvements in exercise efficiency and/or exercise tolerance were detected. In both the ramp-incremental and constant work rate cycling tests, time to task failure did not differ between supplement conditions (**Fig. 7** and **Fig. 17**). These results are somewhat conflicting in the existing literature. For example, Bailey et al. (2015) reported a 12% increase in exercise tolerance at severe-intensity exercise and a 7% enhancement in total sprint work completed after L-citrulline supplementation. Additionally, studies using L-citrulline malate have demonstrated improved muscle force production and contractile efficiency in humans (Giannessini et al. (2011). However, the evidence linking L-citrulline supplementation to improved exercise tolerance remains inconsistent. For example, Hickner et al. (2006) reported impaired exercise capacity during incremental exercise following supplementation in healthy adults. The source of these discrepancies remains unclear, but may be attributed to differences in study design, including dosing regimens and, importantly, exercise testing protocols. Earlier studies used ramp incremental exercise tests, which, although scientifically informative, primarily assess exercise tolerance rather than performance (Jeukendrup et al., 1996). Conversely, previous investigations have utilised constant power-to-fatigue tests to examine volitional exhaustion, an approach widely used in

exercise physiology to evaluate tolerance (Koppo et al., 2009; Vanhatalo et al., 2010). These tests, however, have been criticised for limited ecological validity and poor test-retest reliability (Jeukendrup et al., 1996). When pre-exercise conditions are rigorously standardised and participants are appropriately familiarised, however, constant power tests may yield more reliable performance measures than previously assumed (Amann et al., 2008).

Contrary to our initial hypothesis, the dose-dependent increases observed in plasma concentrations of [Arg], [Orn], and AAI did not translate into measurable effects of L-citrulline on exercise tolerance. This finding does not support the ergogenic efficacy of L-citrulline under the specific conditions investigated. Whether higher doses of L-citrulline, potentially leading to further elevations in AAI and NO production, could be required to elicit performance benefits in healthy, moderately trained young adults, remains uncertain. It is plausible, however, that different results could be expected in other populations, such as untrained adults or clinical groups, in whom impairments in NO synthesis and bioavailability are more pronounced (Moncada and Higgs, 2006). In such cases, the physiological response to L-citrulline may be enhanced, increasing thus the likelihood of a beneficial impact on exercise tolerance and/or exercise efficiency.

This hypothesis, however, is naturally speculative and remains to be studied. It should be noted that alternative NO-boosting supplements, such as dietary nitrate, have been shown to elevate plasma NO_3^- and NO_2^-, reduce the O_2 cost of submaximal exercise, and enhance performance under certain circumstances in healthy, moderately trained young adults (Bailey et al., 2009). Whether a minimum threshold of plasma NO_3^- and NO_2^- elevation is required to induce NO synthesis and bioavailability and performance gains in similar populations remains unclear. Hence, the lack of direct measurement of plasma concentrations of NO_3^- and NO_2^- constitutes an important limitation of our study. In the present investigation, only precursor amino acids (e.g., [Arg]) and indirect indicators such as AAI were assessed, which may not reliably reflect improvements in NO synthesis or bioavailability in the studied population.

6 | Conclusions

In recent years, dietary supplementation with L-citrulline has emerged as a promising nutritional strategy to enhance exercise performance through the augmentation of NO bioavailability. The ergogenic potential of L-citrulline, however, remains equivocal. Discrepancies across studies may be attributed, at least in part, to variations in exercise testing protocols and/or differences in supplementation regimens, including dosage. These factors, however, have not been systematically evaluated, and the existence of a dose-response relationship remains uncertain. To address this knowledge gap in the literature, the present thesis investigated the effects of varying doses of L-citrulline on exercise tolerance and associated physiological responses during ramp-incremental (***Study 1***) and constant work rate cycling tests (***Study 2***) in moderately trained adults. The principal original finding of the present thesis was that blood pressure, pulmonary gas exchange and ventilation, and NIRS-derived muscle oxygenation responses to both ramp-incremental and constant work rate cycling remained unaltered, regardless of the L-citrulline dosage. This absence of effects occurred in contrast to significant dose-dependently elevations in plasma [Arg], [Orn], and AAI. The findings of the current

thesis, therefore, do not support the ergogenicity of L-citrulline under the studied conditions. The study sample, however, consisted exclusively of healthy, moderately trained young male participants, which further limits the generalisability of our results. Future research, therefore, should explore the effects of L-citrulline supplementation across diverse populations, such as untrained individuals, clinical groups, or females. Despite this fact, from a practical standpoint, the current results offer valuable insights regarding the safety and practical applicability of acute L-citrulline supplementation in healthy, trained adults. These findings may inform future research directions and contribute to the development of evidence-based recommendations in the field of sports nutrition and exercise science.

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