AN EXAMINATION OF PSYCHOLOGICAL STRESS AND ITS EFFECTS ON THE MITOCHONDRIAL DNA

PARSA MIRZADEH

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Abstract

Background: With improvements in cellular stress measurement, current findings firmly implicate mitochondrial activity in cell stress reactions. Recent studies have highlighted the effects of psychological stress on mitochondria in terms of oxidative deterioration and crisis signaling. However, these studies have mainly assessed this relationship using the Trier Social Stress Test (TSST), while a variety of methods have been applied in blood sample analyses. This study contributes to the progression of existing studies as a partial replication that employs a different experimental stress protocol (based on the International Affective Picture System) (IAPS), a younger student population and similar blood sample analyses. Specifically, the relationship between cell free mitochondria (via analyses of ccf-mtDNA levels following experimental stress) and acute psychological stress was evaluated in healthy female university-student participants.

Methods: This study partially replicates the Trumpff et al., 2019 study. Fourteen female participants (n=14) were recruited on a university campus via posters and emails. All participants completed baseline questionnaires and two blood draws, with a 10-minute experimental stressor occurring between the pre-stress blood draw and post-stress blood draw. Blood samples were obtained at: i) baseline prior to the stressor and ii) +30 mins post stressor, to evaluate the changes in ccf-mtDNA levels associated with acute psychological stress.

Results: A paired two tailed t-test was used to assess the difference between the pre and post blood samples, There was a statistically significant increase ($P < 0.01$) (a 53% increase) in ccf-mtDNA +30 mins after the psychological stressor.

Conclusion: The present findings suggest that acute psychological stress is associated with a statistically significant increase in cell free mitochondrial DNA (ccf-mt-DNA) in young healthy female university students.

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1. Introduction

1.1. Rationale

In the last decade, multiple investigations have focused on mitochondria, the subcellular structures (or organelles) that function as 'powerhouses' and collectively supply most bodily energy (Castellani et al., 2020; Trumpff et al., 2021). With interests in understanding the conditions under which mitochondria serve physical and mental health, recent studies have highlighted the effects of psychological stress on mitochondria in terms of crisis signaling and possible oxidative stress (Trumpff et al., 2021). In particular, studies on cell free mitochondria DNA offer a pathway for better understanding the physical and mental detriments associated with stressor effects. When mitochondria leave cells and are detected in blood and saliva tests as cell-free mitochondria DNA (ccf-mtDNA), the body appears to be unndergoing an adaptation crisis (Trumpff et al., 2021; Trumpff et al., 2022). Within minutes or hours of carefully defined and delivered experimental stressors, relatively large increases of ccf-mtDNA (2-3 fold) are observed (Trumpff et al., 2019). Comparatively elevated levels of ccf-mtDNA are also observed in studies of individuals who attempted suicide (Lindqvist et al., 2016), and individuals diagnosed with depression (Lindqvist et al., 2018).

Recent ccf-mtDNA studies have occurred in different labs, employing different stress induction protocols and measurement procedures (Trumpff et al., 2021). There are variances in results that present consequent challenges in deriving systematic estimates of the short and long term effects. Nonetheless, for the most part, the effect sizes, i.e. the levels of increased ccf-mtDNA observed, are 2 to 3 fold, suggesting that psychological stress can potentially influence mitochondria-provoked hormone release and oxidative load (Trumpff et al., 2019). Given the multiple problems of stress-related burnout in current society, (especially during the COVID crisis), more precision is required in assessing the effects of psychological stress and how much it alters future coping resources, especially in the context of ongoing heavy workloads.

Mitochondrial health appears to play a key role in multiple psychological and chronic disease conditions. It follows that studies would be undertaken that focus on acute stress impacts. Although multiple studies have focused on "acute stress" effects on mitochondria, they have

mainly employed social anxiety stressors via the Trier Social Stress Test (TSST) (Hummel et al., 2018; Trumpff et al., 2019). These studies require further replications, and partial replications, to address current knowledge gaps. Specifically, no studies have investigated associations between ccf-mtDNA and acute psychological stress, using lab-based stressors other than the TSST. For example, no studies have used images to provoke stress that are associated with the International Affective Picture System (IAPS). The IAPS is a unique development where all photo images have been carefully pre-evaluated for human stress impact, permitting fine-tuned adjustments that can, putatively, raise or lower the stress impact delivered. As an experimental stressor, it differs from the TSST as:1) it requires no deception of subjects, making it amenable to multiple uses and comparisons over time; 2) it elicits multiple forms of anxiety rather than dominantly eliciting social anxiety.

Furthermore, most of the studies that have investigated the role of ccf-mtDNA in psychological or physical disorders have done so by obtaining and measuring ccf-mtDNA via blood plasma as opposed to serum (Trumpff et al., 2021). Recent reviews of ccf-mtDNA isolation protocols favour serum over plasma for accurate measurement, given that plasma is rich in platelets, an abundant source of both mitochondria and cf-mtDNA (Trumpff et al., 2021). The use of clotting agents, in the preparation of serum samples, enables a more accurate measurement of ccf-mtDNA levels (Trumpff et al., 2021).

Lastly, existing studies investigating the ccf-mtDNA and its roles in the body have been mostly male-dominated, with fewer studies that involve a lesser number of female subjects (Hummel et al., 2018; Trumpff et al., 2019). Our study solely focuses on female students to better understand female effects.

1.2. Objectives

This thesis aimed to assess quantities of ccf-mtDNA increases observed in university students confronting experimental stress via an IAPS-based experimental stressor, whereas prior studies involved individuals confronting TSST evoked stress. Although different stress induction protocols were implemented, closely replicated blood draw methods and assays were employed due to the close coordination possible with the Mitochondrial PsychoBiology Lab (MPL) at

Columbia University. The assays undertaken were very similar to those used in prior, published ccf-mtDNA experimental stress studies by members of the MPL at Columbia (Trumpff et al., 2019).

2. Literature Review

2.1. Mitochondria

Because life is energy-dependent, generative interactions of molecules cannot coherently interact without sufficient energy. The structures, produced by such molecular interactions, cannot form or replicate. The supply or flow of energy is therefore a vital differentiating factor between animate organisms and inanimate ones. For sustained life, a constant energy resource is required to support biochemical interactions. This energy is largely obtained through the subcellular structures termed mitochondria, which produce ~ 90% of the cell's energy (Castellani et al., 2020). Biochemical reactions collectively termed 'cellular respiration' produce the required energy as food molecules (in the forms of glucose and fatty acids) combine with oxygen molecules to make adenosine triphosphate (ATP) (Picard & McEwen, 2018). ATP is the energy currency of the cell which releases the energy stored in its chemical bonds (Hinkle & McCarty, 1978).

The optimal functioning of mitochondria also relies on the maintenance of a flow of cellular energy (ATP) (Picard et al., 2018). In other words, a state of 'balanced energy flow' is optimally maintained in mitochondria, through an equilibrium between energy demand and energy production. The energy equilibrium can be disrupted by acute physical and/or psychological stressors (Picard et al., 2018). To gain better understandings of the relationship between stress and mitochondria, it is necessary to fully recognize the functional capacities of mitochondria, as well as how they operate under the conditions of different diseases and disorders and dynamically interact with diverse environmental stressors. These understandings can form a framework for better understanding the unique ways in which normative and psychopathological stress impact mitochondria and overall health.

2.1.1. Mitochondria energy production

Within the membrane-bounded cytosol of eukaryotic cells, multiple mitochondria exist. The most important of the multiple mitochondria functions is, once again, the production of cellular (bodily) energy (~ 90%) (Castellani et al., 2020). The process of energy production (termed oxidative phosphorylation) utilizes the Electron Transport Chain (ETC) which is composed of membrane-embedded proteins and organic molecules located on the inner mitochondrial membrane. The 'chain' consists of four complexes, often identified by numerals I, II, III and IV (Saraste, 1999). In order to generate energy from food intake, the mitochondria undergo biochemical transactions, during which glucose and free fatty acid (FFA) molecules interact and become oxidized to generate NADH and FADH2 and donate electrons to the ETC (Lowell & Spiegelman, 2000). These electrons travel from one protein complex to another (i.e. between I, II, III and IV), moving from higher to lower energy levels while releasing energy (Vercellino & Sazanov, 2022). The released energy pumps hydrogen molecules into the mitochondria's inter-membrane space, forming an electrochemical gradient (Lowell & Spiegelman, 2000). The gradient, produced by hydrogen ions, involves stored energy typically referred to as the "proton motive force". Hydrogen ions then move down the ATP synthase (an enzyme) which functions like a turbine, such that the downward flow of hydrogen ions causes the inner part of the complex to catalyze the transformation of inorganic phosphate into Adenosine Bi-Phosphate (ADP), which later becomes Adenosine Tri-Phosphate (ATP) (Vercellino & Sazanov, 2022).

Generally, the energies stored in ATP molecules are used for multiple processes such as muscle contractions, nerve impulse activities, other chemical syntheses and physical housekeeping tasks (Picard & McEwen, 2018). The ETC ensures the flow of mitochondrial energy and an optimal current of energy essential to human survival, growth, healing and environmental adaptation. Due to the mitochondrial generation of human cell energy and the complex communications with other mitochondria and cells, mitochondria play an essential role in modulating energy (via ATP) during cellular stress events (Lindqvist et al., 2018).

2.2. Evolution of Stress as a Concept

Understandings of stress in the domain of health have evolved over time. In the 20th century, Walter Cannon was one of the first physiologists to explore the concept of stress in relation to

health. His articulation of the "fight or flight" response in relation to Claude Bernard's homeostasis concept considerably advanced current stress understandings (Cannon, 1970; Goldstein & Kopin, 2007). Cannon defined stress as the physiological response to environmental threats, resulting in adaptive responses that maintain internal physiological balances (i.e. homeostasis) that ensure survival (Cannon, 1970).

Another considerable advancement was based on the homeostasis and fight-or-flight constructs, and was articulated by the Hungarian-Canadian endocrinologist, Hans Selye, who observed that rats exposed to the stressors of cold, drugs, or surgical injuries exhibited a common response pattern. He called this the "general adaptation syndrome", later identifying an initial *alarm* phase followed by an *adaptation* phase and, finally, an *exhaustion-death* stage when there were additional physical demands (Selye, 1956, 1946).

Bernard, Cannon and Selye significantly influenced psychology and psychotherapy as their stress conceptualizations involved a psychological component, potentially modifiable by changes of behavior. Modifiable stress experiences were then further investigated by Christina Maslach, who focused on prolonged stress, establishing the concept of "burnout", psychometrically, via the Maslach Burnout Inventory (MBI) (Maslach, 1986). She contributed to current understandings of stress by illustrating how prolonged experiences of stress elevations could lead to physical, psychological and emotional exhaustion.

In recent years, investigations of stress have emphasized the interconnectedness of biopsychosocial factors. Most recently, stress has been focused on as an energy-demanding process, integrating the role of dynamic change in mitochondria. During human encounters with environmental stress, energy demands increase and mitochondria respond to these increased demands (Picard et al., 2018).

Demands for increased energy are responded to (within mitochondria) with ATP, powering the enzymatic reactions that enable survival (Picard et al., 2018). In human organisms, where mitochondria generate ~ 90% of bodily energy, their functioning requires temperatures below

50 °C for the ETC complexes to function optimally and for the mitochondria to function as sites of cellular thermogenesis (Chrétien et al., 2018).

Cells that require more energy usually have a higher mitochondrial content. As such, mitochondrial content is more abundant in the cells of organs that are highly functional during stress responses (e.g. the heart and the brain). For example, the increased heart rate that is typical in responses to stressors involves an increased energy demand in cardiac cells which increases the work of the multiple cells that generate energy through ATP. The depolarization-repolarization necessary for cardiac contractions requires ATP (Schubert et al., 2009; Suga et al., 1993). Similarly, stress responses often demand more rapid cognitive processing, increasing the cellular energy required in multiple brain areas (Magistretti & Allaman, 2015; Picard et al., 2018). This is also the case when the energy demands of skeletal muscles require more ATP from muscle serving mitochondria during fight - flight responding.

Although the stress response is generically responsible for increases in bodily energy demands, different types and durations of stressors have varying biological effects and mitochondrial modifications (Picard et al., 2018).

2.2.1. Stress types and their role in the mitochondrial energy transformation

From the perspective of mitochondrial changes, stress is defined as brain-body responses to threats to the organism's homeostasis which stimulate energy-consuming adaptations (Picard et al., 2018). Stress can be viewed as *good*, *tolerable* and *toxic*, using definitions articulated by Picard et al. *Good* stress occurs when performing tasks that are challenging and desired, such as during public speaking and in games or competitive sports. Feelings of reward accompany success (Picard et al., 2018). During *good* stress, the biochemical stress mediators, such as adrenalin and cortisol, are secreted and 'turned on' when necessary and 'turned off' after the challenge is met. This promotes longer term positive adaptations. *Tolerable* stress occurs when a negative event takes place, such as the death of a loved one, divorce, or the loss of a job. Despite the adversity, the individual has the resources and tools to successfully cope (Picard et al., 2018). During tolerable stress, stress mediators are turned on for different time periods for adaptation

purposes. However, if they stay on for periods beyond a certain length, they pose a threat to overall health and well-being. Nevertheless, by maximizing internal and external resources, a person can minimize the negative effects and ultimately reach adaptive resolutions (Picard et al., 2018). *Toxic* stress follows a negative event that the individual has insufficient internal or external resources to cope with (Picard et al., 2018). While these definitions and related states vary by degree and severity per individual, depending on the control of the perceived stress and the situational challenges faced, they comprise a phenomena known as the "allostatic load" or "allostatic overload" (Sterling, 1988). The concept of allostatic load suggests that the same mediators that aid stress adaptation, when overused or dysregulated, lead to pathophysiology. This is evident when stress regulators are activated for lengthy periods, prompting damage and dysfunction as opposed to homeostatic adjustment (McEwen, 1998; McEwen & Stellar, 1993).

2.2.2. Mitochondrial Allostatic Load (MAL)

The mitochondria have their own (specific) allostatic loads, specifically labeled "Mitochondrial Allostatic Load" (MAL) (Picard & McEwen, 2018). Not surprisingly, the MAL contributes to the allostatic load of the whole individual via a 3-stage process: i) sensing ii) integration iii) signaling (please refer to Figure 1).

In the *sensing* stage, some form of psychosocial stress occurs in an individual's life through ethnic discrimination, Adverse Childhood Experience (ACE), low SES, job strains, social isolation, caregiving tasks and/or internal-interpersonal psychopathology. The individual lacks sufficient internal and/or external resources to cope with the stressor (Picard & McEwen, 2018). As such, a resource challenge is experienced and the mitochondria apparently *sense* that life altering changes are necessary. As a result, stress mediators are systematically activated.
In the *integration* stage, the mitochondria interact with systematic stress mediators in a bi-directional manner (Picard & McEwen, 2018). The stress mediators are often physiological and behavioural. The physiological mediators may include glucocorticoid secretions induced by the hypothalamus-pituitary-adrenal (HPA) axis, catecholamine secretions induced by the sympathetic-adreno-medullar (SAM) axis, sex hormone secretions induced by hypothalamic–pituitary–gonadal (HPG) axis, and the concurrent sympathetic and parasympathetic autonomic

nervous system changes such as those involving Heart Rate (HR) and Heart Rate Variability (HRV), elevated oxidative stress, pro-inflammatory and anti-inflammatory cytokine secretion and brain atrophy (Picard & McEwen, 2018). The parallel behavioural mediators might include reduced exercise and increased sedentary behaviors, increased cognitive-emotional reactivity, poor diet and sleep disturbance (Picard & McEwen, 2018).

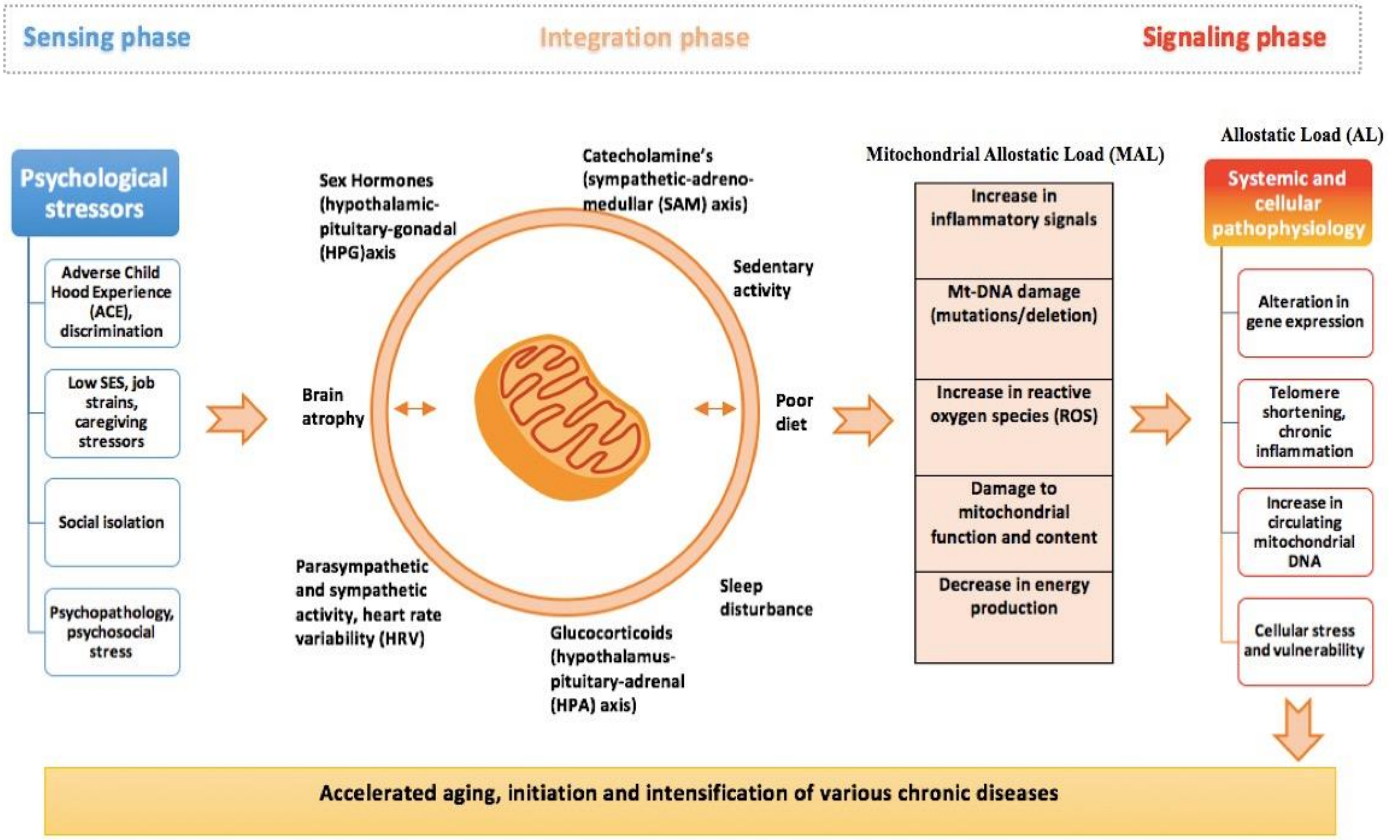
Depending on the type and intensity of the stress, the mitochondria may have different responses. For instance, during times of moderate/controllable stress, stress moderators, interacting with the mitochondria, can stimulate an adaptative response. This is evident in the mitochondrial fusion and fission processes. During a fusion, a healthy mitochondrion rescues a damaged mitochondrion by a sharing or fusion of components that results in a healthier unit (Picard & McEwen, 2018). During a fission process, cleavage of partially damaged mitochondrion components prompts new organelle generation (Chen & Chan, 2006).

Depending on the time duration of the activations of physiological and behavioural mediators, there can be a long-lasting damaging effect on the organelle, defined as a state of mitochondrial allostatic load (MAL). Then various physiological recalibrations occur such as increased inflammatory signals and Reactive Oxygen Species (ROS), which have destructive effects on the mitochondrial DNA (mtDNA) causing genetic mutations/deletions and further changes in function, content and overall energy production capacity (Picard & McEwen, 2018).

In the *signaling* stage of this process, the MAL is transduced via a variety of signaling molecules that affect the body's overall systematic allostatic load leading to systematic cellular changes including: alterations in gene expression, telomere shortening, increases in circulating mitochondrial DNA, chronic inflammations, cellular stress, vulnerability and fatigue (Picard & McEwen, 2018). Ultimately, the induced changes from the MAL and the prolonged activation of systematic allostatic load, result in accelerated aging and the initiation or intensification of various chronic diseases (Picard & McEwen, 2018; Picard & Turnbull, 2013).

Figure 1

Visual Representation of Mitochondrial Allostatic Load and its Contributes to the Systematic Allostatic Load



(Picard & McEwen, 2018)

2.3. Mitochondrial DNA and ROS

Given the consequences of mitochondrial dysfunction, there are various measurements applied to assessing healthy mitochondrial function. One approach is to assess mtDNA copy number (mtDNA-cn), a quantification of the mitochondrial genomes located in each cell. The mtDNA-cn are measured and obtained from peripheral blood mononuclear cells (PBMC), particularly

monocytes, lymphocytes, and dendritic cells that perform immune functions and other body maintenance tasks (Gambardella et al., 2019).

Similar to nuclear DNA, the mtDNA is a double-stranded nucleic acid that encodes for essential genes. However, the mtDNA replicates independently of nuclear DNA and varies from the nuclear DNA in several ways (West & Shadel, 2017). First, mtDNA is much smaller than the nuclear DNA (16,569 bp vs. 3.2 billion bp) and contains and carries less genetic information (West & Shadel, 2017). The mtDNA contains 37 genes, of which 13 encode for the fundamental mechanisms involved in mitochondrial respiration, such as ATP synthase and protein complex activity (I–IV). Unlike nuclear DNA, mtDNA is highly vulnerable to oxidative stress. While it is less protein protected than nuclear DNA, the mtDNA has protective factors that are packaged into its protein–DNA complexes, known as nucleoids. This protective packaging is directed by mtDNA-binding protein transcription factor A, often labelled as mitochondrial TFAM (Bonawitz et al., 2006). Unfortunately, this DNA packaging lacks adequate DNA repair mechanisms (e.g. robust histones) and as a result, the protective proteins can only prevent minor (but not major) mtDNA damage. Because responses to more severe stressors require high rates of oxidative phosphorylation to satisfy the heightened energy demands, the heightened stress, energy demands and responses increase the intracellular Reactive Oxygen Species (ROS) that are located in close proximity to the mtDNA (West & Shadel, 2017). In such high oxidative stress environments, the mtDNA is vulnerable to dysregulation and mutagenesis. Studies have shown that when placed under similar oxidative stress environments, the mtDNA is at higher risks (10- to 200-times higher) for genetic mutations than the nuclear DNA (Habbane et al., 2021). High-energy-demanding cells, such as myocytes and neurons, are particularly vulnerable given their high sensitivity to ROS-mediated damage (Gambardella et al., 2019).

When there is an imbalance between ROS production and cellular antioxidant capacity, the ROS causes damage to the macromolecules within cells (e.g. lipids, DNA, proteins). Previous studies have indicated that individuals with major depression disorders experience substantial increases in oxidative stress. As such, depressed individuals have been shown to have lower levels of plasma antioxidants and altered levels of antioxidant enzymes (Moylan et al., 2014). Essentially, the mitochondria are key actors in regulating oxidative stress but are, simultaneously, a primary

source of ROS production. Their regulatory actions are significant, because endogenous and/or exogenous cellular stress, due to psycho-physical stressors, can substantially increase ROS production. In turn, the accumulation of ROS can damage, oxidize and mutate the mtDNA because of: 1) the close proximity of the ROS to the mtDNA in the mitochondrial matrix; 2) the lack of adequate packaging proteins guarding the mtDNA (West & Shadel, 2017).

2.4. ccf-mtDNA

The unfolding of the events described above is important to this thesis study, because as oxidative damage accumulates beyond the capacities of repair systems, mtDNA is released into the blood and detected in blood plasma as circulating cell-free mtDNA (ccf-mtDNA) (Trumpff et al., 2019). This mtDNA release is measurable as ccf-mtDNA (Moya et al., 2021) and can, theoretically, be linked to identifiable mechanisms.

One of the processes by which this happens, "autophagy", is a process of cell degradation that involve lysosomes, or membrane-bound organelles that contain digestive enzymes (Moya et al., 2021). In autophagy, a structure, known as the autophagosome, is generated, which consists of numerous protein complexes and components of the membrane-based endoplasmic reticulum. The autophagosome can encapsulate mitochondria and eventually fuse its apparatuses to the mitochondrial lysosomal membranes, forming an autolysosome (Moya et al., 2021). Digestive enzymes released from the lysosomes then degrade the mitochondria in various ways. One significant degrading enzyme is DNase II, which degrades the DNA strand through hydrolysis of its phosphodiester backbones. In cases where high ROS levels are detected in the mitochondrial matrix, the nuclease activity of DNase II becomes saturated, preventing it from degrading the mitochondrial DNA and permitting it to escape into the blood circulation as ccf-mtDNA (Moya et al., 2021).

Another mechanism linked to mtDNA release (as ccf-mtDNA) is from the increased permeability of the mitochondria membranes (Moya et al., 2021). This process occurs because high oxidative stress increases intracellular Ca2+ concentration within the mitochondria, which, in turn, increases the diameter of mitochondrial permeability transition pores (mPTP), allowing for mtDNA to escape into the blood stream (Moya et al., 2021).

Lastly, another significant pathway for mtDNA release is through cell apoptosis processes (i.e. cell-death). Although the specific associated mechanisms are not precisely understood, currently, it's plausible that apoptosis leads to the degradation of the cell membrane, with the result that mtDNA is released into the circulating blood as ccf-mtDNA (Moya et al., 2021).

Although the exact biochemical processes that take place during releases of ccf-mtDNA are not fully understood, the circulating DNA segments have been shown to play an important role in many chronic diseases as outlined in the section below.

2.4.1. ccf-mtDNA and Health Risks

The elevated ccf-mtDNA that indicates mitochondrial dysfunction also indicates increased health risk (Gambardella et al., 2019). For example, elevated levels of ccf-mtDNA have been observed in patients with cancer, acute systemic inflammatory responses, chronic inflammation, diabetes, sepsis, physical trauma, myocardial infarction and after post-surgical interventions (as reviewed by (Trumpff et al., 2021). Furthermore, elevated levels ccf-mtDNA levels are associated with a 4 to 8 times increased mortality risk in critically ill hospitalized patients (Nakahira et al., 2013). In addition to physical health problems, ccf-mtDNA has been linked to psychological distress. For instance, significantly higher ccf-mtDNA levels were found in patients with major depressive disorders (MDD) and suicide attempters (Lindqvist et al., 2018). Additionally, ccf-mtDNA levels were observed to stay elevated in patients with MDD who did not respond to selective serotonin reuptake inhibitor (SSRI) pharmacotherapy (Lindqvist et al., 2018). In the study described above, significantly lower ccf-mtDNA levels were found in patients who responded positively to the SSRI treatment vs. non-responders, reflecting possible SSRI therapy effects (Lindqvist et al., 2018). The non-responders demonstrated increases in ccf-mtDNA while the responders showed no change ($p = 0.02$) (Lindqvist et al., 2018). Recent publications have also demonstrated significant ccf-mtDNA increases after exposures to psychological lab stress responses, providing further evidence of theoretically coherent relationships between ccf-mtDNA, psychological health and psychological distress (Picard et al., 2018; Trumpff et al., 2019; Lindquistet al., 2018).

2.4.2. ccf-mtDNA, Inflammation and CVD

It has been recently discovered that mitochondrial mediated inflammation is associated with the progression and pathogenesis of CVD. This occurs as high levels of ROS production induce cell-necrosis, causing the release of mtDNA into the circulating blood as ccf-mtDNA. The ccf-mtDNA contributes to the activation of the immune system by inducing inflammation via three variable mechanisms; 1) inflammasomes, 2) stimulator of interferon genes (STING) pathways and 3) toll-like receptor 9 (TLR9) (as reviewed by (Nie et al., 2020). Inflammation induced via ccf-mtDNA signaling, has been associated with increased risk of CVD-associated conditions such as diabetes mellitus, arterial hypertension and hypercholesterolemia (as reviewed by (Nie et al., 2020).

Inflammation mediated by ccf-mtDNA has been shown to also play a crucial role in patients with acute myocardial infarction (AMI) and Heart Failure (HF) (Arafat et al., 2018; Oka et al., 2012). Following an AMI episode, an initial pro-inflammatory response is induced by innate immune cells, apparently to remove the necrotic cell debris. Next, the adaptive immune system is triggered, resulting in a gathering of inflammatory cytokines. During this stage, ccf-mtDNA levels have been shown to increase by 4-fold due to oxidative damage (Bliksøen et al., 2012). The ccf-mtDNA-induced inflammation after an AMI episode has also been linked to development of HF (Yndestad et al., 2007). The ccf-mtDNA levels have not only been shown to be elevated in HF patients, but such elevation has been associated with increased mortality. In fact, significant elevations in ccf-mtDNA levels have been shown to be much higher in patients who died within 30 days of hospitalization following an HF, as opposed to those who survived (Krychtiuk et al., 2017).

Inflammation that is ccf-mtDNA-mediated also plays a big role in cardiac recovery and remodeling. Recent studies indicate that inflammation levels are reduced in patients following successful heart surgeries such as percutaneous coronary intervention (PCI) and pulmonary bypass (Wang et al., 2015). This is crucial as CVD is a major source of mortality-morbidity risks worldwide, accounting for one-third of all deaths (Nie et al., 2020). Therefore, targeting mitochondrial mediated inflammation, and possibly reducing it, could attenuate progression in pursuit of CVD healing.

2.4.3. ccf-mtDNA, Inflammation and SARS-CoV-2

Mitochondria also play a major role in viral infections such as the acute respiratory syndrome (SARS-CoV-2). The pathological and biochemical features of this virus invoke in the body a state of acute inflammation, cellularly stressing the mitochondria, and elevating ROS production (Valdés-Aguayo et al., 2021). Furthermore, this virus triggers an increase in ferritin (iron) and platelet dysfunction, exacerbating mitochondrial dysfunction through rapid ROS elevations. The inflammatory responses cause the organelles to be damaged, releasing mtDNA into the circulation in the form of ccf-mtDNA (Valdés-Aguayo et al., 2021). As mentioned previously, ccf-mtDNA appears to participate in the immune system by inducing inflammation through mechanisms such as the inflammasomes, the STING pathways and TLR9 (Nie et al., 2020; Valdés-Aguayo et al., 2021). This causes a pro-inflammatory state, intensifying disease severity. As a result, elevated ccf-mtDNA levels are shown to be an early indicator of the severity of SARS-CoV-2 (covid-19) viral infections (Valdés-Aguayo et al., 2021). In the absence of dynamic time-course data literature, such findings have led researchers to believe that ccf-mtDNA is a relatively stable marker of chronic diseases. However, subsequent studies have also shown that ccf-mtDNA is similarly rapidly induced during physical activity and acute stress.

2.5. Disruption and Preservation of Balanced Flow

As previously indicated, a ‘balanced flow’ of cellular energy is required for the mitochondria to function optimally and healthfully. This means that the mitochondria are meeting the energy demands of external stressors by using available oxygen molecules within the cell to produce chemical energy. In states of balanced flow, the production of this chemical energy meets the cellular energy demands.

However, this ‘balanced flow’ of energy can be disrupted by acute and chronic physical and/or psychological stressors (Picard et al., 2018). With ccf-mtDNA levels used as indicators, depending on the type of the stressor, it appears that the mitochondria could be either engaged in adaptation and resilience, or malfunction and disruption (Picard et al., 2018).

2.5.1 Exercise Stress and ccf-mtDNA: Preservation and Disruptions of Balanced Flow

With regards to stress resilience responses and mitochondria activity, exercise training apparently has a significant role in enhancing resilience and adapting to stress. The impact of exercise-induced stress on the mitochondria, however, can vary depending on the type and intensity.

For example, in a study conducted by Stawski et al., the relationships between mitochondrial health (as measured by ccf-mtDNA) and acute physical activities were examined (Stawski et al., 2017). The study involved 11 healthy men who were instructed to engage in repeated bouts of exhaustive treadmill exercise (at 70% of their VO2 max) on three separate occasions, with a 72-hour resting period between each session. Blood samples were collected immediately before and after each treadmill exercise session (Stawski et al., 2017). The findings demonstrated an elevation in ccf-mtDNA levels following each exercise bout, suggesting that short-term, high-intensity exercise may induce a form of detrimental stress in individuals who are not regularly active. Consequently, this could potentially compromise an individual's system overall, leading to allostatic overload and an increased risk of mitochondrial allostatic load (MAL), as evidenced by the rise in ccf-mtDNA blood levels.

Conversely, additional research has indicated that different intensities and types of exercise can induce a beneficial form of stress that promotes mitochondrial adaptation. A study conducted by Shockett et al. (2016) explored the impact of acute physical activity in sample of healthy, moderately trained, young men and discovered a reduction in ccf-mtDNA levels one hour after a bout of moderate intensity exercise (60% VO2 max). Another study undertaken by Nasi et al. suggested that ccf-mtDNA levels decline, but only if the training is highly regular (see Table 1, is positioned after references). Such findings indicate a possibly direct association between regular exercise training and decreases in baseline levels of ccf-mtDNA (Nasi et al, 2016). They suggest that regular physical activity could be a “good” form of stress, where the stress mediators are turned on when necessary and turned off after challenges are completed, resulting in on-off pulsations and durations that promote mitochondrial adaptation and states of balanced flow.

However, most existing studies have demonstrated mixed results associated with brief aerobic exercise bouts in terms of ccf-mtDNA outcomes (Trumpff et al., 2021). Furthermore, many of these studies had different timings and lab procedures of blood processing for DNA isolation (Trumpff et al., 2021). It is important to note that the reductions in the ccf-mtDNA associated with moderate exercise and increases associated after high intensity exercise could be attributed to increases or reductions of ROS related mechanisms. High intensity aerobic exercise has been shown to elevate ROS formation and decrease antioxidant enzymes in bodily (particularly skeletal muscle) tissues. As a result, accumulations of ROS, due to high intensity exercise could damage the mtDNA, elevating releases of ccf-mtDNA in to the blood circulation (Shamsnia, 2023).

2.5.2. Psychological Stress and ccf-mtDNA: Disruptions of Balanced Flow

Evidence that psychological stress is closely linked to mitochondrial health and functioning is related to four factors (Trumpff et al., 2021).

1) Energy is essential at multiple levels of biological functioning (including cellular, organelle, and systemic levels), for the endurance of stress impacts.

2) Specific stress-reactive hormones like steroid hormones and glucocorticoids are produced and processed by mitochondria.

3) Both metabolic and neuroendocrine stress mediators can influence mitochondrial function and related behaviors.

4) Experimental manipulations of mitochondrial functions have demonstrated alterations in individual physiological and behavioural stress responses (Trumpff et al., 2021). Once again, maintaining a balanced flow of cellular energy is crucial for mitochondria to function optimally and the flow balance can be disrupted by acute physical and/or psychological stress. To gain a better understanding of the relationship between stress and mitochondria, it is important to examine studies focusing on acute psychological stresses on mitochondrial function.

In the current research literature, two prior studies have focused on the hypothesis that acute psychological stress triggers ccf-mtDNA release. These studies quantified ccf-mtDNA release in

relation to the psychosocial stress evoked by the Trier Social Stress Test (TSST) an experimental stressor). Hummel et al. tested 20 healthy young men and found a 1.6-fold increase of plasma ccf-mtDNA, two minutes after completion of the TSST stressor exposures (Hummel et al., 2018). Using a similar protocol, Trumpff et al. assessed 50 middle-aged male and female subjects and observed a 2-3-fold increase of serum ccf-mtDNA, within 30 minutes of the TSST exposures (Trumpff et al., 2019). Both studies therefore showed relatively immediately increases of cf-mtDNA (in plasma *and* serum) following completion of the experimental stress protocol. Accordingly, a linkage between psychosocial stress and increased ccf-mtDNA appears likely. These findings suggest that acute psychological stressors can act as a detrimental or "toxic" form of stress, leading to irregularities in stress mediators that disrupt mitochondrial function and result in damaging organelle effects.

Moreover, the occurrence of ccf-mtDNA has been shown in other biofluids than blood, including urine (Han et al., 2021) cerebrospinal fluid (Varhaug et al., 2017) and saliva (Trumpff et al., 2022) indicating that ccf-mtDNA is likely a biomarker in multiple biofluids. More recently, the ccf-mtDNA was measured using saliva samples (Trumpff et al., 2022). In this study, two healthy men were studied at 4 daily time points for between 53 and 60 consecutive days (Trumpff et al., 2022). Although the ccf-mtDNA results were consistently similar for most days, an anomalous spike of ccf-mtDNA results was observed on day 26 for one participant. This occurred on the day before the participant ran a marathon (which took place on Day 27). Such results could be due to anticipatory stress, i.e. anticipating energy mobilization needs. Results like these seems to confirm the importance of ccf-mtDNA in adapting to varying types of acute stress. It is worth mentioning that on the anticipatory day (i.e. day 26) there were no significant spikes in cortisol awakening response (CAR) levels, suggesting a noteworthy divergence between ccf-mtDNA and CAR responses (Trumpff et al., 2022).

Further indications on how mitochondria respond to psychological stressors are located in studies on how chronic psychological stress decreases mitochondrial energy production and alters morphology. Chronic stress impacts were demonstrated in multiple decreases in the enzymatic activity of electron transport chain (ETC) complexes (as in altered rate of oxygen consumption, membrane changes, and changes in intracellular content) and in genomic sequences that code for

essential proteins within the ETC system (Picard et al., 2018). Comparatively few studies have focused on the impact of acute psychological stresses on mitochondria, which explains the focus of our study. The few studies already undertaken are interesting and provocative but are in need of replication and expansion.

2.6. Primary Research Question

Previous studies have investigated the relationship of acute stress and ccf-mtDNA but mainly in middle aged, male-dominated samples with TSST used as the stressor source. Since research on female dominated samples and responses to IAPS photo stimuli are limited, this study was aimed at assessing whether the IAPS photo induced lab stressor is sufficient to cause elevations in post-stressor ccf-mtDNA levels (+30 mins after stressor) in young, healthy female participants.

***2.6.1. Hypothesis 1*:** Study participants will demonstrate significant increases in ccf-mtDNA levels after exposures to IAPS photo lab stressors, within 30 to 45 minutes of the stress exposures.

2.7. Secondary Research Questions

The study we aimed to replicate by Trumpff et al. demonstrated an increase in the negative mood states of participants, which were correlated with their elevations of pre-post ccf-mtDNA levels. Thereby, in this replication study we were motivated to assess whether the IAPS photo-based lab stressors induced lab stressor effects sufficient to cause a significant increase in the negative mood states of participants, in correlation to their elevation in pre-post ccf-mtDNA levels. Moreover, additional research has indicated that different intensities and types of exercise can induce a beneficial form of stress that promotes adaptation in mitochondria (refer to table 1). A previous study by Shockett et al. (2016) explored the impact of acute physical activity in healthy individuals and their results suggested individuals who engaged in regular exercise tended to exhibit lower levels of ccf-mtDNA compared to those who did not. Since there has been a mixture of results in the research literature regarding exercise and ccf-mtDNA. Our study

employed self-administered IPAQ questionnaires to assess physical activity (PA) levels and evaluate PA levels play a role in acute psychological stress and ccf-mtDNA release.

***2.7.1. Hypothesis 2*:** the induced lab psychological stress will demonstrate significant increases in negative mood state profiles, in correlation with elevations of ccf-mtDNA levels.

2.7.2. Hypothesis 3: the more physically inactive subjects will experience a greater difference in ccf-mtDNA levels elevations than those who are moderately or highly active.

3. Methods

3.1. Study Design

This is a clinical study which aims at partially replicating the Trumpff et al. (2019) study. The study design (see figure 2a, page 25), consent form and the recruitment poster were reviewed and approved by the Human Participants Review Committee at York university.

3.2. Sample size and study participants

3.2.1. Sample size calculation

The sample size estimation utilized the G*power software, using a t-test with the following input parameters (0.8 Effect size, 0.05 α error probability, and 0.95 for the power/ 1- β error probability).

3.2.2. Participants and recruitment

A total of 18 female students were recruited in our study, and data from 14 of these subjects (n=14) were used in the final analyses. Four participants were excluded due to confounders discovered after they completed study protocol (n=2), and due to issues related to instrumental limitations (qPCR) encountered during the mitochondrial DNA sampling analyses (n=2) (Please refer to CONSORT diagram, figure 2b). Two blood draws were taken before and after exposure to the lab stress protocol. Participants were recruited through recruitment flyers and posters which were posted at York University (Keele campus). Since many participants are motivated by compensation, a modest incentive of $20 (CAD) was provided for each participant who participated in the study.

3.2.3. Inclusion criteria

1. Females of 20-30 years of age; 2. Residency maintained in Canada; 3. Generic good health as defined by no history of asthma, cancer treatment, myocardial infarction, and/or systematic immune diseases (Trumpff et al., 2019); 4) Non-smokers, as ascertained by self-report; 5) Completion of informed consent procedures approved by the Human Participant Research Committee of York University.

3.2.4. Exclusion criteria

1. Pregnant and/or lactating women; 2. Current or recent indications of mental health problems (during the last 3 months) ascertained by self-report; 3. No history of chronic or acute physical disabilities or injuries; 4. No current intake of prescribed medications that could potentially alter endocrine, nervous and immune system functioning (oral contraceptives were excepted).; 4. No antibiotic use and self-report of infection and vaccination symptoms; 5) No recent tattoos (as of 2 weeks before the scheduled blood withdraw).

These inclusion-exclusion criteria were largely aligned with Trumpff et al. (2019) excepting the inclusion of solely females and the narrower-younger age criteria.

3.3. Psychometric assessments/measures

3.3.1. Self-Administered Comorbidity Questionnaire (SCQ) (see Appendix A)

The Self-Administered Comorbidity Questionnaire (SCQ) was used to evaluate the presence and severity of comorbidities in individuals (Sangha et al, 2003). The SCQ is a self-administered tool that can be completed without assistance from health care professionals (Sangha et al, 2003). It includes 13 generic medical health conditions and it has been shown to be valuable in research settings where medical records are not accessible (Sangha et al, 2003).

3.3.2. International Physical Activity Questionnaire (IPAQ) (see APPENDIX B)

The IPAQ (International Physical Activity Questionnaire) is a self-administered, tool used to assess physical activity behaviors undertaken during the previous seven days (Craig et al., 2017). The IPAQ measures the intensity of physical activity that individuals perform as a part of their daily lives in order to estimate their metabolic equivalent (MET) minutes per week (Craig et al., 2017). The IPAQ has a high test-rest reliability (r = .80) and has demonstrated adequate levels of predictive, concurrent, convergent, criterion, and discriminant validity (Craig et al., 2017; Warren et al., 2010).

3.3.3. The Profile of Mood States (POMS)-2A (See APPENDIX C)

The POMS (Profile of Mood States) is a widely used psychological assessment tool to evaluate a spectrum of transient mood states per individual. The mood states measured are tension, depression, anger, vigor, fatigue, and confusion (Heuchert & McNAir, 2012). It utilizes 65 statements that describe different emotions. Each statement is ranked on a Likert scale, ranging from 0 (not at all) to 4 (extremely) (Heuchert & McNAir, 2012). The POMS has been widely used in a variety of health field domains such as clinical research and medical treatments setting and sport performance settings (Heuchert & McNAir, 2012). The POMS has been shown to have very strong levels of internal consistency (assessed with Cronbach's Alpha) (.83-0.97) and good test-retest reliability (r = .48-.72) for normative samples (Heuchert & McNAir, 2012). POMS were assessed using T-scores obtained with the online Multi-Health Systematic (MHS) assessment protocol derived by Heuchert & McNAir (2012).

3.3.4. International Affective Picture System (IAPS)

The IAPS image stress task (IAPS) is based on 60-photographs selected from the International Affective Picture System (IAPS) database reflecting fear, sadness, anger, or frustration with the intention of evoking stressful autonomic activation (Lang et al., 1997). For the stress phase, a series of 60 negative emotion-evoking images from the IAPS were presented consecutively at 10-second intervals. Based on safety considerations, all images were carefully screened and approved by the clinical psychologist supervising this thesis. Each of the emotion-evoking images was set at the same height of 16.51cm high and 20.32cm wide. The computer screen luminance was set to night settings with blue light removed and brightness standardized and set at 60%. These lab stress exposures were followed by a 5 minutes' recovery phase where the sole visual stimulus was a moving fixation cross on a computer screen that randomly changes locations within a 9-square grid every 10-seconds. The sequential ID numbers of the 60 negative emotion-evoking images utilized from the IAPS database are listed as follows: 1670, 7016, 2352.1, 2352.2, 2770, 1620, 3001, 1920, 3000, 1617, 1750, 2900, 3030, 3051, 1610, 9043, 6250, 3101, 5600, 9400, 9405, 1560, 3266, 1590, 3050, 3060, 1630, 6530, 3103, 9041, 1710, 1999, 7521, 9163, 1274, 9427, 5781, 3195, 3110, 6838, 3170, 3019, 5825, 2880, 3022, 9910, 3010, 1410, 1525, 1120, 3071, 6837, 3100, 1111, 2655, 9187, 9570, 6520, 5001, 5665.

3.4. Procedures:

3.4.1. Prior to the study:

This study was conducted on consecutive weekdays from Monday–Friday (with one day assigned to each participant). The study took place each day from 9am - 12pm, with the purposeful timing of the stress exposures aimed at avoiding potential confounding daily stress factors related to circadian rhythms as well as diet-related factors (Trumpff et al., 2019).

Prior to this session, participants were asked to abstain from non-prescription medications and strenuous physical activities for 24 hours and to abstain from alcohol consumption for 48 hours (Trumpff et al., 2019). Furthermore, participants were asked to fast from food and caffeine on the morning of the experiment (Trumpff et al., 2019). Lastly, participants were instructed to meet at the lab testing room (Bethune Building 123) on the scheduled date of the study for 9:40 am via email prior to the study date.

3.4.2. Baseline Questionnaire Administration

Upon their arrival, participants were initially required to fill out the informed consent form, approved by the Human Participant Research Committee of York University. Subsequently, they proceeded to complete a set of questionnaires that included: A) assessment of covid-19 screening (See Appendix D); C) Self-Administered Comorbidity Questionnaire (SCQ) (See Appendix A) and current physical activity levels using the short term- International Physical Activity Questionnaire (IPAQ) (See Appendix B).

3.4.3. Baseline (1st blood draw)

Following the completion of consent forms and baseline questionnaires, participants were asked to prepare for the baseline blood draw, where a blood sample (of 6ml) was collected from the arm (front part of elbow) using a standard venipuncture technique. The aim of the first blood test was to measure the ccf-mtDNA levels at baseline (see figure 3, positioned on page 27). Following this rest period and the experimental stress exposure, the participant's mood states were evaluated using the Profile of Mood States questionnaire (POMS) (See Appendix C).

3.4.4. Lab Stress Assessment Procedure

At this stage in the unfolding protocol, participants were directed to a room inside the Bethune 123 Laboratory that was isolated from outside noise and other distractions to undergo the stress test protocol. They were exposed to the Emotional Image Stress Task (EIST) which is based on 60-images from the International Affective Picture System (IAPS) database reflecting fear, sadness, anger, or frustration with the intention of evoking stressful autonomic activation. Prior to the EIST, participants were given a 5-minute rest period (the resting period). Participants were instructed to keep their eyes open and view the slideshow of 60 images designed to invoke stress. Immediately following the Emotional Image Stress Task (EIST) exposure, participants entered the recovery phase of 5 minutes duration where the sole visual stimulus was a moving fixation cross on the watched computer screen that randomly changes locations within a 9-square grid every 10-seconds. The entire stress task protocol was conducted in a standardized dimly lit room (e.g., lux = 71) that was sound attenuated with use of noise cancelling headphones. Following the 5 minutes' recovery phase, the participants entered a 25-minute resting and sitting phase, where they were instructed to abstain from using any electronic devices or engaging in other intentional activities.

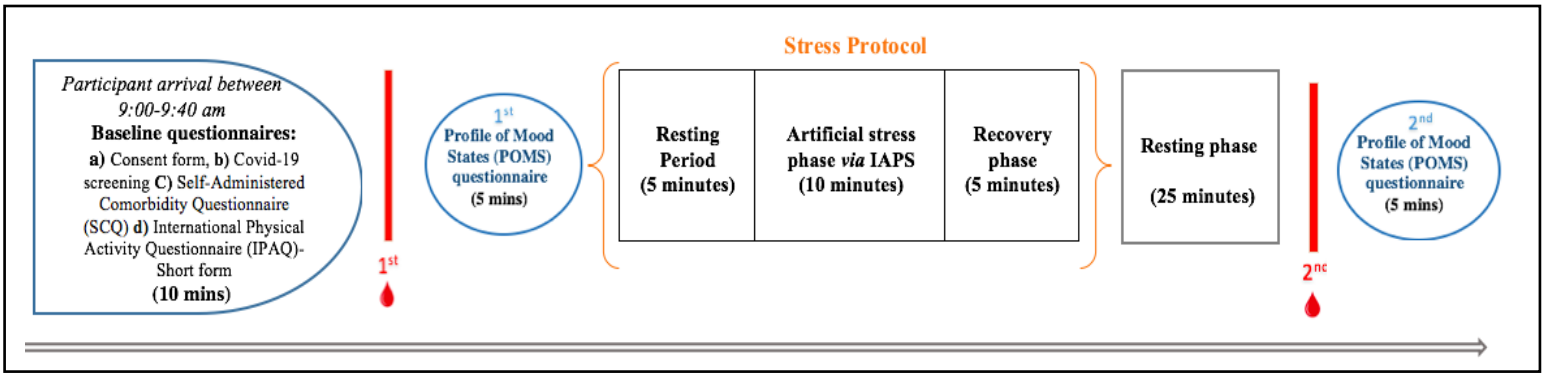
3.4.5. 2nd Blood draw

All subjects engaged in a 2nd blood draw designed to assess changes in ccf-mtDNA (time 2), at 30-40 minutes following the lab stress protocol. Similar to the baseline blood draw, a blood sample (6ml) was collected from the arm (front part of elbow) using standard venipuncture technique. A POMS questionnaire was re-administered again to assess the possible impacts of the lab stressor. All blood draws and stress protocol exposures occurred during mornings between 10:00-11:00 am to control for possible diurnal differences in metabolism and blood composition.

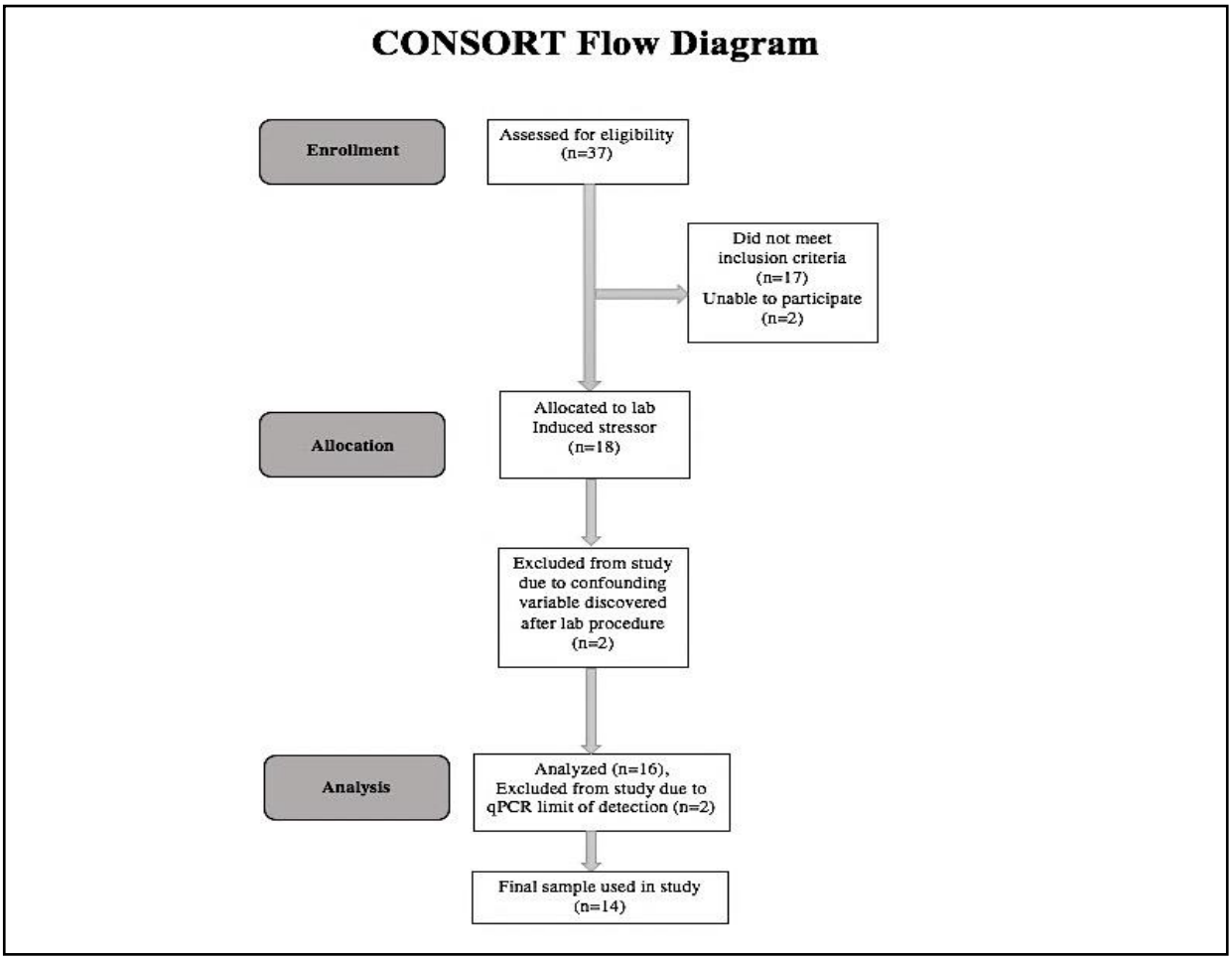
Figure 2

Visual Representation of Study Procedures

A.



B.



Note: Figure 2, A) Represents the step by step procedure of the study, B) Represents the CONSORT flow diagram of the study.

3.5. Serum DNA extraction and quantification considerations

3.5.1. Nuclear DNA measurement

Most previous studies on the psychopathology and ccf-mtDNA literature only focused on measuring the cell free mitochondrial DNA while neglecting to also assess the cell free nuclear DNA (cf-nDNA). In this study, similar to Trumpff et al. (2019), we measured the circulating levels of both cf-mtDNA and cf-nuclear DNA (nDNA). Assessment of the levels of cf-nDNA enable a more specific assessment of the specific release of ccf-mtDNA (Trumpff et al., 2021). Parallel increases in ccf-mtDNA and cf-nDNA suggest tissue damage, whereas increases in ccf-mtDNA without cf-nDNA suggest a process specific to psychological stress that does not include tissue damage reactivity (Trumpff et al., 2021).

3.5.2. Serum vs. plasma collection and analyses

While plasma reflects the liquid fraction of whole blood obtained from anti-coagulated blood, serum is the liquid fraction of the blood after a clotting process has been undertaken (Trumpff et al., 2020). This is vital when assessing ccf-mtDNA levels because platelets found in plasma are an abundant source of mitochondria and ccf-mtDNA. The inclusion of platelets can inflate the apparent ccf-mtDNA levels assessed in ways not comparable to serum findings (Trumpff et al., 2021). Furthermore, the use of clotting factors, when preparing serum samples, reduces the probabilities of blood degradation, yielding more reliable results (Trumpff et al., 2021). Therefore, we opted for serum sampling (over plasma) to reduce both the chances of blood degradation and the possibly inflated ccf-mtDNA measurements from platelet contamination.

3.5.2. Centrifugation time and speed

We employed the most updated standard techniques suggested by Michelson et al. (Michelson et al., 2023) from the Mitochondrial Psychobiology Lab at Columbia University (M. Picard, Director) although they differed minimally from the processes employed by Trumpff et al.

(2019). Specifically, instead of employing a 2 spin centrifugation process, we followed the 3 spin protocol suggested by Michelson et al., (2023). It is important to note, that mtDNA in circulation could be detected in packaged vesicles, bound to proteins or intact genomes. As a result, the mt-DNA assessed could have different physiological properties. The addition of extra spin in the centrifuge/aliquot steps represents an effort to get the "purest" serum sample (including ccf-mtDNA fragments) by preventing the assessment of extra cell layers or debris that could contaminate our results.

3.6. Serum isolation protocol for ccf-DNA measurements

This study collected serum from the blood samples. The isolation protocols for the serum ccf-mtDNA measurements proceeded as follows (see Figure 3 positioned on page 28):

1. Blood was collected from participants via serum clot activator tubes. These blood tubes contain a gel-formed clot activator, where upon blood entry into the tube activator prompts a coagulation cascade initiating the blood to clot. The blood samples were set to clot in serum clot activator tubes for 30 mins.
2. After the 30 min clot, the serum tubes were spun immediately via centrifuge (1,000 X G for 5 min at room temperature). [**Spin 1**]
3. After this spinning, in order to separate the serum from the red blood cells, 80% of the serum was transferred from the serum clot activator tubes to new 15ml conical tubes.
4. Then serum was spun again, at 2,000 X G for 10 min at 4°C. [**Spin 2**]
5. Then 80% of the serum was transferred to a new 15ml conical tube, and spun at 2,000 X G for 10 min at 4°C. [**Spin 3**]

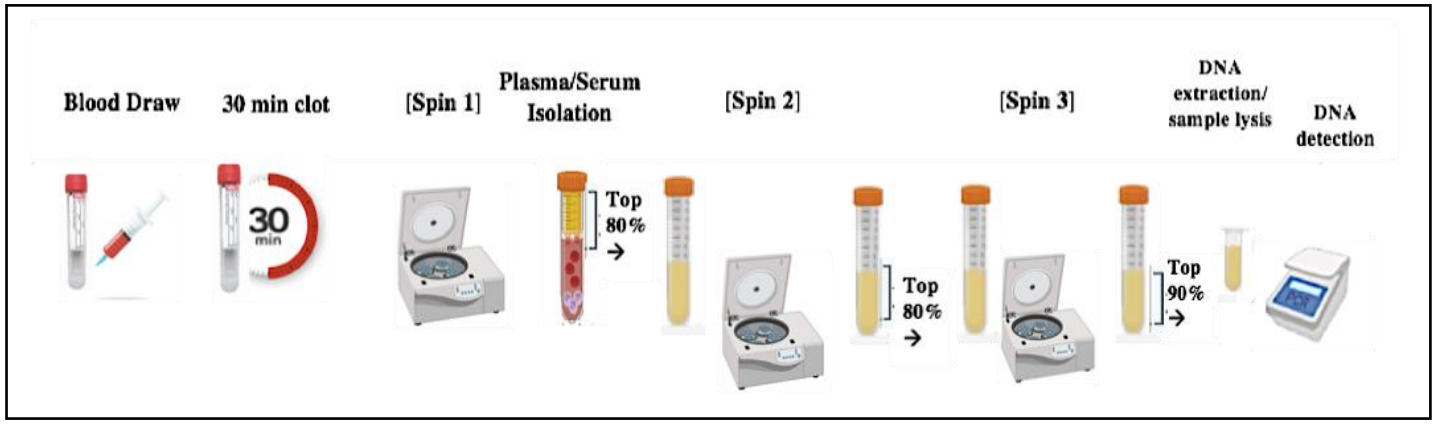
   (The purpose of spin 2 and 3, and the transfer of 80-90% of serum to new tubes is prevent any extra cell layers or debris from contaminating the results)

6. Then 90% of the serum was transferred to new graduated 1.5ml conical falcon centrifuge tubes, and, subsequently, mixed and aliquoted, and stored at -80°C.

7. Blood assay quantification and analysis were performed at an external lab location, and undertaken by David Shire, PhD of the Mitochondrial PsychoBiology Lab (M. Picard, PhD, Director) of Columbia University.

Figure 3

Visual Representation of Serum Isolation Protocol



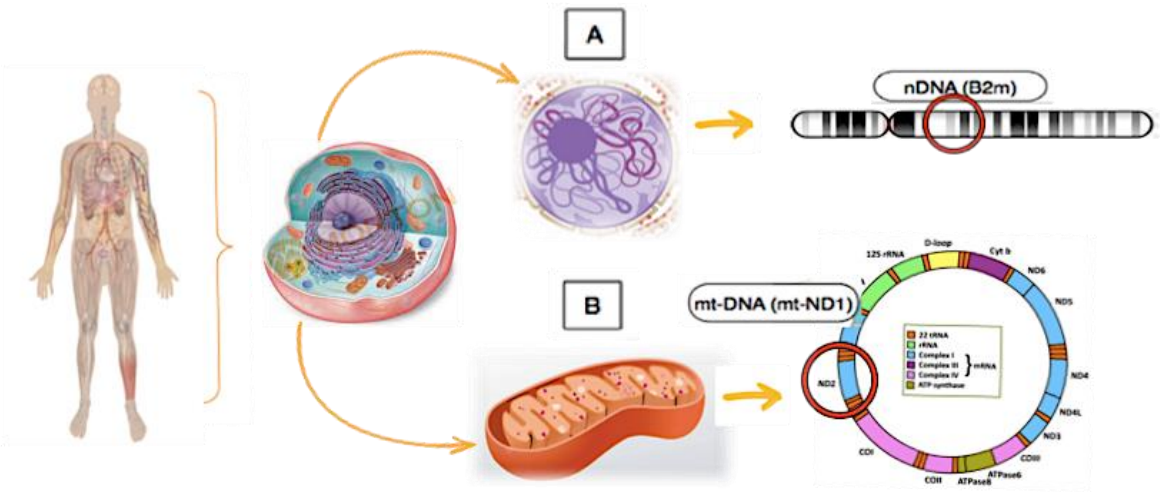
4. Results

4.1. Descriptive Statistics

A total of 14 healthy, young female adults (mean age = 25 y) were studied. Serum samples were collected at two different time points: i) baseline prior to the emotional image stress task (EIST); and ii) post: + 30 min or 30 minutes after the end of the stress task (Fig. 2). Following the serum isolation protocol (outlined above in section 3.6), the serum samples were then used to assess the levels of nuclear DNA (nDNA) and cell-free mitochondrial DNA (cfmtDNA). To do so, one mtDNA (ND1: mtDNA) and one nuclear DNA (B2m: nDNA) were measured using qPCR analyses to determine the levels of nDNA and ccf-mtDNA associated with both the pre and post stress task status of each participant (Fig.4).

Figure 4

Visual Representation of Nuclear and Mitochondrial DNA Amplicons in Assessing ccf-mtDNA



Note: Figure 4, A) is a representation of human nuclear DNA cells and the amplicon nuclear DNA (B2m) used in this study to represent nDNA release. B) is a representation of mitochondrial DNA cells and the amplicon mtDNA (ND1) assessed in this study to represent mtDNA release.

4.2. Primary outcomes

4.2.1. Induced Acute Psychological Stress Increases in ccf-mtDNA Levels

The female participants were exposed to a 10-minute psychological stress task involving a series of 60 negative emotional images from the International Affective Picture System (IAPS) presented consecutively at 10-seconds intervals. To validate the efficacy of this task as an induced experimental psychological stressor, we examined the individual's mood states prior to and after stressor exposure using the Profile of Mood States questionnaire.

Confirming hypothesis 1, there was a significant increase in ccf-mtDNA content post-stress task exposure. The majority of the included participants (93%) collectively demonstrated a mean 52.5% increase in their ccf-mtDNA collected after stressor exposure. A paired two tailed t-test was used to assess the difference between the pre and post blood samples. The mean ccf-mtDNA levels increased from the baseline value of 126.99 µl to 193.68 µl at 30 min post-stress, exhibiting a statistically significant increase ($t(13) = -4.40$, $P < 0.01$). This is otherwise quantified as a 53% increase in ccf-mtDNA levels in response to a 10-minute psychological stressor followed by a 30-minute waiting period. The stress-induced ccf-mtDNA release difference observed was in a similar direction to the social evaluative stress induction results obtained by Trumpff et al. (2019). The photo image-based stressor resulted in a statistically significant increase in ccf-mtDNA levels 30+mins after stressor exposure.

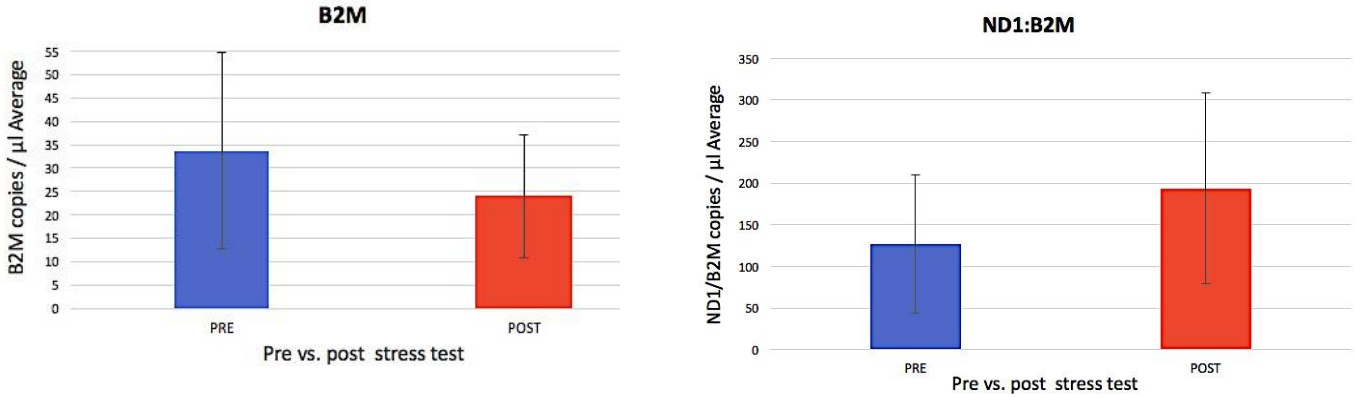
4.2.2. Stress Selectively Increases ccf-mtDNA

The serum samples were also analyzed for nuclear DNA content to examine whether the increased ccf-mtDNA was specific to mitochondria or due to a general increase in circulating cellular genomic material. We found that the cellular genomic material (nuclear DNA (B2m: nDNA) did not increase after the stressor test but significantly decreased from average value of 33.65 µl to 23.92 µl ($P < 0.05$). This decrease in nDNA content and increase in mtDNA content further suggest that the increase in ccf-mtDNA levels was specific to mitochondria genomic material and not related to a concomitant increase in total cellular genomic material.

Figure 5

Bar Graph Representation of Nuclear DNA Versus ccf-mtDNA Pre-Post Stressor Test

A **B**



Note: Figure 5, A) demonstrates the decrease in nuclear DNA (B2m: nDNA) content pre.vs.post the stress test, while B) demonstrates the increase in ccf-mtDNA (ND1/B2M) content pre.vs.post the stress test.

4.3. Secondary Outcomes

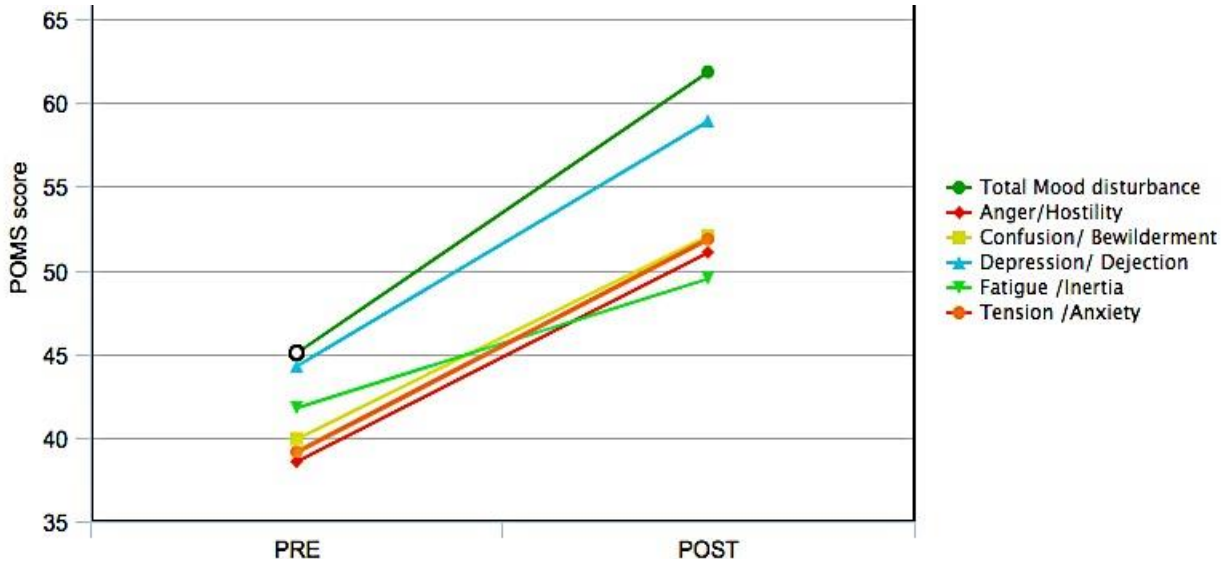
4.3.1. Induced Acute Psychological Stress Increases Negative Mood States

Consistent with the intended partial study replication (Trumpff et al., 2019), the stressor exposure was associated with a significant increase in negative mood profile states (e.g. anxiety and anger). Furthermore, there was a significant increase in other negative mood profile states such as confusion, depression, fatigue, and total mood disturbance (Figure 6). Thereby, the intended manipulation of psychological states was apparently achieved.

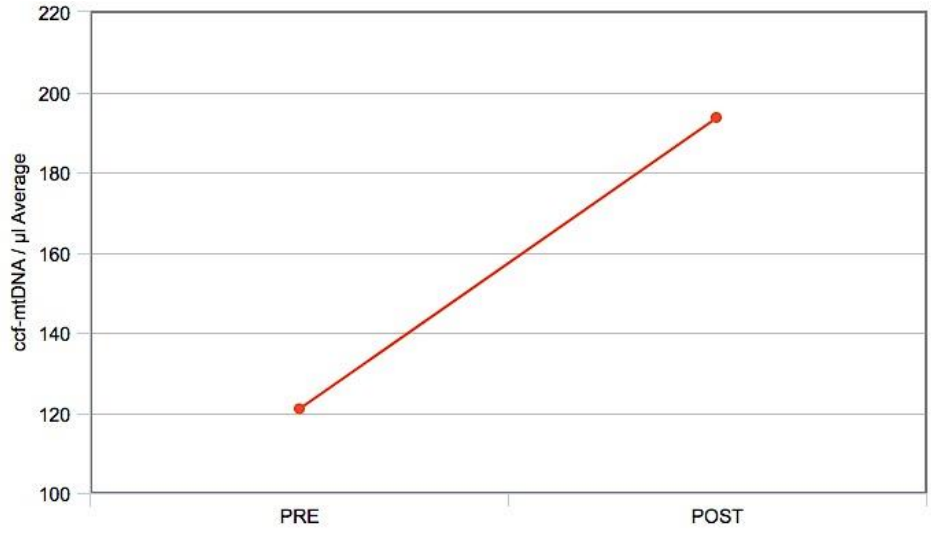
Figure 6

Linear Graph representation of Increase in Negative Mood States and the Increase in ccf-mtDNA from Pre to Post Stressor Test

A.



B.



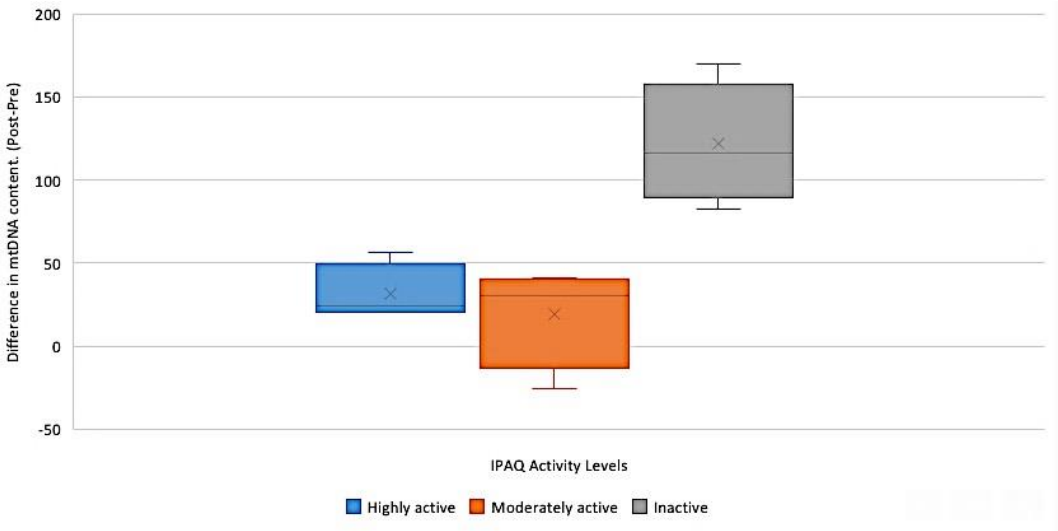
Note: Figure 6 A) visual representation of the change in mean POMS T-scores from pre- to post- stress task in 6 assessed mood states. B) Line graph representation of the mean change in ccf-mtDNA levels from pre (baseline) to post stressor task (+30mins).

4.3.2. Physical Activity Levels are Significantly Associated with the Release of ccf-mtDNA during Induced Psychological Stress

Based on each participant's (n=14) responses to the short-term IPAQ questionnaire, administered prior to the stressor (at baseline), each participant was assigned a categorical PA level using median-MET minutes of energy expenditure per self-report of their last 7 days of PA. The three categorical groups were as follows: i) Group 1; inactive (n=6); ii) Group 2; sufficiently to moderately active (n=4); iii) Group 3; highly active (n=4). An analysis of the variance-based group comparison in terms of ccf-mtDNA release levels and a Tukey post hoc test suggested significant differences (Figure 7). Specifically, there was a significant difference between the highly active group (n=4) and the inactive group (n = 6) with the difference of 90.50 ($P < 0.05$). There was also a significant difference between the moderately active group (n = 4) and the inactive group (n = 6) with the difference value of 102.61 ($P < 0.005$). The comparison between the highly active and the moderately active group was not significant with a difference value of 12.10 ($P < 0.05$).

Figure 7

Bar Graph Representation of IPAQ Physical Activity Groups and their Difference in Increase of ccf-mtDNA Levels



Note: Figure 7 three IPAQ physical activity groups (n=14) and their difference in increased ccf-mtDNA (ND1/B2M) content pre.vs.post the stress test.

4.3.3. Pre and Post Stressor Data: Correlations amongst Mood States and between Mood States and ccf-mtDNA

To better understand the lab stress effects on mood and their possible relationships to stress effects on ***ccf-mtDNA*** levels, Pearson r correlations were estimated on the five mood states, the Total Mood Disturbance scores and the ***ccf-mtDNA*** levels assessed. The correlations were estimated using SPSS and the full findings are represented in Figure 8 (below). As seen in the pre-stress data, the n = 5 specific (negative) mood states and the Total Mood Disturbance score were negatively but non-significantly correlated at **baseline** with ccfmtDNA levels. In terms of directionality, such negative correlations suggest more intense (more negative) mood experiences (n = 5) were associated with lower levels of ccfMtDNA released in the blood. This pattern was repeated with the Total Mood Disturbance score which demonstrated the highest negative correlation (-.485) but a correlation that only approached statistical significance (p = .079).

As seen in the post-stress data, amongst the n = 5 (negative) mood states and the Total Mood Disturbance, only one mood state was negatively correlated with the levels of ccfMtDNA released into the blood (i.e. depression), while the other N = 4 mood states and the Total Mood Disturbance score were insignificantly but positively correlated. Although within these data, the dominant pattern of negative correlations was altered to a dominant pattern of positive correlations, the absence of statistical significance make statements about these data preliminary and descriptive (e.g. a negative correlation pattern changing to a positive correlation pattern).

In summary, there were no statistically significant relationships between mood state (pre-to-post stressor) and ccfMtDNA released into the blood. Given the absence of statistical significance, all associations, including those of directional association, must be considered tentative.

Additional Pearson r correlations were estimated between the n = 5 specific mood states and the Total Mood Disturbance scores, as assessed in the pre-stressor and post-stressor conditions. As might be envisioned, multiple significant correlations were observed in both pre-stress and the

post-stress conditions. Altogether, in the pre-stress condition, there were positive, significant correlations observed between Anger-Tension, Anger-TMD, Confusion-Depression, Confusion-Tension, Confusion-TMD, Depression-TMD, Confusion-Anger and Tension-TMD. In the post-stress condition, positive, significant correlations were observed between Anger – Confusion, Anger – Depression, Anger – Fatigue, Anger – Tension, Anger – TMD, Confusion-Depression, Confusion-Fatigue, Confusion-Tension, Confusion-TMD, Depression-Anger, Depression-Fatigue, Depression-Tension, Depression-TMD, Fatigue-Tension, Fatigue-TMD, Tension-Confusion and Tension-TMD. The presence of these significant correlations suggest sufficient power to detect other significant correlations were they present in the data matrix.

Figure 8

Pearson r Correlations Amongst Mood States and Between Mood States and ccf-mtDNA, Pre to Post Stressor Test

A

Correlations

		Anger	confusion	depression	fatigue	tension	TMD	ccfmtDNA
Anger	Pearson Correlation	1	.472	.176	.444	.669**	.581*	-.313
	Sig. (2-tailed)		.089	.548	.112	.009	.029	.276
	N	14	14	14	14	14	14	14
confusion	Pearson Correlation	.472	1	.718**	.428	.557*	.883**	-.288
	Sig. (2-tailed)	.089		.004	.127	.039	<.001	.319
	N	14	14	14	14	14	14	14
depression	Pearson Correlation	.176	.718**	1	.109	.229	.780**	-.417
	Sig. (2-tailed)	.548	.004		.710	.430	<.001	.138
	N	14	14	14	14	14	14	14
fatigue	Pearson Correlation	.444	.428	.109	1	.520	.460	-.318
	Sig. (2-tailed)	.112	.127	.710		.057	.098	.268
	N	14	14	14	14	14	14	14
tension	Pearson Correlation	.669**	.557*	.229	.520	1	.745**	-.345
	Sig. (2-tailed)	.009	.039	.430	.057		.002	.227
	N	14	14	14	14	14	14	14
TMD	Pearson Correlation	.581*	.883**	.780**	.460	.745**	1	-.485
	Sig. (2-tailed)	.029	<.001	<.001	.098	.002		.079
	N	14	14	14	14	14	14	14
ccfmtDNA	Pearson Correlation	-.313	-.288	-.417	-.318	-.345	-.485	1
	Sig. (2-tailed)	.276	.319	.138	.268	.227	.079	
	N	14	14	14	14	14	14	14

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

B

Correlations

		anger	confusion	depression	fatigue	tension	TMD	ccfmtDNA
anger	Pearson Correlation	1	.749**	.710**	.786**	.707**	.871**	.085
	Sig. (2-tailed)		.002	.004	<.001	.005	<.001	.773
	N	14	14	14	14	14	14	14
confusion	Pearson Correlation	.749**	1	.768**	.841**	.809**	.866**	.210
	Sig. (2-tailed)	.002		.001	<.001	<.001	<.001	.471
	N	14	14	14	14	14	14	14
depression	Pearson Correlation	.710**	.768**	1	.839**	.709**	.905**	-.014
	Sig. (2-tailed)	.004	.001		<.001	.005	<.001	.962
	N	14	14	14	14	14	14	14
fatigue	Pearson Correlation	.786**	.841**	.839**	1	.854**	.923**	.007
	Sig. (2-tailed)	<.001	<.001	<.001		<.001	<.001	.981
	N	14	14	14	14	14	14	14
tension	Pearson Correlation	.707**	.809**	.709**	.854**	1	.899**	.021
	Sig. (2-tailed)	.005	<.001	.005	<.001		<.001	.942
	N	14	14	14	14	14	14	14
TMD	Pearson Correlation	.871**	.866**	.905**	.923**	.899**	1	.084
	Sig. (2-tailed)	<.001	<.001	<.001	<.001	<.001		.776
	N	14	14	14	14	14	14	14
ccfmtDNA	Pearson Correlation	.085	.210	-.014	.007	.021	.084	1
	Sig. (2-tailed)	.773	.471	.962	.981	.942	.776	
	N	14	14	14	14	14	14	14

**. Correlation is significant at the 0.01 level (2-tailed).

Note: Figure 8 A) Pearson r correlations amongst Mood States and between Mood States and ccf-mtDNA using SPSS, prior to the stressor task. Figure 8 B) Pearson r Correlations amongst Mood States and between Mood States and ccf-mtDNA using SPSS, after the stressor task.

5. Discussion

5.1. ccf-mtDNA as a Mitokine

Our ability to adapt to stress depends on multiple molecular factors secreted within our bodies that ensure growth, reproduction and survival (Castellani et al., 2020). While the majority of hormones, cytokines and proteins have been thoroughly investigated in this area, the roles of the mitochondria and its genome have been more modestly investigated. Studies on cell free mitochondria DNA therefore offer a pathway for better understanding the role of the mitochondria in stress adaptation. The present findings, in this partial replication study, suggest that acute psychological stress causes a statistically significant increase in cell free mitochondrial DNA (***ccf-mt-DNA***). The current study findings support hypotheses proposed by Trumpff et al., notably that mitochondria play a signaling role when psychological threat conditions are experienced in humans. This theory indicates that the mitochondria genome (ccf-mtDNA) likely functions as a "*mitokine*", or a stress-induced cytokine, released in response to psychological and cellular stress.

5.2. Acute stress, POMS, Exercise and ccf-mtDNA

Our findings in the current study were similar to the study we aimed to partly replicate (Trumpff et al., 2019). The findings confirmed all three of the hypotheses tested. Consistent with the first hypothesis, a statistically significant and notable increase (53%) in ccf-mtDNA levels was found within 30+ minutes of the stress exposure. Although the magnitude of the mitochondrial DNA increase was greater in the study done by Trumpff et al. (~85% vs. 53%), our findings (in terms of direction) are similar and, again, a statistically significant increase in ccf-mtDNA levels was observed. These results were also similar (in direction) to the previous findings of Hummel et al. who found a 70% increase in ccf-mtDNA levels in response to a psychological stressor in a sample of 20 healthy young men.

Consistent with our second hypothesis, the induced lab stress was associated with a statistically significant increase in both i) negative mood state profiles, and ii) elevations of ccf-mtDNA levels. Moreover, these results were consistent with the study being replicated (Trumpff et al., 2019), as our acute lab stressor, although apparently milder in impact, caused statistically significant increases in negative mood profile states such as anxiety and anger (also consistent with the Trumpff et al., 2019 study).

In relation to the third hypothesis, there was a significant difference in ccf-mtDNA levels in physically inactive subjects when compared to moderately or highly physically active subjects. These findings add to current knowledge on exercise in relation to the dynamics of the mitochondria genome. Subjects who were physically inactive demonstrated a much greater increase in ccf-mtDNA levels than those deemed moderately or highly active. Although exercise was not a manipulated variable in our study, the self-report IPAQ questionnaires from participants suggest that reported exercise levels may act as a covariate in the release of ccf-mtDNA levels from psychological stress. This relationship can be further assessed in future studies.

5.3. ccf-mtDNA in Psychiatric and Healthy groups

Our research findings also add to the current knowledge on psychiatric studies that have investigated the dynamic response of the mitochondria to the organism's psychological state. The current study findings on the elevation of circulating ccf-mtDNA levels in response to acute psychological stress adds to the group of studies that have focused on both the psychiatric and healthy populations.

With regards to the role of ccf-mtDNA in *psychiatrically-diagnosed* populations, two cross-sectional studies (Lindqvist et al., 2016; Lindqvist et al., 2018) respectively focused on patients with major depressive disorder and non-violent suicide attempters. They showed elevated levels of ccf-mtDNA in these patients when compared to matched controls.

With regards to the role of ccf-mtDNA in *healthy* populations, only two studies have focused on the hypothesis that acute psychological stress triggers ccf-mtDNA release and both used the identical experimental stressor (Trier Social Stress Test) (TSST). Hummel et al., tested 20 healthy young men and found a 0.7-fold plasma ccf-mtDNA increase after TSST exposure (Hummel et al., 2018). In line with these findings, Trumpff et al. assessed 50 middle-aged male and female subjects and observed Cohen's *d* values ranging from 0.85–1.23 in serum ccf-mtDNA elevation within 30 minutes of TSST exposures (Trumpff et al., 2019). Both studies therefore showed a relatively quick increase of ccf-mtDNA (in plasma and serum) following completion of the experimental stress protocol.

5.4. ccf-mtDNA and Blood Quantification/Analysis

Expanding upon previous findings, our study aimed to address previous literature gaps and limitations about the responsiveness of ccf-mtDNA to psychological status. Two studies by Lindqvist and colleagues (Lindqvist et al., 2016; Lindqvist et al., 2018) focused on psychiatric populations, while the Hummel et al., study focused on healthy men with measurements of ccf-mtDNA that used plasma samples as opposed to serum. This is a vital difference, since plasma reflects the liquid fraction of the whole blood obtained from anti-coagulated blood. In contrast, serum is the liquid fraction of the blood after a clotting process (Trumpff et al., 2020). This is relevant when measuring the ccf-mtDNA, as platelets are an abundant source of mitochondria and ccf-mtDNA. The use of clotting agents in serum preparations reduce the platelet ccf-mtDNA contamination and potential interference from nuclear DNA. As platelets contain abundant mitochondria and mtDNA, plasma findings could inflate the apparent ccf-mtDNA levels, yielding results not comparable to serum findings (Trumpff et al., 2021). Furthermore, the use of clotting factors when preparing serum samples reduces the probability of blood degradation, yielding more reliable results (Trumpff et al., 2021). Therefore, our study, similar to the Trumpff el al., study, measured ccf-mtDNA using serum samples. However, our blood processing and centrifugation were also somewhat different from Trumpff, as we employed the most updated techniques suggested by Michelson et al. (Michelson et al., 2023). Instead of employing a 2-spin centrifugation process, we followed the 3-spin protocol suggested (Michelson et al., 2023). It is important to note, that mt-DNA in circulation could be detected in packaged vesicles, bound to proteins or intact genomes which could have different physiological properties. The addition of an extra spin in the centrifuge/aliquot steps is an effort to obtain the "purest" serum sample (ccf-mtDNA fragments) and to prevent any extra cell layers or debris that could contaminate our results.

5.5. ccf-mtDNA and Age Differences

In the undertaking of a partial replication, inevitable differences occur of which only a critical subset can be meaningfully controlled. Notably using a highly similar blood analysis procedure was key in this study although the population varied, as the Trumpff et al. study investigated 50 middle aged participants (30 males and 20 females) in the age range of 41-58 yrs, while our study included 14 female students in the age range of 20-30 years. It is notable that previous studies indicated that ccf-mtDNA measurements seemed to increase with age. For example, in a study that investigated 831 healthy individuals, it was found that mtDNA levels increased significantly after the fifth decade of life (Pinti et al., 2014). Although the experimental stress effects were intended to be similar, several procedures were not exactly replicable. The lesser pre-post differences may therefore be attributable to the more youthful responses of a population and to the fact that several of the students recruited and assessed were graduate students familiar with lab conditions (i.e. environments similar to the settings where assessments occurred) and with blood draw procedures. Therefore, the whole contact (aside from the experimentally invoked stress) might have been less disturbing and stressful than for individuals naïve to such environments. A sample of more naïve individuals were assessed in the Trumpff et al., 2019 study.

5.6. Lab induced stressor tests (IAPS .vs. TSST)

What has been labelled, psychotherapeutically, as anticipatory anxiety may be considered a component of the intra-organismic registration of future increased energy needs. Therefore, an experimental stressor may evoke a mitochondrial response to the degree it signals needs for future energy generation and dispersion. This aligns with notable findings from Trumpff and colleagues (Trumpff et al., 2022) where the ccf-mtDNA was measured using *saliva* samples. In this study, two healthy men were assessed at 4 daily timepoints for 53–60 consecutive days. Although the ccf-mtDNA results were consistently similar on most days, an anomalous increase in ccf-mtDNA results was observed on day 26 for one participant. This occurred on the day preceding the participant's engagement in a marathon run on Day 27 (Trumpff et al., 2022). It is notable that the ccf-mtDNA results on the day of the marathon were more aligned with those obtained in prior assessment days. Such results could be interpreted as the result of 'anticipatory stress', which underlines the significance of ccf-mtDNA in relation to psychological stress of multiple kinds.

The specific stressor test used in two previous studies (Trumpff et al., 2019; Hummel et al., 2018) was the TSST, which induces social types of defensiveness in subjects. It also likely evokes associated situations where socially defensive events were evoked in the past, and generally signals a need to prepare and enact social defenses. In contrast the IAPS photos are anxiety-provoking and thus induce a generic defensiveness but not necessarily a socially-based defensiveness. Therefore, they may not signal (as focally) needs to generate energy for a future event. While the TSST stressor is widely known to be a more invasive and evocative procedure than other alternatives, it is limited with respect to representing varying stress levels. Although the IAPS images may not be as stress evoking as the TSST, they provide a framework for exploring dose-response relationships. This is because prior research with the IAPS has resulted in each photograph being associated with a stress evocation level derived from exposures to a range of research subjects. Thus an associated IAPS manual labels each picture with a metric scale-range that reflects the emotional reactions of former subjects across the three emotional dimensions of valence, arousal and dominance. Thus, using the IAPS photographs, the researcher can employ empirical findings to examine a potential dose-response relationship between stressor and ccf-mtDNA release. He or she can alter the 'dose' of the induced stress via purposeful image selection.

5.7. Lab induced stressor Mood Effects

In summary, there were no statistically significant relationships between mood state (pre-to-post stressor) and ccfMtDNA released into the blood. Given the absence of statistical significance, all associations, including those of the directional associations, must be considered tentative. Although positive and significant correlations were observed between the 5 specific mood states and Total Mood Disturbance scores (in the pre-stress and post-stress conditions), such correlations are somewhat predictable given the similar content of the POMS questionnaire used in pre-stress and post-stress conditions.

5.8. ccf-mtDNA and Sex differences

The current study also addressed the gender challenges in prior research in relation to studying the role of the mitochondrial DNA and stress, given the lack of sufficient female representation in this research field. As mentioned above, the only studies conducted on acute stress and its relation to the circulating mtDNA have largely involved male subjects. The Hummel et al. study assessed 20 healthy young men, and the Trumpff et al. study assessed 50 middle aged participants and was mainly male dominated (n = 30 males; 20 females). Thereby, this study aimed to mitigate the existing gender imbalance by only including female participants. The minor difference between the current findings and the study we aimed to replicate may also be attributed to the gender differences in the population studied as Trumpff et al. observed a greater stress-related increase of ccf-mtDNA in male participants than in female participants. Although further research is needed to elaborate on such differences and the underlying biochemical evidence behind them, it is important to mention that numerous aspects of the biology of mitochondria, including its sensitivity permeability, apoptotic signaling, respiratory capacity, and ROS production have been shown to be different in males than females. Altogether, the mitochondria in males appear much more vulnerable and susceptible to impairment and damages than those in females (Ventura-Clapier et al., 2017).

5.7. Strength and Limitations

While our study aimed to investigate the effects of acute stress on ccf-mtDNA levels and provide valuable additions to the existing literature, it is important to address the strengths and limitations that should be considered when referring to findings, especially in considering future research directions.

5.7.1. Strengths

With regards to study strengths, we appeared to successfully manipulate the independent variable (lab-induced stress) using a well-known standardized stressor (IAPS) by selecting 60 images that were screened and approved by the clinical psychologist supervising this thesis. Notably, we were also able to ascertain that the images evoked negative emotional, stress inducing effects by employing 2 sets of POMS questionnaires: i) before the 1st blood draw, at baseline ii) after the stressor task and before the second blood draw. The statistically significant increase in the total mood disturbance from the pre-to-post POMS analysis validated the induction of a more stressful psychological state, and further indicated that the induced psychological state was likely due to the lab stressor imposed. While there might have been stress due to potential fears of needles and blood draws, that stress would likely be present during the baseline POMS and not likely increased at the follow up POMS. One interpretation to the contrary would be that the individual was reacting to needle and/or blood draw fears and that anticipating the second blood draw caused the increased stress captured on the POMS.

Furthermore, the methodology of the current study suggests strengths in several areas, notably the inclusion-exclusion criteria, the standardized (morning) scheduling and the protocol instructions to participants designed to avoid potential confounds with diet-related and daily stress factors related to circadian rhythms. The use of self-administered IPAQ questionnaires enabled exploration of potential exercise-based covariates that might have contributed to variability in the observed dependent variable.

Lastly, the current study employed the most suitably updated technique of DNA processing and centrifugation via encompassing a 3-spin method as opposed to a 2 spin method during blood analysis. We used this method to attain the "purest" possible serum sample based on the recent evidence of optimal blood processing and centrifugation (Michelson et al., 2023). This helped to eliminate external 'noise' in our blood sampling analyses via limiting extra cell layers and/or debris that could contaminate results.

5.7.2. Limitations

With regards to limitations, the investigation of 14 female participants constitutes a relatively small sample. This was mainly due to time constraints as most participant recruitment, participation and data collection occurred within a 2-month time period (March-April, 2023). The initial recruitment, which occurred during the winter semester's final exams and assignments, was hampered by many students being occupied with exam preparation and assignment completion. Many potential subjects experienced a scheduling issue as the study could only be scheduled for 9:30 am (to avoid potential confounding) which often conflicted with class schedules. As we were limited to recruit female York university students who had voluntarily viewed study posters across campus, and met several screening requirements, recruitment was challenging.

Another key limitation was the absence of a control group who might have been studied via two similar blood draws and questionnaire administrations but without exposure to the lab stressor. The absence of a control group was partially due to the difficulties encountered in recruiting participants. As the main study focus was a partial replication of the Trumpff et al. study (which also did not include a control group), incorporation of a control group in the study design awaits future efforts in this research area.

6. Conclusion

Overall, this study adds to recent evidence that acute psychological stress is associated with elevations of serum ccf-mtDNA levels in human blood circulation, with results aligning with a prior study where the blood analyses undertaken and other methodological elements were similar (Trumpff et al., 2019). These findings suggest that the mitochondrial genome plays a significant role in psychological stress reactivity, in combination with the apparently significant role in chronic illnesses such as cancer, inflammation, diabetes, sepsis and physical trauma. This study adds to the current research on acute stress and mitochondrial DNA release by indicating that other lab stressor tests appear associated with a temporally-proximal elevations in serum ccf-mtDNA levels.

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Table 1) Studies evaluating physical activity and exercise to cf-mtDNA levels

Types of exercise study	Study groups	Data collection	Results
Regular exercise training: Healthy volleyball players Nasi et al., 2016	Exercise group: 12 healthy volleyball players *compared* to Controls:12 age matched non-athlete males for two seasons. (n=24)	Blood samples were collected for 2 seasons, 5 times per each season: once at baseline, once at the preseason, and three times during the regular season.	Baseline: Control had lower cf-mtDNA levels. Season 1: the exercise group cf-mtDNA levels decreased from the baseline gradually until the end of the first season. Second 2: cf-mtDNA levels stayed below baseline but did not decrease further than its value from the first season.
Acute Physical activity: 90 mins treadmill exercise (60% VO2 max) Shockett et al., 2016	7 healthy, moderately trained, young men were used for both exercise and control trials. (n =7)	Blood samples were collected 4 times: 1) Right before the exercise 2) During the exercise at + 18 mins 3) during the exercise at +54 mins 4) immediately after exercise at + 90 mins	The cf-mtDNA levels dropped at two points (compared to control trials): 1) exercise at +54 mins 2) immediately after exercise at + 90 mins
Acute Physical activity: Repeated bouts of exhaustive treadmill exercise Stawski et al., 2017	11 healthy men were asked to run on treadmill at 70% of their VO2 max for three different occasions with 72 hours of resting between each. (N=11)	At each occasion, blood samples were taken right before and immediately after the treadmill exercise.	An increase in cf-mtDNA levels after each bout. However, these values were only significant for after the second and third bout. The pre-exercise cf-mtDNA levels dropped following each bout of exercise ($1^{st} - 2^{nd}$, $2^{nd} - 3^{rd}$, $1^{st} - 3^{rd}$)

APPENDIX A:

PROBLEM	Do you have the problem?		Do you receive treatment for it?		Does it limit your activities?	
	No (0)	Yes→ (1)	No (0)	Yes (1)	No (0)	Yes (1)
Heart disease	N	Y	N	Y	N	Y
High blood pressure	N	Y	N	Y	N	Y
Lung disease	N	Y	N	Y	N	Y
Diabetes	N	Y	N	Y	N	Y
Ulcer or stomach disease	N	Y	N	Y	N	Y
Kidney disease	N	Y	N	Y	N	Y
Liver disease	N	Y	N	Y	N	Y
Anemia or other blood disease	N	Y	N	Y	N	Y
Cancer	N	Y	N	Y	N	Y
Depression	N	Y	N	Y	N	Y
Osteoarthritis, degenerative arthritis	N	Y	N	Y	N	Y
Back pain	N	Y	N	Y	N	Y
Rheumatoid arthritis	N	Y	N	Y	N	Y
Other medical problems (please write in)						
	N	Y	N	Y	N	Y
	N	Y	N	Y	N	Y

APPENDIX B:

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

Think about all the vigorous activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?

_____ days per week

No vigorous physical activities Skip to question 3

2. How much time did you usually spend doing vigorous physical activities on one of those days?

_____ hours per day

_____ minutes per day

Don't know/Not sure

Think about all the moderate activities that you did in the last 7 days. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ days per week

No moderate physical activities Skip to question 5

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

4. How much time did you usually spend doing moderate physical activities on one of those days?

_____ hours per day

_____ minutes per day

Don't know/Not sure

Think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

_____ days per week

No walking Skip to question 7

6. How much time did you usually spend walking on one of those days?

_____ hours per day

_____ minutes per day

Don't know/Not sure

The last question is about the time you spent sitting on weekdays during the last 7 days. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the last 7 days, how much time did you spend sitting on a week day?

_____ hours per day

_____ minutes per day

Don't know/Not sure

APPENDIX C:

Directions: Describe HOW YOU FEEL RIGHT NOW by circling the most appropriate number after each of the words listed below

Item	Not at all	A little	Moderate	Quite a bit	Extremely
1. Friendly	1	2	3	4	5
2. Tense	1	2	3	4	5
3. Angry	1	2	3	4	5
4. Worn out	1	2	3	4	5
5. Unhappy	1	2	3	4	5
6. Clear-headed	1	2	3	4	5
7. Lively	1	2	3	4	5
8. Confused	1	2	3	4	5
9. Sorry for things done	1	2	3	4	5
10. Shaky	1	2	3	4	5
11. Listless	1	2	3	4	5
12. Peeved	1	2	3	4	5
13. Considerate	1	2	3	4	5
14. Sad	1	2	3	4	5
15. Active	1	2	3	4	5
16. On edge	1	2	3	4	5
17. Grouchy	1	2	3	4	5
18. Blue	1	2	3	4	5
19. Energetic	1	2	3	4	5
20. Panicky	1	2	3	4	5
21. Hopeless	1	2	3	4	5
22. Relaxed	1	2	3	4	5
23. Unworthy	1	2	3	4	5
24. Spiteful	1	2	3	4	5
25. Sympathetic	1	2	3	4	5
26. Uneasy	1	2	3	4	5
27. Restless	1	2	3	4	5
28. Unable to	1	2	3	4	5
29. Fatigued	1	2	3	4	5
30. Helpful	1	2	3	4	5
31. Annoyed	1	2	3	4	5
32. Discouraged	1	2	3	4	5
33. Resentful	1	2	3	4	5
34. Nervous	1	2	3	4	5
35. Lonely	1	2	3	4	5
36. Miserable	1	2	3	4	5
37. Muddled	1	2	3	4	5
38. Cheerful	1	2	3	4	5
39. Bitter	1	2	3	4	5

Please continue on the next page….

40. Exhausted	1	2	3	4	5
41. Anxious	1	2	3	4	5
42. Ready to fight	1	2	3	4	5
43. Good-natured	1	2	3	4	5
44. Gloomy	1	2	3	4	5
45. Desperate	1	2	3	4	5
46. Sluggish	1	2	3	4	5
47. Rebellious	1	2	3	4	5
48. Helpless	1	2	3	4	5
49. Weary	1	2	3	4	5
50. Bewildered	1	2	3	4	5
51. Alert	1	2	3	4	5
52. Deceived	1	2	3	4	5
53. Furious	1	2	3	4	5
54. Effacious	1	2	3	4	5
55. Trusting	1	2	3	4	5
56. Full of pep	1	2	3	4	5
57. Bad-tempered	1	2	3	4	5
58. Worthless	1	2	3	4	5
59. Forgetful	1	2	3	4	5
60. Carefree	1	2	3	4	5
61. Terrified	1	2	3	4	5
62. Guilty	1	2	3	4	5
63. Vigorous	1	2	3	4	5
64. Uncertain about things	1	2	3	4	5
65. Bushed	1	2	3	4	5

Please check to see that you have responded to all the items….thank you

APPENDIX D:

COVID-19 Screening Form

Participant Name: _____

Have you tested positive for COVID-19 during the past 14 days? Yes__ No___

Do you have any of the following new or worsening symptoms? (*symptoms should not be chronic or related to other known causes or conditions*)

Fever or Chills	Yes__	No___
Difficulty breathing or shortness of breath	Yes__	No___
Cough	Yes__	No___
Sore throat	Yes__	No___
Runny nose/stuffy nose or nasal congestion	Yes__	No___
Decrease or loss of smell or taste	Yes__	No___
Nausea, vomiting, diarrhea, abdominal pain	Yes__	No___
Not feeling well, extreme tiredness, sore muscles	Yes__	No___

Have you travelled outside of Canada in the past 14 days? Yes__ No___

Have you had close contact with a confirmed or probable case of COVID-19 during the past 14 days? Yes__ No___

Participant signature: _____ Date: _____