The Effects of Exogenous Cardiolipin on Skeletal Muscle, Aerobic Exercise and Anxiety-Related Behaviour

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ABSTRACT

The Effects of Exogenous Cardiolipin on Skeletal Muscle, Aerobic Exercise and Anxiety-Related Behaviour

Amanda Laplante

Cardiolipin is a phospholipid found in the inner mitochondrial membrane and, consequently, is involved in energy metabolism. Cardiolipin is released from necrotic cells following myocardial ischemia and reperfusion injury. Furthermore, increased levels of anxiety have been seen in individuals following myocardial infarction. Despite its various effects on liver, smooth and cardiac muscle, the impact of increased cardiolipin on skeletal muscle and anxiety-like behaviour remains unknown. **PURPOSE**. This two-part project investigated the effects of exogenous cardiolipin on voluntary exercise metrics, forced exercise training, functional aerobic capacity, anxiety-related behaviors, and mitochondrial respiration of skeletal muscle in a mouse model. **METHODS**. C57BL/6 mice were randomized to an experimental and a control group. The mice were injected 2x/week with 0.1ml of cardiolipin (0.25mg/ml or 0.5mg/ml) or placebo solution. In-cage running wheels measured voluntary running, while high-intensity interval training was used as a method of exercise training. Aerobic capacity was assessed by maximal endurance or maximal graded exercise tests. Open-field test and elevated plus maze served to measure anxiety-related behaviours. Lastly, mitochondrial respiratory capacity of the vastus lateralis muscle was measured by high-resolution respirometry. **SIGNIFICANCE.** Overall, this research has significant implications for advancing our understanding of cardiolipin biology, cardiovascular physiology, skeletal muscle function, and mental health, with potential implications for developing novel therapeutic interventions and improving patient outcomes.

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CONTRIBUTION OF AUTHORS

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Dr. Konrad Schoettner: Conceptualization, methodology, project administration, resources, supervision, formal analysis, writing (review and editing)

All authors reviewed the final manuscript and approved of the contents.

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CHAPTER I

Introduction

THEORETICAL CONTEXT

Cardiolipin

Cardiolipin (CL), a phospholipid predominantly found in the inner mitochondrial membrane (IMM), constitutes approximately 25% of the total phospholipid content in cardiomyocytes (Gasanoff et al., 2021). Moreover, it has been detected in human serum, suggesting that CL exists in the extracellular space as a component of plasma lipoproteins (Deguchi et al., 2000; Murray et al., 2022). Its structure is distinct from other phospholipids as it contains four acyl chains and a negatively charged head group composed of two phosphate groups and a glycerol bridge, resulting in a conical shape (Dudek, 2017; Gasanoff et al., 2021). In turn, CL applies lateral pressure within the bilayer membrane, prompting negative curvature and leading to the bending of the inner monolayer of the IMM, forming cristae (Gasanoff et al., 2021). Consequently, this phospholipid plays an imperative role in mitochondrial function and membrane fluidity (Bradley et al., 2016).

The de novo synthesis of CL is facilitated by mitochondrial enzymes and involves a four-step process that largely aligns with the phosphatidylglycerol pathway. However, the final step is uniquely catalyzed by CL synthase to yield the CL archetype (Gonzalvez and Gottlieb, 2007). In this reaction, the enzymes exhibit no selectivity for a particular acyl chain length. Therefore, due to the extensive variety of potential acyl chain combinations, variations in CL molecular species are observed across organisms and tissues (Gonzalvez and Gottlieb, 2007). Eukaryotic CL is composed of 18-carbon (C18) chains and in human heart and skeletal muscle, tetralinoleoyl-cardiolipin (L4-CL) stands as the most prevalent species (Shen et al., 2015). After synthesis, a maturation process replaces the original acyl chains with specific unsaturated C18 equivalents.

This involves a cyclic sequence of two reactions, starting with the hydrolysis of one original acyl chain to yield monolysocardiolipin (MLCL) with three acyl groups, followed by reacylation of MLCL with an appropriate C18 acyl chain (Gonzalvez and Gottlieb, 2007).

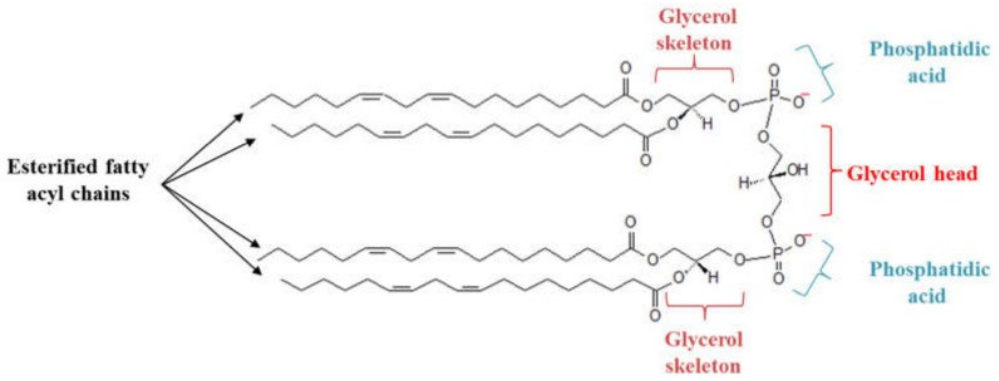

Figure 1. Cardiolipin structure (Ahmadpour et al., 2020).

Mitochondrial Electron Transport Chain

A crucial function of CL within the mitochondria is its involvement in the electron transport chain (ETC), which is responsible for producing energy in the form of adenosine triphosphate (ATP). This process, known as oxidative phosphorylation (OXPHOS), is the primary means of energy generation under aerobic conditions. Within the inner mitochondrial membrane, electrons are transferred by carriers along the ETC from complex I to IV. Concurrently, protons are moved from the mitochondrial matrix to the intermembrane space, creating an electrochemical gradient. The ultimate electron acceptor, complex IV, transfers the electrons to an oxygen molecule while protons flow back across the membrane through ATP synthase (complex V) which releases energy to generate ATP from ADP and inorganic phosphate (Djafarzadeh & Jakob, 2017). Cardiolipin interacts with various mitochondrial proteins, including complex I, complex III, complex V and cytochrome c, making it an essential element of this process (Schlame et al., 2000).

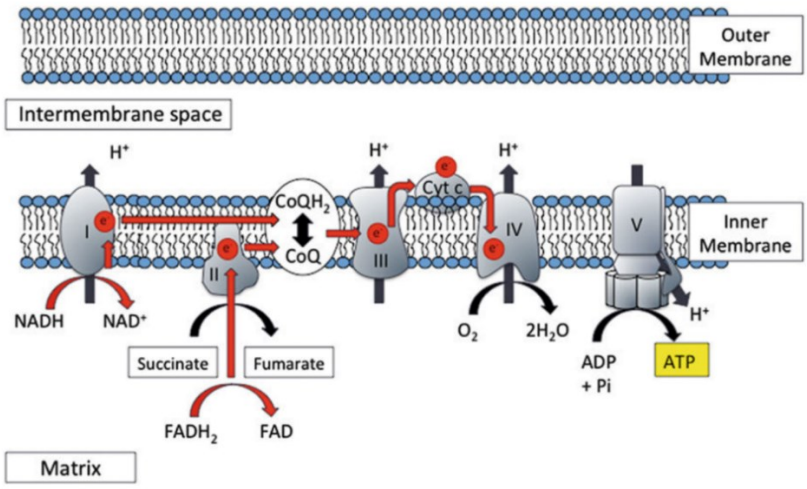

Figure 2. The mitochondrial electron transport chain (de Villiers et al., 2018).

Cardiolipin and Mitochondrial-Dependent Apoptosis

In addition to its implication in bioenergetics and cristae morphology, CL plays a multifaceted role in mitochondria-dependent apoptosis. This process first requires the permeabilization of the outer mitochondrial membrane (OMM) which is induced by pro-apoptotic signals originating from the cytosol (Gonzalvez and Gottlieb, 2007). In response, CL is translocated from the IMM to the OMM and serves as an indicator of mitochondrial dysfunction (Dudek et al., 2019; Pizzuto and Pelegrin, 2020). Cardiolipin then becomes an essential binding platform for pro-apoptotic factors such as Caspase-8, BCL2 associated X protein (Bax) and BH3 interacting domain death agonist (Bid), ultimately leading to pore formation in the OMM (Dudek et al., 2019; Pizzuto and Pelegrin, 2020). Cytochrome c, which is normally involved in ATP generation through electron transfer from complex III to complex IV, is predominantly bound to the outer leaflet of the IMM via specific interactions with CL (Gonzalvez & Gottlieb, 2007). However, oxidation of the phospholipid causes the detachment of cytochrome c from the IMM, allowing it to travel from the intermembrane space to the cytosol through the previously formed pored in the OMM (Ott et al., 2007; Pizzuto and Pelegrin, 2020). Here, the free cytochrome c triggers apoptosome formation and activates the caspase cascade, ultimately leading to cell degradation (Ott et al., 2007; Pizzuto and Pelegrin, 2020).

Cardiolipin Dysregulation and Associated Pathological Conditions

Changes in the composition and abundance of CL significantly influence mitochondrial efficiency. A deficiency in CL leads to a reduction in both the length and number of cristae, as well as a decrease in the overall surface area of the IMM, diminishing ATP production (Gasanoff et al., 2021). Conversely, as seen in the heart, tissues with an elevated concentration of CL and a denser arrangement of cristae in the IMM display more efficient ATP synthesis and successfully support greater energetic needs (Gasanoff et al., 2021).

Given its role in various mitochondrial and cellular processes, modifications in the quantity and structure of the phospholipid are associated with a wide range of pathological conditions, including Barth syndrome, diabetic cardiomyopathy and myocardial ischemic-reperfusion injury (Claypool and Koehler, 2012; Gasanoff et al., 2021; Shen et al., 2015)

Barth Syndrome

Barth syndrome is a rare X-linked genetic disorder with a wide variety of clinical phenotypes ranging from symptom-free presentations to cardiomyopathy, skeletal myopathy, neutropenia and growth retardation (Jefferies, 2013; Shen et al., 2015). Biochemically, this condition is characterized by an 80% decrease of CL skeletal muscle as well a 20% decrease in cardiac tissue (Shen et al., 2015). Tafazzin is a phospholipid transacylase located in the inner leaflet of the mitochondrial membrane that partakes in CL remodeling (Jefferies, 2013). It catalyzes the transition from immature CL after initial synthesis to mature CL, which is predominantly L4-CL. More than 160 different mutations in the tafazzin gene have been identified as the cause of Barth Syndrome (Shen et al., 2015). Tafazzin dysfunction leads to a rise in MLCL, the intermediate species of the phospholipid. Therefore, patients with Barth Syndrome present with an increased MLCL:L4-CL ratio (Jefferies, 2013). In turn, a destabilization of mitochondrial respiratory complexes is observed which hinders energy production and results in skeletal muscle impairments leading to compromised functional exercise capacity and reduced daily activity (McKenzie et al., 2006; Sabbah, 2021)

Diabetic Cardiomyopathy

Diabetes is a metabolic disorder characterized by prolonged elevated blood glucose levels, resulting from either the autoimmune-mediated destruction of pancreatic β-cells leading to inadequate insulin production (Type I) or peripheral resistance and defective insulin secretion (Type II) (Ndisang et al., 2017). Diabetes significantly increases the risk of cardiovascular disease, whereby diabetic cardiomyopathy emerges as the leading cause of mortality in people with this chronic disease (Leon & Maddox, 2015). Altered lipid composition and mitochondrial dysfunction in cardiomyocytes are typical diabetic comorbidities (Han et al., 2000). Trends of decreased total cardiac CL levels have been seen in the early stages of both Type I and Type II diabetes (Han et al., 2005). Therefore, these findings suggest a potential association between CL content, mitochondrial dysfunction and the development and progression of diabetic cardiomyopathy (Shen et al., 2015).

Myocardial Ischemia-Reperfusion Injury

Myocardial ischemia, which is characterized by insufficient blood flow to the myocardium, as well as the subsequent reperfusion of heart tissue lead to irreversible injury and cell death (Shen et al., 2015). Throughout the process of obstruction and restoration of blood flow, an overproduction of reactive oxygen species (ROS) occurs, triggering lipid peroxidation and damage of cellular macromolecules, including CL (Bugger and Pfeil, 2020; Shen et al., 2015). The combination of reduced ATP demand during ischemia, which results in a build-up of NADH, along with damaged ETC complexes heightens the probability of nonspecific electron leakage. This results in the generation of superoxide, the principle free radical produced by this process (Bugger and Pfeil, 2020; Kehrer et al., 2010). Unsaturated CL acyl species are especially susceptible to oxidative damage, leading to decreased total CL levels and increased peroxidized CL (Shen et al., 2015). These modifications in the CL profile result in altered bioenergetic parameters, including reduced rates of oxygen consumption and decreased activity of ETC complexes I, III, and IV (Bugger and Pfeil, 2020; Shen et al., 2015). Consequently, a feedback loop emerges during ischemia-reperfusion injury, where ROS-induced damage to CL impairs the

ETC complexes, leading to further ROS generation and exacerbating mitochondrial dysfunction and myocardial injury.

Cardiolipin Antibodies and Myocardial Infarction

Myocardial infarction (MI), commonly known as a heart attack, arises from diminished blood flow to the heart muscle. Consequently, the oxygen deprivation leads to injury and death of cardiomyocytes (Lu et al., 2015). The presence of antibodies to CL (aCL) in the blood following MI suggests that CL may be released into circulation upon some occurrences of MI. A longitudinal study involving 62 survivors of MI under the age of 45 measured aCL levels at 3-, 12-, and 36-months post-event. Twenty one percent of patients exhibited elevated aCL levels on at least two out of the three sampling occasions, with eight patients displaying titres five times higher than in voluntary blood donors (Hamsten et al., 1986). Similarly, 37 of 597 MI patients tested positive for aCL at a mean of 27.6 days post-MI (Sletnes et al., 1992). Additionally, a significant association was found between MI and aCL positivity, with 11.1% of MI patients testing positive for aCL IgG 6 to 10 weeks post-MI compared to 1.3% of control participants (Grosso et al., 2019). These findings highlight the link between MI and the presence of aCL, warranting further exploration into the potential role of elevated CL levels in the body.

Mitochondrial Efficiency and Aerobic Performance

Mitochondrial efficiency plays a significant role in aerobic performance as the generation of ATP is required for sustained exercise. Decreased mitochondrial respiration can disrupt OXPHOS and lead to a reduction in energy production. Contrarily, individuals with greater rates of respiration exhibit enhanced aerobic performance (Jacobs & Lundby, 2013). Multiple ways exist to quantify mitochondrial efficiency, which is essential in determining the overall energy output and health of a tissue or an organism under different conditions. For instance, measurements of cellular respiration, such as oxygen consumption, provide insight on respiratory capacity across complexes I-IV by comparing relative fluxes through the ATP synthase and proton leak pathways (Brand & Nicholls, 2011; Djafarzadeh & Jakob, 2017). As such, the acceptor control ratio (ACR)

can be used to assess coupling efficiency across complex I, whereas the respiratory control ratio (RCR) compares maximal values of OXPHOS and LEAK respiration to determine overall mitochondrial health (Brand & Nicholls, 2011).

High-Intensity Interval Training and Mitochondrial Adaptations

High-Intensity Interval Training (HIIT) is a form of exercise characterized by brief and repeated bouts of vigorous activity performed at a workload typically corresponding to 90% of VO_2 max or, in mice, 90% of the rodents' maximal running speed (Buchheit and Laursen, 2013). This method has shown to induce physiological adaptations and health benefits comparable to Moderate-Intensity Continuous Training (MICT), but with less time commitment (Atakan et al., 2021; Gray et al., 2016).

Research done on animals and humans has found HIIT to promote health by exerting favorable effects on various risk factors associated with cardiometabolic diseases (Batacan et al., 2016). These include improvements in insulin sensitivity, lipid profiles, mitochondrial biogenesis, glucose control, and weight loss. In mice, HIIT has been associated with beneficial effects on glucose metabolism, metabolic dysfunctions, grip strength, treadmill endurance and gait speed (Chavanelle et al., 2017; Seldeen et al., 2018; Wang et al., 2017). In humans, the cardiovascular benefits of HIIT are evident in reduced mean heart rate and improvements in heart rate variability (Munk et al., 2010). Furthermore, HIIT is associated with significant enhancements in quality-of-life parameters, including physical function, and general and mental health (Mangiamarchi et al., 2017).

Studies have demonstrated increased exercise capacity and skeletal muscle adaptations following HIIT (Chrøis et al., 2020; Little et al., 2010). Mitochondrial adaptations include an increase in complex IV abundance as well as protein content and maximal activity of citrate synthase (CS) and cytochrome c oxidase (COX), which are required for the efficient production of ATP (Chrøis et al., 2020; Little et al., 2010). Additionally, HIIT training has resulted in an elevation in the protein content of mitochondrial transcription factor A (Tfam) and a substantial increase in total sirtuin 1 (SIRT1) content (Little et al., 2010). Both play imperative roles as regulators of mitochondrial biogenesis.

High-Intensity Interval Training and Anxiety

Fear is as an adaptive mechanism designed for alarm (Dean, 2016). However, when fear negatively affects everyday life by exceeding necessary levels, arising in inappropriate contexts or persisting to a chronic extent, it is identified as anxiety (Dean, 2016). This intricate emotional state can be divided into various categories such as state and trait anxiety or normal and pathological anxiety. It is primarily characterized by feelings of worry, fear and uneasiness alongside increased heart rate, muscle tension and restlessness (Bourin et al., 2007). While anxiety can manifest itself in various contexts, increased levels have been seen in individuals following myocardial infarction (Feng et al., 2016). The heightened incidence is often associated with concerns of future fatal and nonfatal cardiac events, hospital readmission and cardiac mortality (Feng et al., 2016).

Rodent models of anxiety are categorized into two subclasses: (1) conditioned responses to stressful or painful events, and (2) spontaneous and natural reactions to stressful stimuli that do not involve pain, such as exposure to a novel or highly illuminated test area (Kumar et al., 2013). Observable behaviours and physiological markers are used to study anxiety in mice. Behavioural manifestations include altered exploratory behaviour, avoidance of open spaces and exaggerated startle responses (Bourin et al., 2007).

The relationship between anxiety and HIIT has been explored in both human and animal studies. In humans, HIIT exhibits fast-acting and sustained treatment effects on Generalized Anxiety Disorder (Plag et al., 2020). In rodents, HIIT was capable of reducing anxiety-like behaviour in diabetic rats, in obese mice that were following a high-fat diet, and in mice with Alzheimer's disease (Foroozan et al., 2021; Orumiyehei et al., 2022; Ying et al., 2023).

RATIONALE AND OBJECTIVES

Cardiolipin plays a critical role in both membrane fluidity and mitochondrial function (Bradley et al., 2016). However, its precise role when released into the blood after MI remains unknown. Studies have shown that increased concentrations of CL mimicking post-infarction conditions negatively affect endothelial cell migration and proliferation as well as angiogenic sprouting, ultimately hindering blood vessel formation (Carnevale and Bergdahl, 2015). Moreover, acute increases in CL have resulted in a decrease in mitochondrial respiration in vascular smooth muscle, impairing the tissue's ability to oxidize glucose and fatty acid substrates (Galambo and Bergdahl, 2023). Despite these findings, the impacts of heightened CL levels on skeletal muscle remain unexplored.

Objective 1: To investigate the effects of exogenous cardiolipin on voluntary exercise metrics, functional aerobic capacity, and mitochondrial respiration in skeletal muscle.

Cardiolipin falls within the category of glycerophospholipids, which are brain membrane lipids and hypothesized to be promising molecules for the treatment of stress-related disorders (Müller et al., 2015). Moreover, increased levels of anxiety have been reported following myocardial infarction (Feng et al., 2016). However, the impact of CL on anxiety remains unexplored.

Additionally, HIIT has shown to effectively reduce anxiety-like behaviours in both humans and rodents (Orumiyehei et al., 2022; Plag et al., 2020), and exploring the effects of exercise training will further our understanding of the impact of CL on skeletal muscle.

Objective 2: To investigate the effects of exogenous cardiolipin on voluntary exercise metrics, forced exercise training, functional aerobic capacity, anxiety-related behaviors, and mitochondrial respiration in skeletal muscle.

HYPOTHESIS

It is hypothesized that the release of CL during myocardial infarction plays a role in alleviating the heart's workload, thus aiding in its recovery. Reduced mitochondrial respiration in vascular smooth muscle leads to a decline in ATP production, resulting in decreased vasoconstriction. This, in turn, enhances blood flow to tissues to compensate for reduced ejection efficiency. In addition, cellular processes such as proliferation, migration, and angiogenic sprouting rely on ATP. Temporarily decreasing these activities reduces the demand for ATP, enabling resources to be reallocated elsewhere.

Additionally, it is hypothesized that extract oxygen will increase in skeletal muscle, consequently alleviating the workload of the heart by reducing oxygen demand in the periphery. The addition of exogenous CL is anticipated to improve mitochondrial respiratory capacity, enhance functional aerobic capacity and voluntary running metrics. Lastly, it is hypothesized that HIIT exacerbates these effects while decreasing anxiety levels.

SIGNIFICANCE

Overall, this thesis has significant implications for advancing our understanding of CL biology, cardiovascular physiology, skeletal muscle function, and mental health, with potential implications for developing novel therapeutic interventions and improving patient outcomes.

Exploring the effects of exogenous CL on skeletal muscle function provides valuable insights into its role beyond the cardiovascular system. Understanding how CL influences mitochondrial respiration and functional aerobic capacity in skeletal muscle can have implications for exercise physiology and supplementation.

Understanding how CL supplementation and exercise training may influence anxiety levels could have implications for mental health and stress management strategies, particularly in individuals recovering from myocardial infarction.

Lastly, investigating the effects of CL on peripheral tissue following myocardial infarction provides insights into its role in cardiovascular health and disease progression. This understanding could lead to the development of novel therapeutic strategies and inform clinical practices to mitigate the adverse effects of myocardial infarction and improve recovery.

CHAPTER 2

Manuscript I

Intraperitoneal Administration of Cardiolipin Enhances Mitochondrial Respiration in Skeletal Muscle of Male Mice

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ABSTRACT

Cardiolipin, a phospholipid located in the inner mitochondrial membrane, is released following myocardial ischemia and reperfusion injury. Despite its various effects on other tissues, the impact of increased cardiolipin on skeletal muscle remains unknown. This study aimed to examine the effects of cardiolipin on aerobic capacity and mitochondrial respiration of skeletal muscle using a mouse model. Male C57BL/6 mice were randomized to an experimental (n=11) and a control group (n=12). The mice were injected 2x/week for 6 weeks with 0.1ml of cardiolipin (0.5mg/ml) or placebo solution. Concurrently, voluntary running distance was also measured. At baseline, week 3 and week 6, aerobic capacity was assessed by recording time to exhaustion on a rodent treadmill at a speed of 16m/min. At the end of the intervention, the mice were euthanized, the vastus lateralis muscle was extracted and mitochondrial respiratory capacity was measured by high-resolution respirometry. The results show that exogenously elevated levels of cardiolipin lead to increased oxygen consumption in skeletal muscle. Mitochondrial density, determined by immunoblotting, was similar between groups, suggesting that the difference observed is likely due to improved respiratory efficiency. These findings allow for a better understanding of the implications of cardiolipin on energy generation and aerobic performance. Future considerations include the effects of cardiolipin and imposed exercise training on respiratory capacity in skeletal muscle.

INTRODUCTION

Cardiolipin (CL) is a phospholipid that constitutes up to 20% of the inner mitochondrial membrane (IMM) (Bradley et al., 2016; Tatsuta and Langer, 2017). Its structure is distinct as it contains four acyl chains and a negatively charged head group composed of two phosphate groups and a glycerol bridge, resulting in a conical shape (Dudek, 2017; Gasanoff et al., 2021). In addition to its implication in cristae morphology, CL plays a multifaceted role in mitochondrial-dependent apoptosis by facilitating the recruitment of essential factors (Dudek, 2017; Gonzalvez and Gottlieb, 2007; Pizzuto and Pelegrin, 2020).

Cardiolipin plays a crucial role by interacting with key proteins in the electron transport chain (ETC), such as cytochrome C, as well as complex I, III and V (Schlame et al., 2000). These interactions are essential for efficient energy production within the mitochondria.

Mitochondria play a significant role in aerobic performance as the generation of adenosine triphosphate (ATP) is required for sustained exercise. Reduced mitochondrial respiration can impair OXPHOS, resulting in decreased energy production. Conversely, individuals with higher rates of respiration typically demonstrate improved aerobic performance (Jacobs and Lundby, 2013).

Changes in CL composition and abundance impact mitochondrial efficiency and have been linked to several disorders. In conditions characterized by a loss of CL, such as Barth syndrome, a destabilization of mitochondrial respiratory complexes hinders energy production and results in reduced aerobic capacity (McKenzie et al., 2006). Similarly, a reduction in CL through modulation of the very-long chain fatty acid (VLCFA) pathway results in decreased coupled respiration in skeletal muscle (Prola et al., 2021).

Conversely, elevated levels of anti-CL antibodies have been found in young post-myocardial infarction patients, suggesting that CL is released into the blood upon cardiomyocyte necrosis (Hamsten et al., 1986). Increased concentrations of CL mimicking post-infarction physiological conditions have been shown to negatively affect endothelial cell migration and proliferation as well as angiogenic sprouting, therefore hindering blood vessel formation (Carnevale and Bergdahl, 2015). Moreover, an acute increase in CL resulted in a decrease in mitochondrial

respiration in vascular smooth muscle, impairing the tissue's ability to oxidize glucose and fatty acid substrates (Galambo and Bergdahl, 2023).

Although CL has been shown to have various effects on body tissues, the impacts of an increase in CL on skeletal muscle have not yet been explored. The purpose of this study was to examine the effects of a chronic increase in CL levels on functional aerobic capacity and mitochondrial respiration in skeletal muscle. It was hypothesized that exogenously increased CL concentrations would negatively affect oxygen consumption and ATP production, consequently decreasing aerobic performance.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice aged 6-7 months were obtained from the Concordia University breeding colony. Females were excluded to eliminate the potential impact of estrous cycle variations on exercise performance (Aguiar et al., 2018). The mice were screened and selected based on their ability and willingness to run on a rodent treadmill at a speed of 4m/min before random assignment to the control (CON) or cardiolipin (CL) group (Bouganim and Bergdahl, 2017). The animals were housed individually in cages equipped with a running wheel and kept in a thermoneutral environment (22°C) while subjected to a 12:12 light/dark photoperiod. Food and water were available ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (#30000259) and performed in compliance with guidelines set forth by the Canadian Council on Animal Care.

Experimental protocol

Over a 6-week period, the mice received two intraperitoneal injections per week of 0.1 mL containing CL at a concentration of 0.5mg/mL for the CL group or placebo solution containing physiological salt solution [135.5 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl, 11.6 mM glucose, 11.6 mM HEPES (pH 7.35)] for the CON group. This quantity of CL resembles physiological concentrations (Deguchi et al., 2000).

Voluntary wheel running

Running wheels were connected to a bike computer and gathered data on voluntary running activity. Prior to injection, total distance run was recorded. The data was then assessed on a meters per day basis.

Functional aerobic capacity assessment

An adapted rodent treadmill was used to carry out maximal endurance tests at baseline, week 3 and week 6 (Bouganim and Bergdahl, 2017). The mice were subjected to a 10-minute warm-up period consisting of three 2-min bouts of exercise at a speed of 4m/min, 8m/min and 12m/min, respectively, followed by a 4-min bout at 16m/min. After a 2-min rest with the treadmill off, the treadmill was turned on for a 2-min rest period with auditory acclimatization. Then, three 2-min bouts of exercise at a speed of 4m/min, 8m/min and 12m/min, respectively, were repeated. Thereafter, the speed was increased to 16m/min and time to exhaustion was recorded to the nearest minute. Exhaustion was defined as the moment when the mouse remained in contact with the brush found at the bottom of the treadmill for 10 seconds or more despite tactile motivation.

Tissue permeabilization

After the 6-week period, the mice were euthanized by isoflurane anaesthesia, CO_2 asphyxiation and cervical dislocation. The vastus lateralis muscle was immediately extracted and permeabilized for mitochondrial respiratory assessment. Upon extraction, the vastus lateralis muscle was rid of connective tissue and the fibers were gently separated with forceps. The isolated tissue incubated for 30 minutes on ice in 50 µg/mL saponin and 2mL BIOPS buffer solution [2.77mM CaK_2EGTA, 7.23mM K2EGTA, 5.77mM Na2ATP, 6.56mM MgCl2·6H2O, 20mM Taurine, 15mM Na2Phosphocreatine, 20mM Imidazole, 0.5mM Dithiothreitol, 50mM MES (pH 7.1) (Fontana-Ayoub et al., 2016). Subsequently, the tissue underwent two consecutive 10-minute washes in MiR05 buffer [0.5mM EGTA, 3.0mM MgCl2·6H2O, 60mM K-lactobionate, 20mM Taurine, 10mM KH2PO4, 20mM HEPES, 110mM Sucrose, 1g/L BSA (pH 7.1)] (Kuznetsov et al., 2008).

Mitochondrial respiratory measurements

Mitochondrial oxygen consumption was measured via high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Approximately 2-2.5 mg of muscle tissue were added to 2mL of MiR05 buffer in each chamber. The experiments were carried out at 37°C in a hyper-oxygenated environment to prevent oxygen limitation. Malate (2mM), glutamate (10 mM) and pyruvate (6 mM) were added in succession to stimulate LEAK respiration across complex I and to build the proton gradient across the inner mitochondrial membrane. ADP (5mM) was then incorporated to initiate complex I-dependent OXPHOS respiration. The outer mitochondrial membrane integrity was assessed upon the addition of cytochrome c (10µM). Subsequently, succinate (10mM) was added to measure maximal respiration, followed by oligomycin (2µg/mL) to inhibit ATP synthase and observe maximal LEAK respiration. Lastly, an FCCP (0.25µM) titration was carried out to assess maximal uncoupled respiration. The high-resolution respirometry values were obtained from stabilized O2 flux per mass (pmol/s/mg) readings following the addition of each substrate. Data was analyzed by DatLab 7.0 software. The acceptor control ratio (ACR) was determined by dividing ADP by pyruvate values while the respiratory control ratio (RCR) was calculated by dividing succinate by oligomycin values.

Protein extraction, immunoblotting, and immunofluorescence

Immunoblotting with an antibody specific for the voltage-dependent anion channel (VDAC) was used to determine mitochondrial density. Cell lysates were extracted in lysis buffer containing (in mmol/L) NaCl 250, HEPES 50, glycerol 10%, Triton X-100 1%, MgCl2 1.5, EGTA 1, Na4P2O7 10, NaF 1, Na3VO4 800 mol/L, pH 7.5 and centrifuged at 13 000g for 10 min. Supernatant was collected and equal volumes of lysates were separated on a 10% SDS–PAGE and transferred to a nitrocellulose membrane (0.02 µm, Biotrace NT Nitrocellulose) using 10 mmol/L sodium tetraborate buffer. The membranes were blocked in 3% BSA in TBS-T buffer (10 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20) for 1h at room temperature followed by overnight incubation at 4 °C with primary antibody VDAC (1:2000, ab14734 Abcam). The blots were washed 2x10 minutes in TBS-T, incubated with horseradish-peroxidase-conjugated secondary antibodies (anti-mouse, ab6728; Abcam), and visualized with a chemiluminescence

system (Immun-Star Chemiluminescent; 1705070; Bio-Rad, Mississauga, Ontario, Canada). The bands were analyzed using the ImageJ software.

Statistical analysis

Data are presented as mean ± standard error (SE) except immunoblotting, which is mean ± standard error of the mean (SEM). Baseline refers to the period before the intervention, whereas Week 1 indicates the first week of injections. Normality was assessed using the Shapiro-Wilk normality test. Independent-sample two-tailed t-tests were carried out to compare data between the control and cardiolipin groups for mitochondrial respiratory capacity measurements, acceptor control ratio, respiratory control ratio and mitochondrial density. To quantify the magnitude of the differences between groups for the mitochondrial respiratory measurements, Cohen's d was calculated as a measure of effect size. A mixed-model ANOVA was used to compare the results between groups and across time for voluntary running distance and functional aerobic capacity. Post hoc comparisons were conducted using Tukey's HSD test. A p-value of < 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics (Version 28.0).

RESULTS

Voluntary Running Distance

There was no significant difference in daily voluntary running distance between the CON (n=12) and CL (n=11) groups at any of the time points (Fig. 1). The mixed-model ANOVA revealed a statistically significant main effect of time, whereby both groups experienced a statistically significant increase in daily running distance at all timepoints when compared to week 1 of the experiment ($F= 31.04$, $p<0.0001$). Values increased from 39.74 ± 5.85 m/day and 46.85 ± 8.25 m/day during week 1 to 86.53 ± 11.91 m/day and 85.11 ± 6.28 m/day during week 2 for the CON and CL mice, respectively. Thereafter, the CON mice yielded daily running distances of 93.10 ± 8.51 m/day, 98.12 ± 8.36 m/day, 96.86 ± 7.85 m/day and 91.13 ± 7.15 m/day for weeks 3 to 6,

respectively. Similarly, the values for the CL group were 94.15 ± 6.97 m/day, 92.14 ± 5.93 m/day, 94.34 ± 7.52 m/day and 87.81 ± 6.40 m/day.

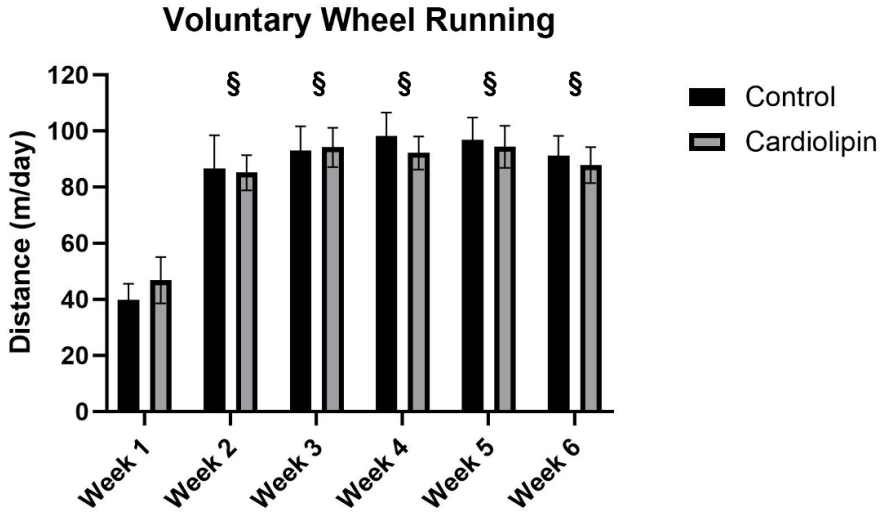

Figure 1. Voluntary running distance expressed in meters per day. No significance differences were found between the control group (n=12) and the cardiolipin group (n=11). § denotes a statistically significant difference compared to week 1, where week 2 to week 6 mean values for both groups were found to be significantly increased. Values are expressed as mean ± SE. §$p<0.05$.

Functional Aerobic Capacity

No significant differences in running capacity were reported between the two groups at any of the three time points (Fig. 2). The mixed-model ANOVA revealed a statistically significant main effect of time ($F=28.16$, $p<0.0001$). Both the CON and CL groups showed a significant increase in running time at week 3 (CON: 161.92 ± 26.28 min, CL: 145.73 ± 23.49 min) and week 6 (CON: 197.75 ± 24.63 min , CL: 192.00 ± 29.92 min) compared to the baseline functional aerobic capacity test (CON: 71.33 ± 9.11 min, CL: 100.18 ± 12.99 min) ($p<0.05$). Additionally, the CL animals ran significantly longer at week 6 compared to week 3 ($p= 0.03$).

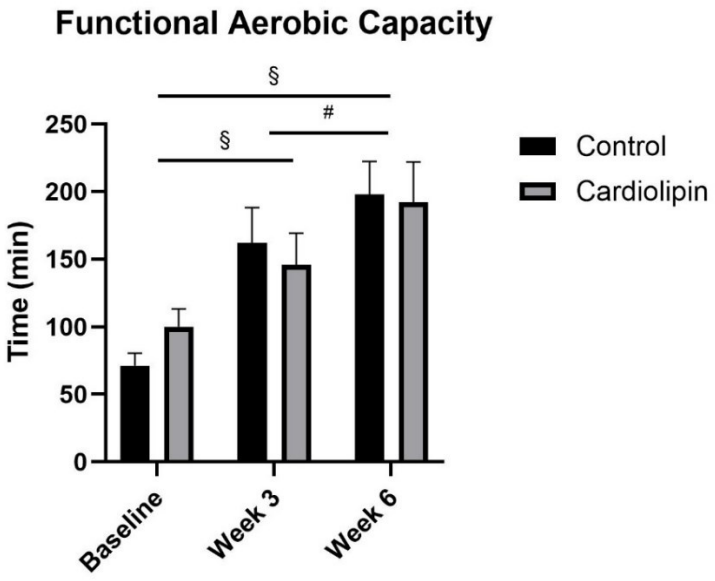

Figure 2. Time to exhaustion in minutes during the functional aerobic capacity assessment. No significant differences in running capacity were observed between the cardiolipin group (n=11) and the control group (n=12). The increase in running capacity was significant over time. Values are expressed as mean ± SE. § denotes a significant change over time for both groups ($p< 0.05$). # denotes a significant change over time for the cardiolipin group only ($p<0.05$).

Mitochondrial Respiratory Assessment

High resolution respirometry showed a statistically significant increase in oxygen consumption for the animals that received exogenous CL (n=11) versus CON (n=12) (Fig 3. displays the results, Fig 4. is a representative graph). The LEAK respiration following sequential addition of malate (CON: 0.63 ± 0.33 pmol/(s·mg) vs. CL: 3.04 ± 0.45 pmol/(s·mg), p= 0.0004, Cohen's d= 1.3), glutamate (CON: 4.11 ± 0.35 pmol/(s·mg) vs. CL: 8.04 ± 0.94 pmol/(s·mg), p= 0.002, Cohen's d= 2.3) and pyruvate (CON: 6.78 ± 0.54 pmol/(s·mg) vs. CL: 12.67 ± 1.73 pmol/(s·mg), p= 0.007, Cohen's d= 4.2) was significantly higher for the CL group compared to the CON group.

ADP-induced Complex I-dependent respiration was also significantly greater in the CL group (30.68 ± 4.15 pmol/(s·mg)) than the CON group (46.39 ± 5.39 pmol/(s·mg)) (p= 0.032, Cohen's d = 16.1). Maximal respiratory capacity, assessed upon the addition of succinate, showed to be

significantly higher in CL animals (68.70 ± 5.74 pmol/(s·mg)) than CON (45.40 ± 5.45 pmol/(s·mg)) (p= 0.008, Cohen's d= 19.0). Similarly, maximal LEAK respiration induced by oligomycin yielded a mean oxygen consumption rate of 38.55 ± 2.29 pmol/(s·mg) for the CL mice, which was significantly higher than 28.32 ± 1.88 pmol/(s·mg) for the CON group (p= 0.003, Cohen's d= 7.1). Lastly, FCCP-induced maximal uncoupled respiration resulted in a mean respiration rate of 63.84 ± 4.82 pmol/(s·mg) for the CL animals, which was again significantly higher than CON (48.92 ± 4.24 pmol/(s·mg)) (p= 0.030, Cohen's d= 15.3). All independent t-tests revealed a large effect size, suggesting that the administration of exogenous CL had a substantial impact on mitochondrial respiratory capacity.

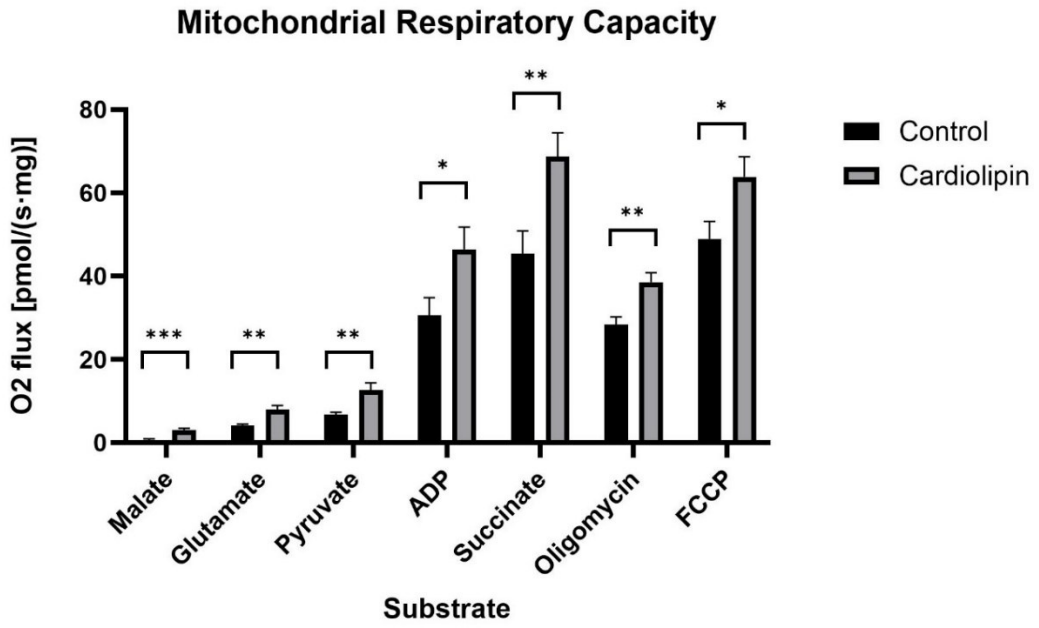

Figure 3. Mitochondrial respiratory capacity of permeabilized vastus lateralis muscle fibers measured by O_2 flux per milligram of tissue [pmol/(s·mg)]. The cardiolipin group (n=11) showed significantly increased rates of respiration for all substrates when compared to the control group (n=12). For each substrate, independent t-tests were carried out to compare O_2 flux between groups. Values are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001

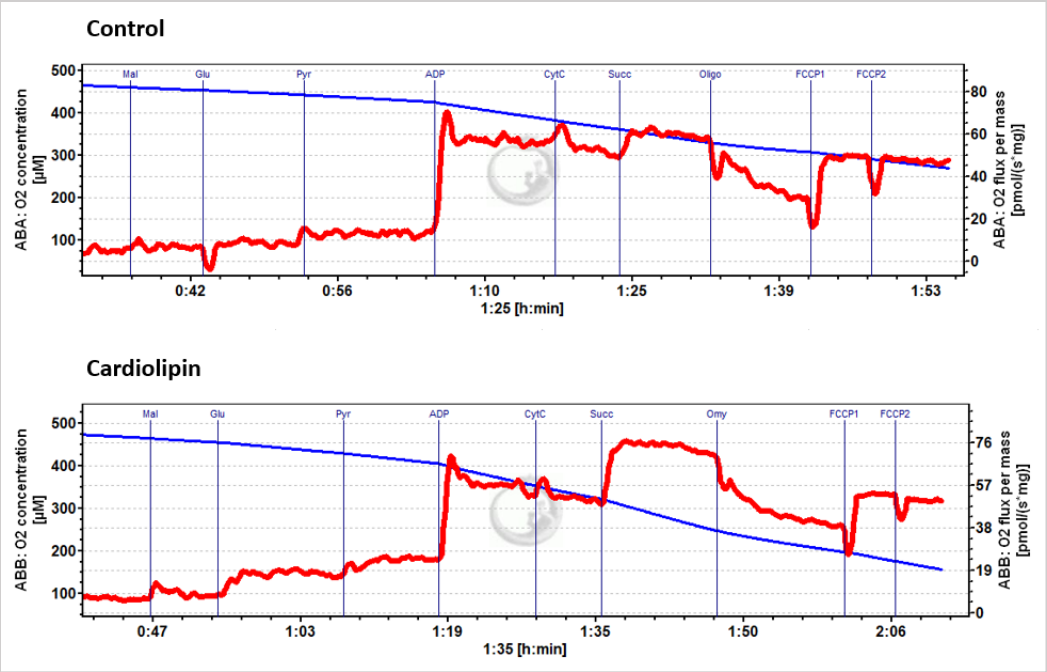

Figure 4. Representative trace of the respiratory flux from the mitochondrial respiratory assessment by high-resolution respirometry. The blue line (y-axis, left) is oxygen concentration in the chamber. Red line (y-axis, right) is oxygen flux in the chamber. Addition of substrates and inhibitors as indicated in the figure. Mal: malate; Glu: glutamate; ADP: adenosine diphosphate; CytC: cytochrome C; Succ: succinate; Omy: oligomycin; FCCP1 and FCCP2 represent an FCCP titration.

No significant changes were observed in the ratio between oxidative phosphorylation and LEAK respiration across complex I. The ACR for the CON group was of 4.79 ± 0.72 whereas the mice who received exogenous CL yielded a ratio of 4.31 ± 0.74 (p= 0.65) (Fig. 5A). Similarly, assessment of overall mitochondrial health via RCR yielded no significant difference between groups, with a ratio of 1.63 ± 0.18 for the CON mice and 1.81 ± 0.14 for the CL group (p= 0.43) (Fig. 5B).

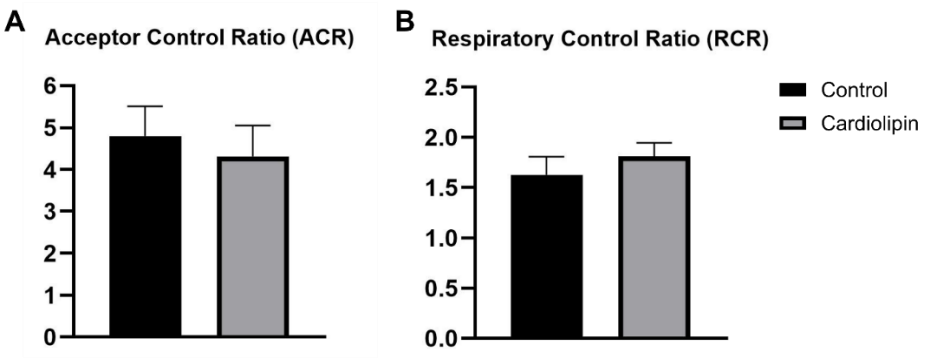

Figure 5. Acceptor control ratio (ACR) (A) and respiratory control ratio (RCR) (B). Both showed no difference between the control group (n=12) and the cardiolipin group (n=11). An independent t-test was carried out to compare the ratios between groups. Values are expressed as mean ± SEM.

Mitochondrial Density

Immunoblotting with an antibody specific VDAC revealed no significant difference in mitochondrial content between the two groups (n=11) (p=0.55) (Fig. 6). One CL sample was excluded due to methodological errors.

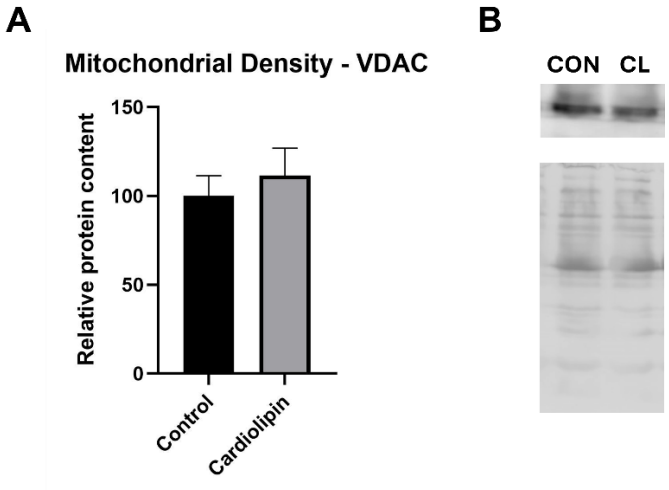

Figure 6. Determination of mitochondrial density using the voltage-dependent anion channel (VDAC) expression. No significant difference was observed between the control (n=11) and cardiolipin (n=11) groups (A). Representative blot and loading control (B). Values are expressed as mean ± SEM.

DISCUSSION

This study set out to examine the effects of exogenously increased CL levels on aerobic capacity and skeletal muscle mitochondrial respiration in a male mouse model. Contrary to our hypothesis, the main finding of this research is that mice that received CL via intraperitoneal injection exhibited an increase in mitochondrial respiratory capacity in the vastus lateralis.

In accordance with previous findings that CL supplementation can rescue respiratory coupling efficiency in isolated mitochondria with CL deficiency, our study highlights that CL content plays a prominent role in mitochondrial function in skeletal muscle (Prola et al., 2021). The overall increase in OXPHOS (Fig. 3) further supports that CL has a positive effect on bioenergetics. It can be inferred that CL contributes to the generation of the proton gradient given the significant increase in oxygen consumption observed in response to malate, glutamate and pyruvate. This aligns with previous findings which suggest that CL plays a unique role as a proton trap within the mitochondrial membrane (Haines and Dencher, 2002). As such, the sequestration of hydrogen ions contributes to building the proton gradient, which may also explain the significant increase in LEAK respiration seen upon the addition of oligomycin, an inhibitor of ATP synthase. Similarly, a greater concentration of hydrogen ions within the intermembrane space would result in a greater amount of uncoupled respiration (Ledesma et al., 2002). This explains the significantly increased rates of oxygen consumption observed in the CL group upon the addition of FCCP.

Cardiolipin plays a central role in the formation and stability of mitochondrial supercomplexes, which are structures that increase the efficiency of the ETC and reduce the rate of reactive oxygen species production (Mileykovskaya and Dowhan, 2014; Novack et al., 2020). The presence of exogenous CL may enhance electron flow, leading to significant increases in ADP-stimulated complex I respiration and succinate-induced maximal respiration observed throughout this experiment.

The ACR and RCR, which compare oxidative phosphorylation to LEAK respiration across complex I and complex I+II, respectively, indicated no change in overall mitochondrial health between the two groups (Fig. 5A, 5B). It can be proposed that exogenous CL affects both active

and resting respiration proportionally, resulting in similar ratios as those seen in the control group.

Higher mitochondrial respiration rates are a result of increases in mitochondrial density and/or efficiency (Brand et al., 2005). Given that the immunoblotting indicated that there was no significant difference in expression between the groups (Fig. 6), it can be concluded that the elevated rates of respiration are due to increased effectiveness. Consequently, it can be proposed that added CL may enhance the generation of the proton gradient and improve mitochondrial membrane integrity, ultimately leading to higher respiratory rates (Haines and Dencher, 2002; Schlame et al., 2000).

While running wheels do not require a familiarization period, it has been shown that total running distance per day can increase progressively and reach a plateau after 2-5 weeks (Manzanares et al., 2018a). Additionally, the average day-to-day distance can vary from 17-25% (Manzanares et al., 2018a). These tendencies align with the significant increase observed in daily voluntary running distance after week 1, followed by a more stable activity level for the remainder of the experiment by both groups (Fig. 1).

Despite the absence of a significant difference in functional aerobic capacity between the two groups at any of the timepoints, the opportunity to voluntarily exercise likely contributed to the significant increase in time-to-exhaustion during the treadmill tests at week 3 and week 6 (Fig. 2). Voluntary running has been shown to result in endurance-like adaptations in skeletal and cardiac muscle after 4 weeks (Manzanares et al., 2018a). In addition, increases in endurance capacity of approximately 90% on treadmill tests have been seen in mice subjected to voluntary wheel running (Manzanares et al., 2018a).

The lack of an increase in functional aerobic capacity despite improvements in mitochondrial respiration can be explained by the fact that maximal oxygen uptake (VO_2 max), which is a determinant of endurance performance, also depends on cardiac output (Bassett and Howley, 2000; De Cort et al., 1991). In turn, increases in mitochondrial efficiency alone may not be sufficient to induce a performance-related phenotype.

Estrogen has been shown to reduce mitochondrial function by decreasing glucose uptake, the expression of genes necessary for bioenergetic demands, and mitochondrial respiration (Yin et

al., 2015). Consequently, including female mice in this study could have introduced a confounding variable that may interfere with the results of the mitochondrial respiratory assessment and functional aerobic capacity test. Further exploration with female mice is warranted to investigate the effects of the estrogen cycle on the outcomes of this study.

Physiological adaptations associated with endurance exercise can take up to 12 weeks or more to manifest (Hughes et al., 2018). Consequently, a possible limitation of this study is the duration of the experimental period, whereby the longer-term impacts of cardiolipin on functional capacity might not have been fully captured within the study's six-week timeframe. Additionally, the absence of a direct measure of cardiac output may have restricted our ability to comprehensively evaluate the effects of cardiolipin supplementation. Without conducting a VO2 max test, the skeletal muscle tissue may not have been adequately challenged to exhibit enhancements in performance.

In conclusion, the present results indicate that exogenously elevated levels of CL significantly increase skeletal muscle mitochondrial respiratory capacity in male mice. No differences in daily voluntary running distance or functional aerobic capacity were observed. Exploring the effects of exogenous CL on skeletal muscle function provides valuable insights into its role beyond the cardiovascular system. Our findings allow for a better understanding of the implications of this phospholipid on energy generation and aerobic performance, which can have implications for exercise physiology and supplementation. Future considerations include exogenous CL administration paired with imposed exercise training, such as high-intensity interval training (HIIT), to further investigate changes in mitochondrial capacity and performance.

CHAPTER 3

Manuscript II

The Impact of Exogenous Cardiolipin and High-Intensity Interval Training on Exercise Performance, Mitochondrial Respiration, and Anxiety-Related Behavior in Mice

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ABSTRACT

Cardiolipin is a phospholipid found in the inner mitochondrial membrane and functions in energy metabolism. Cardiolipin is released following myocardial infarction and that patients have higher levels of anxiety post-event. Although it has shown to have various effects on different tissues, the impacts of an increase in exogenous cardiolipin paired with exercise training on skeletal muscle and anxiety-like behaviour remain underexplored. This study aimed to examine the effects of exercise training and cardiolipin on aerobic capacity, mitochondrial respiration of skeletal muscle and anxiety-like behaviour in a mouse model. Male C57BL/6 mice were randomized to a control (n=9), LOW (n=9) or HIGH (n=9) concentration cardiolipin group. The mice were injected 2x/week for 4 weeks with 0.1ml of cardiolipin (LOW: 0.25mg/ml, HIGH: 0.5mg/ml) or placebo solution. Concurrently, voluntary running distance was measured. The mice were also subjected to high-intensity interval training 3x/week for the duration of the intervention. At baseline and week 4, aerobic capacity was assessed by maximal graded exercise test on a rodent treadmill. At these same timepoints, anxiety-like behaviour was assessed by open field test and elevated plus maze. At the end of the intervention, the mice were euthanized, the vastus lateralis muscle was extracted and mitochondrial respiratory capacity was measured by high-resolution respirometry. The acceptor control ratio (ACR) and respiratory control ratio (RCR) were calculated. Results show that when paired with exercise, the administration of exogenous cardiolipin increases maximal respiratory capacity, maximal LEAK respiration and

uncoupled respiration in skeletal muscle, without affecting functional aerobic capacity or anxiety-like behaviour. These findings allow for a better understanding of the implications of cardiolipin in cardiovascular physiology, skeletal muscle function and a potential path to its role in mental health. Future considerations include assessing mitochondrial density and quantifying complex abundance to gain a deeper understanding of how exogenous cardiolipin affects mitochondrial respiration in this tissue.

INTRODUCTION

Cardiolipin (CL), a phospholipid predominantly found in the inner mitochondrial membrane (IMM), constitutes approximately 25% of the total phospholipid content in cardiomyocytes (Gasanoff et al., 2021). It has also been detected in human serum, suggesting that CL exists in the extracellular space as a component of plasma lipoproteins (Deguchi et al., 2000). Given its role in various mitochondrial and cellular processes, modifications in the quantity and structure of the phospholipid are associated with a wide range of pathological conditions, including Barth syndrome, diabetic cardiomyopathy and myocardial ischemic-reperfusion injury (Claypool and Koehler, 2012; Gasanoff et al., 2021; Shen et al., 2015).

Myocardial infarction (MI), commonly known as a heart attack, arises from diminished blood flow to the heart muscle and consequential oxygen deprivation which leads to injury and death of cardiomyocytes (Lu et al., 2015). The presence of antibodies to CL (aCL) in the blood following MI suggests that CL may be released into circulation upon some occurrences of MI. (Grosso et al., 2019, 2019; Hamsten et al., 1986; Sletnes et al., 1992). These findings warrant further exploration into the potential role of elevated CL levels in the body.

Fear is classified as anxiety when it negatively impacts daily life by exceeding necessary levels of response, emerging in inappropriate contexts, or persisting to a chronic extent (Dean, 2016). Increased levels of anxiety have been seen in individuals following myocardial infarction, most often associated with concerns of future fatal and nonfatal cardiac events, hospital readmission and cardiac mortality (Feng et al., 2016). Observable behaviours and physiological markers, such

as altered exploratory behaviour, avoidance of open spaces and exaggerated startle responses, are used to study anxiety in mice (Bourin et al., 2007).

The relationship between anxiety and High-Intensity Interval Training (HIIT) has been explored in both human and animal studies. HIIT is a form of exercise characterized by brief and repeated bouts of vigorous activity performed at a workload typically corresponding to 90% of maximal running speed in rodents (Buchheit and Laursen, 2013). Studies have demonstrated increased exercise capacity and skeletal muscle adaptations following HIIT (Chrøis et al., 2020; Little et al., 2010). Mitochondrial adaptions in response to this type of training include an increase in complex IV abundance as well as mitochondrial protein content and maximal activity of citrate synthase and cytochrome c oxidase, which are required for the efficient production of ATP (Chrøis et al., 2020; Little et al., 2010). In rodents, HIIT was capable of reducing anxiety-like behaviour in diabetic rats, in obese mice that were following a high-fat diet, and in mice with Alzheimer's disease (Foroozan et al., 2021; Orumiyehei et al., 2022; Ying et al., 2023).

Although CL has shown to have various effects on different body tissues, such as endothelium and vascular smooth muscle, the impacts of an increase in CL on skeletal muscle remain underexplored (Carnevale and Bergdahl, 2015; Galambo and Bergdahl, 2023). The purpose of this study was to investigate the effects of exogenous cardiolipin on voluntary exercise metrics, forced exercise training, functional aerobic capacity, anxiety-related behaviors, and mitochondrial respiration in skeletal muscle. It was hypothesized that the addition of exogenous CL would improve mitochondrial respiratory capacity, thereby enhancing functional aerobic capacity and voluntary running metrics. Moreover, it was thought that HIIT would exacerbate these effects while decreasing anxiety levels.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice aged 6-7 months were obtained from the Concordia University breeding colony and Charles River Laboratories. The mice were screened and selected based on their ability and willingness to run on a rodent treadmill at a speed of 4m/min before random assignment to the control (CON), low cardiolipin (LOW) or high cardiolipin (HIGH) group

(Bouganim and Bergdahl, 2017). The animals were housed individually in cages equipped with a running wheel (Actimetrics, Wilmette, IL, USA) and placed in light-proof cabinets in a thermoneutral environment (22°C) while subjected to a 12:12 light/dark photoperiod. Food and water were available ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (#30000259) and performed in compliance with guidelines set forth by the Canadian Council on Animal Care.

Experimental protocol

Over a 4-week period, the mice received two intraperitoneal injections of 0.1 mL containing CL at a concentration of 0.5mg/mL for the HIGH group, of 0.25mg/mL for the LOW group or placebo solution containing physiological salt solution [135.5 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl, 11.6 mM glucose, 11.6 mM HEPES (pH 7.35)] for the CON group. Given that circulating levels of CL vary amongst individuals post-MI, these quantities resemble physiological concentrations of the phospholipid (Deguchi et al., 2000).

Voluntary Wheel Running

Wheel running was recorded and analyzed using Clocklab 6 (Actimetrics, Wilmette, IL, USA). Total running distance and active phase running distance were collected, analyzed from summed activity counts (10-minute bins), displayed in terms of kilometers covered per day and compared weekly.

Circadian Rhythm

Circadian rhythm analysis was performed using daily running activity patterns. Intra-daily variability (IV) was used to quantify the degree of fragmentation of the activity-rest periods. It is measured on a scale from 0 to 2, where 0 signifies the absence of fragmentation, meaning a consistent activity period followed by a consistent rest period. Conversely, 2 indicates shorter periods of interspaced rest and activity, resulting in a more fragmented rhythm (Gonçalves et al., 2014). Inter-daily stability (IS) was used to assess the consistency of the activity pattern across different days, quantifying rhythmic synchronization to the light-dark cycle. It is measured on a

scale from 0 to 1, whereby 0 indicates a random activity pattern with no stability and 1 represents a highly consistent pattern with perfect stability (Gonçalves et al., 2014).

Maximal Graded Exercise Test

At baseline and week 4, a maximal graded exercise test (protocol adapted from Caru et al., 2019) was performed using a rodent treadmill (Bouganim and Bergdahl, 2017). The mice were subjected to a 3-minute warm up period at a speed of 0.8 km/hr. Thereafter, the speed was increased by approximately 0.2 km/hr every 1 minute (Table 1) until exhaustion, and maximum speed was recorded. Exhaustion was defined as the point when a mouse remained in contact with the brush found at the bottom of the treadmill for 10 consecutive seconds despite tactile motivation. The entirety of the test was carried out at an incline of 10 degrees.

Table 1. Maximal graded exercise test protocol

Stage	Speed (km/hr)	Duration (min)
1	0.8	3
2	1.0	1
3	1.2	1
4	1.4	1
5	1.6	1
6	1.8	1
7	2.0	1
8	2.2	1
9	2.4	1
10	2.6	1

High-Intensity Interval Training (HIIT)

The HIIT program was carried out on an adapted rodent treadmill (Bouganim and Bergdahl, 2017) and consisted of three 25-minute sessions per week for 4 weeks (protocol adapted from Caru et al., 2019). Each session began with a 5 min warm-up, followed by 5 sets of 1 minute at an intensity of 80-90% maximal running speed, calculated based on the baseline exercise

capacity test. A 2-minute active recovery bout was carried out between each set and a 5-minute cool-down period ended the test. The warm-up, active recovery and cooldown were carried out at an intensity of approximately 60% maximal running speed.

Table 2. HIIT protocol

Stage	Speed (% maximal speed)	Duration (min)
Warm up	60	5
1	80-90	1
Recovery	60	2
2	80-90	1
Recovery	60	2
3	80-90	1
Recovery	60	2
4	80-90	1
Recovery	60	2
5	80-90	1
Cool down	60	5

Open Field Test (OFT)

The OFT was conducted at baseline and week 4 to assess anxiety-related behavior using an automated tracking system (Panlab, Barcelona, Spain). The animals were subjected to a 30-minute habituation period in the experimental room prior to the start of the test, which was conducted 2 hours after the beginning of the light cycle (ZT 2). The Plexiglas arena (44 cm × 44 cm × 30 cm) is equipped with a single frame containing infrared beams for subject detection. Mice were placed facing the wall at one corner of the open field, and the Actitrack software (Panlab, Barcelona, Spain) monitored spontaneous horizontal activity for each animal independently for a duration of 15 minutes. Latency of entry into the center, resting time and permanence time in the central area of the open field were recorded along with total distance travelled (Schoettner et al., 2022).

Elevated Plus Maze (EPM)

The EPM test was conducted at baseline and week 4 of the intervention. The animals were subjected to a 30-minute habituation period in the experimental room prior to the start of the test, which was conducted 2 hours after the beginning of the light cycle (ZT 2). A white cross-shaped maze with a central area (6cm x 6cm), two open arms and two black-walled closed arms (6cm x 29.5cm each) was elevated 40cm above the ground (Schoettner et al., 2022). Each animal was placed at the center of the maze facing the closed arm. A phone was mounted above the EPM to video record the animal for 5 minutes. The video files were converted into 1-second image sequences using VirtualDub1.10.4, compiled into multi-Tiff files in FIJI and analyzed by the ImageEP (Ver. 1,201,112) plugin (Komada et al., 2008; Schilders et al., 2013). Distance travelled as well as time spent and number of entries into the open arms was assessed (Schoettner et al., 2022).

Tissue Extraction and Permeabilization

After the 4-week period, the mice were euthanized by isoflurane anesthesia, CO2 asphyxiation and cervical dislocation. The vastus lateralis muscle was immediately extracted and permeabilized for mitochondrial respiratory assessment. Upon extraction, the vastus lateralis muscle was rid of connective tissue and the fibers were gently separated with forceps. The isolated tissue incubated for 30 minutes on ice in 50 µg/mL saponin and 2mL BIOPS buffer solution [2.77mM CaK2EGTA, 7.23mM K2EGTA, 5.77mM Na2ATP, 6.56mM MgCl2·6H2O, 20mM Taurine, 15mM Na2Phosphocreatine, 20mM Imidazole, 0.5mM Dithiothreitol, 50mM MES (pH 7.1) (Fontana-Ayoub et al., 2013). Subsequently, the tissue underwent two consecutive 10-minute washes in MiR05 buffer [0.5mM EGTA, 3.0mM MgCl2·6H2O, 60mM K-lactobionate, 20mM Taurine, 10mM KH2PO4, 20mM HEPES, 110mM Sucrose, 1g/L BSA (pH 7.1)] (Kuznetsov et al., 2008).

High-Resolution Respirometry

Mitochondrial oxygen consumption was measured via high-resolution respirometry (Oxygraph2k, Oroboros Instruments, Innsbruck, Austria). Approximately 2-2.5 mg of muscle tissue were added to 2mL of MiR05 buffer in each chamber. The experiments were carried out at

37°C in a hyper-oxygenated environment to prevent oxygen limitation. Malate (2mM), glutamate (10 mM) and pyruvate (6 mM) were added in succession to stimulate LEAK respiration across complex I and to build the proton gradient across the inner mitochondrial membrane. ADP (5mM) was then administered to initiate complex I-dependent respiration. The integrity of the outer mitochondrial membrane was assessed with the addition of cytochrome c (10μM). Subsequently, succinate (10mM) was added to measure maximal respiration. Oligomycin (2μg/mL) was used to inhibit ATP synthase allowing for maximal LEAK respiration to be recorded. Lastly, an FCCP (0.25μM) titration was carried out to assess maximal uncoupled respiration. The high-resolution respirometry values were obtained from stabilized O2 flux per mass (pmol/s/mg) readings following the addition of each substrate. Data was analyzed by DatLab 7.0 software. The acceptor control ratio (ACR) was determined by dividing ADP by pyruvate values while the respiratory control ratio (RCR) was calculated by dividing succinate by oligomycin values.

Statistical analysis

Data are presented as mean ± standard error (SE). Baseline refers to the period before the intervention, whereas Week 1 indicates the first week of injections. Normality was assessed using the Shapiro-Wilk normality test. A one-way ANOVA was used to compare data between the control and cardiolipin groups for mitochondrial respiratory capacity measurements, acceptor control ratio, respiratory control ratio. A two-way repeated measures ANOVA was used to compare the results between groups and across time for OFT, EPM, IS, voluntary running distance and maximal graded exercise test. Lastly, because of missing data values, a mixed-model ANOVA was used to analyze IV results. Post hoc comparisons were conducted using Tukey's HSD test. A P value of < 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics (Version 28.0).

RESULTS

Voluntary Wheel Running

The two-way repeated measures ANOVA revealed a statistically significant main effect of time during the active phase (dark phase), with running distance decreasing ($F=3.28$, $p=0.024$) (Fig. 1). However, no significant differences were observed between groups ($n=9$ per group) or across time for running distance. Similarly, a statistically significant main effect of time was seen with decreased total running distance over a 24-hour period ($F=3.67$, $p=0.015$), but no significant differences were observed between groups.

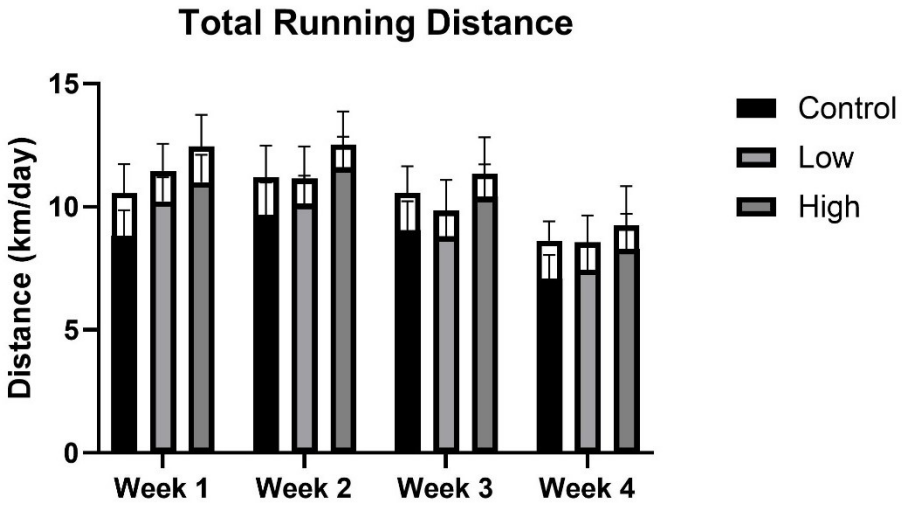

Figure 1. Voluntary wheel running. Active period running distance is represented by the solid portion of each bar, while entire length represents total daily running distance. Both parameters showed no significant differences between groups or over time. A two-way ANOVA was carried out to compare results. Values are expressed as mean ± SE.

Circadian Rhythm

No significant differences were seen across groups for IV (Fig 2A), indicating that all animals had a similar level of fragmentation within their activity patterns. However, the mixed-model ANOVA revealed a statistically significant main effect of time ($F=11.01$, $p=0.0001$).

A significant decrease in IV was seen in the HIGH group from week 1 (0.68 ± 0.08) to week 2 (0.54 ± 0.09) (p=0.040), suggesting a decrease in variability, which then steadily increased over the last two weeks of the intervention.

Similarly, no significant differences were seen across groups for IS (Fig. 2B), indicating that all animals had a comparable level of stability in their activity patterns, yet the two-way repeated measures ANOVA revealed a statistically significant main effect of time (F=4.88, p=0.011). The LOW group experienced a significant decrease in IS from week 3 (0.71 ± 0.04) to week 4 (0.59 ± 0.05) (p=0.001), suggesting a reduction in the synchronization to the light-dark cycle towards the end of the intervention.

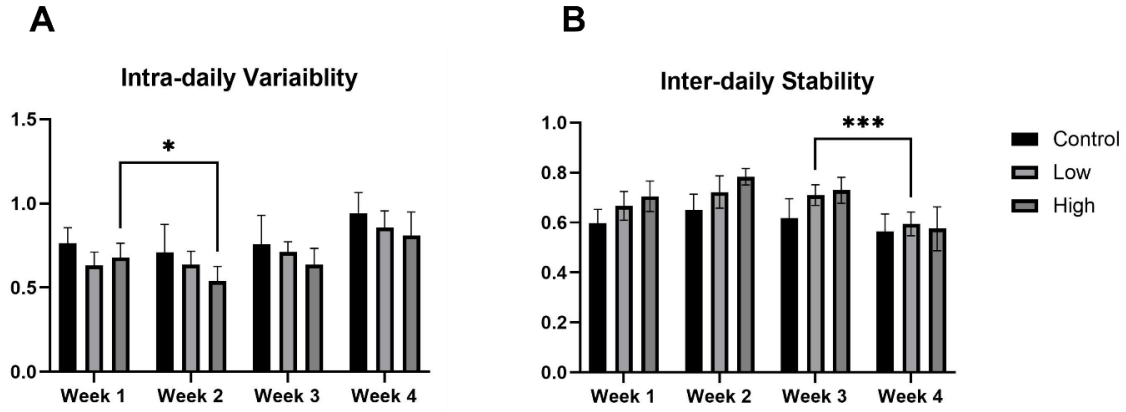

Figure 2. Intra-daily variability (IV) (2A) and inter-daily stability (IS) (2B). A decrease in variability in the HIGH group was seen from week 1 to week 2, and a reduction in stability in the LOW group was seen from week 3 to week 4. No significant differences were observed between groups. Sample size n=9 for all groups. Values are expressed as mean ± SE. *P<0.05, **P<0.01, ***P<0.001.

Maximal Graded Exercise Test

The maximal graded exercise test showed no difference in functional capacity between groups (Fig. 3). However, the two-way repeated measures ANOVA revealed a statistically significant main effect of time (F=20.97, p=0.0001). All three groups saw a statistically significant increase in maximal running speed during the final treadmill test, when compared to baseline. The mean running speed increased from 1.87 ± 0.15 km/hr to 2.18 ± 0.22 km/hr (p=0.033) for CON (n=9),

from 1.76 ± 0.10 km/hr to 2.20 ± 0.08 km/hr (p=0.0035) for LOW (n=9) and from 2.00 ± 0.11 km/hr to 2.33 ± 0.09 km/hr (p=0.023) for HIGH (n=9).

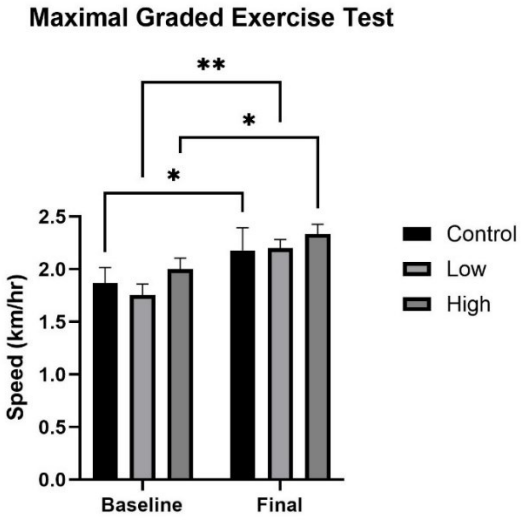

Figure 3. Maximal graded exercise test. Results showed a significant increase in running speed over time for all three groups. No difference between groups was observed. Values are expressed as mean ± SE. *P<0.05, **P<0.01.

Open Field Test (OFT)

Five OFT parameters were analyzed to assess anxiety-like behaviour (n=9 per group for all measurements). No statistically significant differences were observed for permanence time in the center of the open field (Fig. 4C) between groups or across time.

Total distance travelled remained similar at both time points for the LOW and HIGH animals (Fig. 4A). However, the CON group saw a statistically significant increase from 3520.49 ± 364.17 cm at baseline to 4127.94 ± 261.19 cm during the final test (p= 0.027). This suggests that there was an increase in the exploratory behaviour of the CON group over time. No significant differences were observed between groups.

Overall trends of decreased resting time in the center of the open field were observed for all three groups when comparing baseline to the final trial, corresponding to a statistically significant main effect of time (F=13.87, p=0.0011) (Fig. 4D). The CON animals were alone in showing a

statistically significant decrease from 42.36 ± 10.46 sec to 11.63 ± 2.30 sec (p=0.0008). This suggests that all animals were more avoidant of remaining immobile in the center of the field during the final OFT. No significant differences were observed between groups.

The two-way repeated measures ANOVA revealed statistically significant main effects of time (F=6.03, p=0.022) and treatment (F=4.13, p=0.029), as well as a significant interaction between these main effects on latency to enter the center (F=5.91, p=0.008). Compared to the CON and HIGH groups, delay to enter the center was significantly longer in the LOW group at baseline (CON: 11.63 ± 3.02 sec (p= 0.0004), LOW: 48.69 ± 11.91 sec, HIGH: 18.00 ± 7.15 sec (p=0.0033)) (Fig. 4B). However, this measure significantly decreased to 10.96 ± 3.03 sec (p= 0.0004) for the LOW group, yielding no significant differences between groups at the final timepoint. While the LOW group was more hesitant to leave the periphery of the field at baseline, this inhibition was lost, suggesting equal exploratory behaviour into the center of the field in the final test.

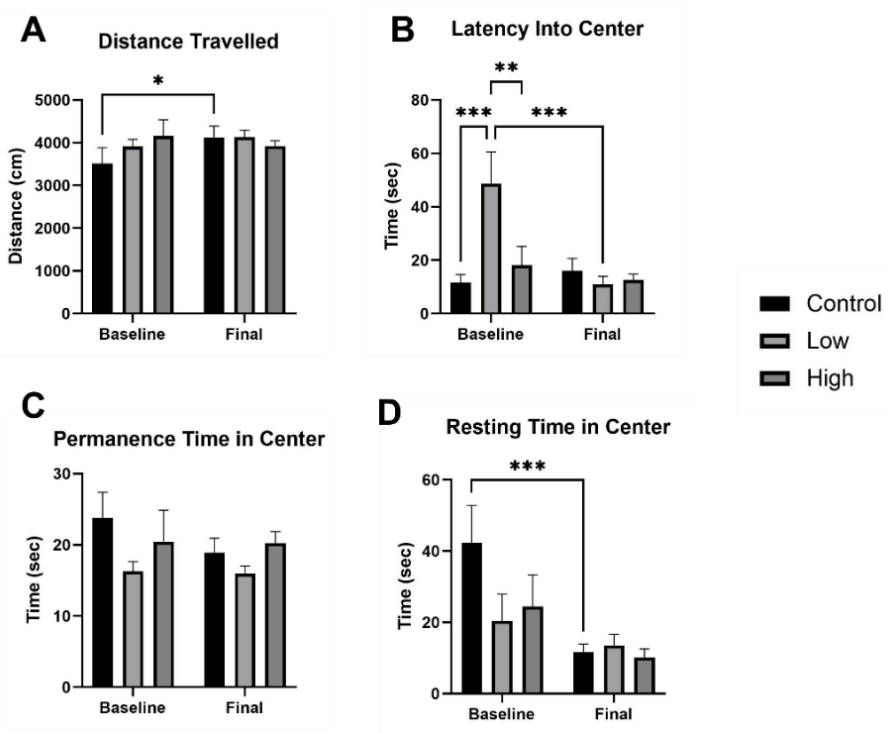

Figure 4. Open Field Test (OFT). Results revealed group specific differences in anxiety-like behaviour over time. No significant differences were observed between groups. Sample size n=9 for all groups. Values are expressed as mean ± SE. *P<0.05, **P<0.01, ***P<0.001.

Elevated Plus Maze (EPM)

Total distance travelled, stay time in open arms and number of open arm entries during the EPM were used to assess anxiety-like behaviour (n=9 per group for all measurements). Two-way repeated measures ANOVA revealed statistically significant main effects of time for all three measures. Total distance increased over time ($F=13.16$, $p=0.0013$), while open arm stay time ($F=14.94$, $p=0.0007$) and open arm entries ($F=25.56$, $p<0.0001$) decreased.

Results showed no difference between groups for all parameters. Total distance covered (Fig. 5A) by the CON group significantly increased from 828.50 ± 55.45 cm at baseline to 1070.26 ± 52.89 cm during the final EPM test ($p=0.027$). Once again, this suggests that there was an increase in the exploratory behaviour of the CON group over time.

A statistically significant decrease was observed over time in all groups for percent time spent in open arms (Fig. 5B), with mean values going from 23.00 ± 4.69 % to 10.12 ± 1.54 % for CON ($p=0.034$), from 22.08 ± 4.96 % to 9.63 ± 1.21 % for LOW ($p=0.040$) and from 23.86 ± 9.41 % to 10.89 ± 3.11 % for HIGH ($p=0.033$). Similarly, the mice entered the open arms significantly less during the final EPM trial. The mean percent of open arm entries (Fig. 5C) decreased from 43.56 ± 3.13 % to 25.76 ± 3.29 % for CON ($p=0.002$), from 42.59 ± 2.97 % to 31.00 ± 1.51 % for LOW ($p=0.037$) and from 41.68 ± 7.71 % to 26.02 ± 4.75 % for HIGH ($p=0.0056$). Together, theses results suggest higher anxiety-like behaviour for all groups during the final EPM trial.

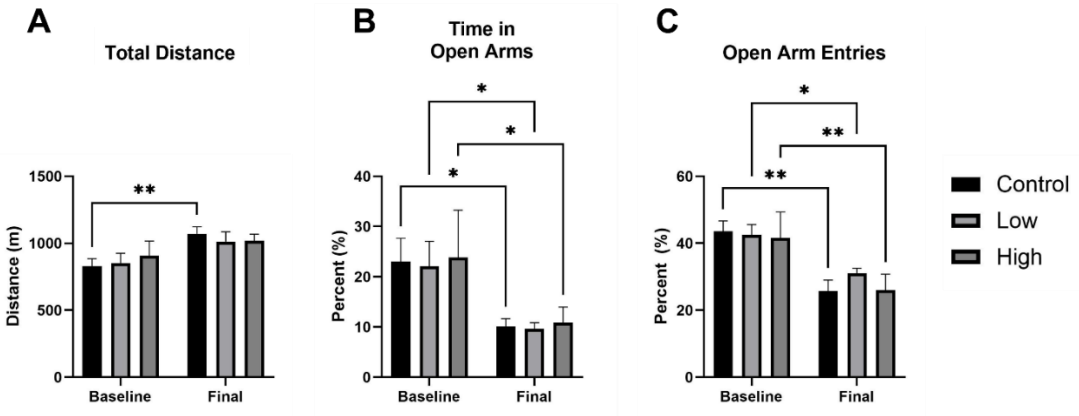

Figure 5. Elevated plus maze (EPM). Results showed increased anxiety-like behaviour over time. The control animals experienced a significant increase in total distance travelled during final EPM. No significant differences were observed between groups. Sample size n=9 for all groups. Values are expressed as mean ± SE. *P<0.05, **P<0.01.

Mitochondrial respiratory capacity

High resolution respirometry (Fig. 6) revealed significantly increased levels of complex I+II-dependent mitochondrial respiration upon the addition of succinate in the HIGH group (39.91 ± 6.07 pmol/(s·mg)) (n=9) compared to CON animals (21.52 ± 3.09 pmol/(s·mg), p=0.0018) (n=9). While rates of oxygen consumption in the HIGH group were also higher than the LOW group (n=9), the difference was not statistically significant. Oligomycin-initiated maximal LEAK respiration was significantly increased in the HIGH group (34.18 ± 6.01 pmol/(s·mg)) compared to both CON (15.84 ± 1.68 pmol/(s·mg), p=0.0019) and LOW (18.60 ± 1.68 pmol/(s·mg), p=0.010) groups. Similarly, oxygen consumption rates were significantly higher in the HIGH group (55.33 ± 8.24 pmol/(s·mg)) during FCCP-induced maximal uncoupled respiration compared to CON (28.61 ± 3.63 pmol/(s·mg), p<0.0001) and LOW (34.12 ± 4.46 pmol/(s·mg), p=0.0003) animals. No statistically significant differences were seen between the groups during complex I-dependent respiration initiated by malate, glutamate, pyruvate and ADP.

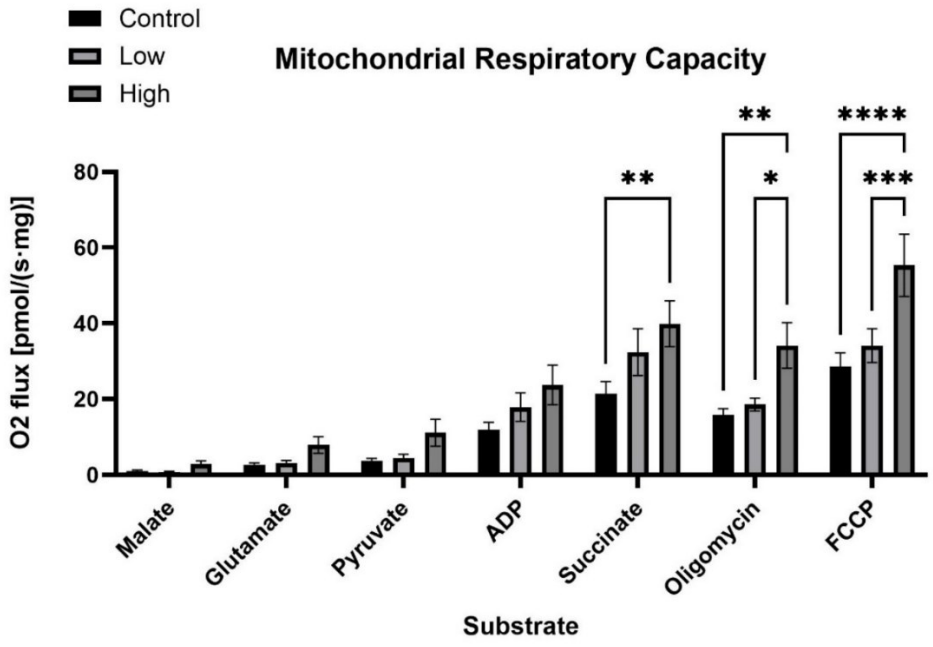

Figure 6. Mitochondrial respiratory capacity of permeabilized vastus lateralis muscle fibers measured by O_2 flux per milligram of tissue [pmol/(s·mg)]. Animals receiving the HIGH concentration of exogenous cardiolipin exhibited increased levels of complex I+II-dependent respiration. For each substrate, a one-way ANOVA was carried out to compare O_2 flux between groups. Values are expressed as mean ± SE. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

No significant changes were observed in the ratio between oxidative phosphorylation and LEAK respiration across complex I (CON: 3.95 ± 0.91, LOW: 4.09 ± 0.52, HIGH: 2.60 ± 0.48) (Fig. 7A). Similarly, assessment of overall mitochondrial health via RCR yielded no significant difference between groups (CON: 1.41 ± 0.21, LOW: 1.70 ± 0.23, HIGH: 1.27 ± 0.16) (Fig. 7B).

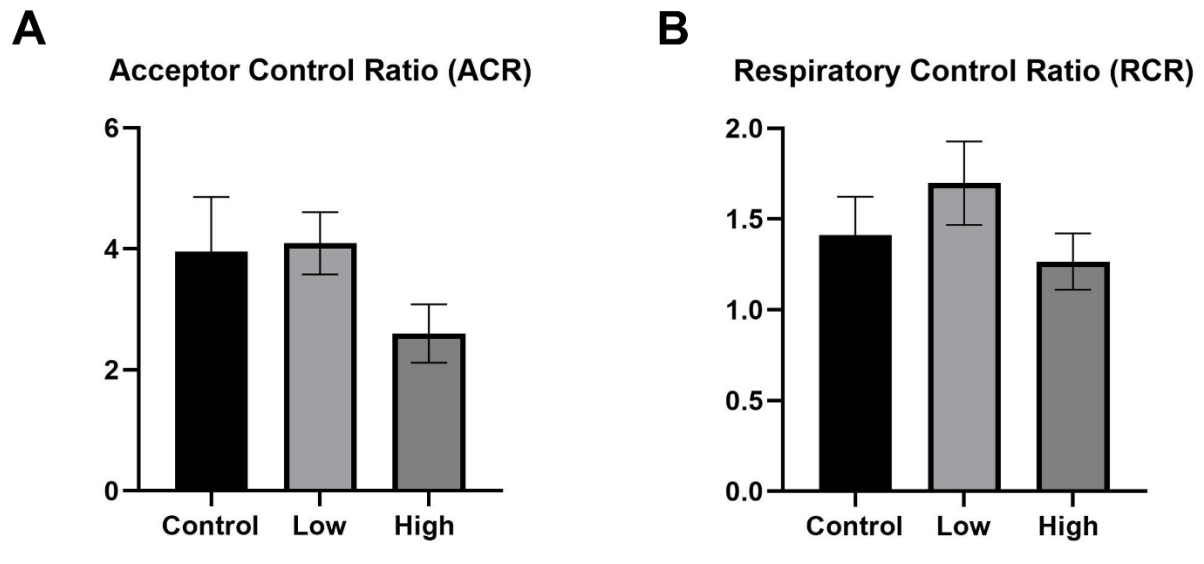

Figure 7. Acceptor control ratio (ACR) (A) and respiratory control ratio (RCR) (B). Both showed no difference between the control (n=9), the low cardiolipin (n=9) or the high cardiolipin (n=9) groups. A one-way ANOVA was carried out to compare the ratios between groups. Values are expressed as mean ± SE.

DISCUSSION

This study explored the effects of exogenous CL on voluntary exercise metrics, forced exercise training, functional aerobic capacity, anxiety-related behaviors, and mitochondrial respiration in skeletal muscle. Remarkedly, when paired with exercise training, heightened levels of the phospholipid increase maximal respiration, maximal LEAK respiration and uncoupled respiration in a dose-dependent manner.

CL and Skeletal Muscle

No significant differences in voluntary running distance were observed between the groups or across time during the active phase (Fig. 1A) and over a 24-hour period (Fig. 1B), indicating that the addition of exogenous CL did not differentially affect overall activity levels. Similarly, the lack of significant differences in the IV (Fig. 2A) and IS (Fig. 2B) measures across groups suggests that all animals maintained a comparable pattern and stability in their activity rhythms. Voluntary wheel running has shown to stabilize the circadian system in mice via feedback effects on the suprachiasmatic nucleus (SCN) in the hypothalamus of the brain, which is the main circadian pacemaker (Ripperger et al., 2011; Weinert and Gubin, 2022). Additionally, exercise is known to affect the peripheral clocks in tissues such as skeletal muscle, liver and kidney (Tanaka et al., 2023). This could explain the uniformity of the activity patterns across the three groups in this intervention given that all animals participated in voluntary wheel running and forced exercise training. However, the significant decrease in IV observed in the HIGH group from week 1 to week 2 (Fig. 2A) suggests a transient decrease in activity variability, which then normalized in the subsequent weeks. Additionally, the significant decrease in IS in the LOW group from week 3 to week 4 (Fig. 2B) indicates a reduced synchronization to the light-dark cycle towards the end of the intervention. Seeing that exercise affects activity patterns, slight inconsistencies in the onset of the HIIT workouts could account for the observed differences within groups over time (Wolff and Esser, 2019).

The maximal graded exercise test (Fig. 3) showed no differences between groups, but a significant increase in maximal running speed was observed for all groups by the end of the four weeks. This suggests an overall improvement in exercise performance irrespective of group

allocation. This finding can be attributed to increased fitness levels resulting from voluntary wheel running and HIIT. Voluntary running has shown to induce endurance-like adaptations in skeletal and cardiac muscle, while HIIT contributes to improvements in cardiovascular efficiency, aerobic and anaerobic capacity, muscle adaptations, VO2 max and energy utilization efficiency (Chavanelle et al., 2017; Chrøis et al., 2020; Manzanares et al., 2018b; Seldeen et al., 2018; Wang et al., 2017). Collectively, these physiological enhancements can lead to better performance in maximal graded exercise tests.

The mitochondrial respiratory capacity results revealed similar complex I-dependent respiration (malate, glutamate, pyruvate, ADP) across all groups (Fig. 6). However, significant increases in complex I+II-dependent respiration (succinate), maximal LEAK respiration (oligomycin) and uncoupled respiration (FCCP) were observed exclusively in the HIGH group (Fig. 6). These findings indicate dose-dependent effects, with the low dose of CL being insufficient to induce significant adaptations. Given that there were no differences in functional exercise capacity, it can be implied that exercise training induced comparable adaptations in complex I across all groups, but that exogenous CL exerted a distinctive impact on respiration when both complexes I and II were involved. HIIT enhances overall oxidative capacity in muscle cells by, for example, promoting mitochondrial biogenesis and increasing the content of mitochondrial proteins (MacInnis and Gibala, 2017). This generalized improvement may overshadow the specific enhancements provided by CL to ubiquinone oxidoreductase (complex I).

Cardiolipin is also known to play a role in stabilizing and enhancing the function of different electron transport chain (ETC) components (Mileykovskaya and Dowhan, 2014). The higher concentration of exogenous CL may have particularly increased the stabilization of succinate dehydrogenase (complex II), which would explain the significantly increased rates of oxygen consumption observed after the addition of succinate. Additionally, this phospholipid is crucial for optimal ETC complex interaction and may have facilitated electron transfer between complexes I and II through ubiquinone (Vos et al., 2017). This would explain the enhanced levels of overall respiration and the less pronounced impact of exogenous CL when assessing complex I in isolation.

Moreover, CL is thought to enhance the generation of the proton gradient, which may account for the significant increases in maximal LEAK respiration and uncoupled respiration in the HIGH

group (Haines and Dencher, 2002). Lastly, no differences were observed across the groups for ACR and RCR, suggesting that exogenous CL and the exercise interventions in this study affected both active and resting respiration proportionally.

CL and Anxiety-Related Behaviour

The open field test (OFT) and elevated plus maze (EPM) assessments provided insights into anxiety-like behavior. Both tests indicated group-specific alterations in exploratory tendencies. In the OFT, the CON group exhibited a significant increase in total distance traveled (Fig. 4A) and a decrease in resting time in the center of the field (Fig. 4D), suggesting enhanced exploratory behaviour (Kraeuter et al., 2019). The EPM results corroborated these findings whereby, once again, the CON group displayed a significant increase in total distance traveled (Fig. 5A). In the OFT, the LOW group initially had a higher latency to enter the center of the field at baseline, but this parameter significantly decreased by the final test, resembling the LOW and HIGH groups (Fig. 4B).

Results from the EPM suggest an increase in avoidance of open spaces across all groups. The test indicated a significant decrease in percent open stay time (Fig. 5B) and open arm entries (Fig. 5C), suggesting heightened anxiety-like behavior during the final trial (Kraeuter et al., 2019). This stress-induced change in behavior could be attributed to intervention-related environmental factors such as being housed individually, manipulation during injection and treadmill sessions (Hohoff, 2009). Overall, the assessments indicate that some anxiety-related behaviors increased, irrespective of exogenous CL presence.

In conclusion, when paired with exercise, the administration of exogenous CL increases maximal respiratory capacity, maximal LEAK respiration and uncoupled respiration in skeletal muscle, without affecting functional aerobic capacity or anxiety-like behaviour. Future research should focus on assessing mitochondrial density and quantifying complex abundance to gain a deeper

understanding of how exogenous CL affects mitochondrial respiration in this tissue. Overall, this research has significant implications for advancing our understanding of CL in cardiovascular physiology, skeletal muscle function, and mental health. This insight could lead to the development of novel therapeutic strategies, have implications for exercise physiology and inform clinical practices to mitigate the adverse effects of myocardial infarction to, ultimately, improve recovery.

CHAPTER 4

Conclusion

The objective of this thesis was to enhance our understanding of cardiolipin's impact on the body, particularly after myocardial infarction, by examining the effects of exogenously elevated levels of the phospholipid on various aspects of skeletal muscle function and related behaviors.

Manuscript I demonstrated a significant increase in skeletal muscle mitochondrial respiratory capacity upon cardiolipin administration in mice. However, no significant differences were observed in daily voluntary running distance or functional aerobic capacity. Limitations to this study include the duration of the experimental period, which may have been too short to fully capture the effects of cardiolipin on functional capacity within the six-week timeframe. Moreover, the lack of a direct measure of cardiac limits our ability to thoroughly assess the impact of cardiolipin supplementation on performance. Additionally, without performing a VO2 max test, the skeletal muscle tissue may not have been sufficiently challenged to demonstrate potential improvements in running capacity.

In Manuscript II, when combined with exercise training, the injection of cardiolipin enhanced maximal respiratory capacity, maximal LEAK respiration, and uncoupled respiration in skeletal muscle, while functional aerobic capacity and anxiety-like behavior remained unaffected. However, when the concentration of cardiolipin was halved, no significant differences in mitochondrial respiration were observed. This study's limitations include the lack of a cardiac output measure, which hinders our ability to draw definitive conclusions about performance. Additionally, slight variations in the timing of the HIIT training may have affected the animals' activity patterns. Lastly, individual housing and physical manipulation are known to induce stress in mice, which may have influenced the results of the behavioural tests and taken away from any possible effects of cardiolipin.

In summary, these findings suggest that exogenous cardiolipin supplementation enhances mitochondrial respiration in a dose-dependent manner, thereby potentially improving energy production. Future research should explore different doses of cardiolipin to ascertain optimal levels for maximal benefits. Additionally, investigating its impact on cardiac output could provide further insights into its broader physiological effects and impacts on performance. To

further understand how the phospholipid affects the body, the impact of exogenously increased levels of cardiolipin on mitochondrial respiration should also be explored in other tissues, such as the brain.

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