

T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHYSIOLOGY

**INVESTIGATION OF EFFECTS OF REPEATED
HYPERTHERMIA ON SKELETAL MUSCLE AND
MOTOR FUNCTION IN MICE**

MASTER THESIS

CANSU BAŞIŞ

İSTANBUL-2023

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İSTANBUL-2023

APPROVAL

Institute : Yeditepe University Institute of Health Sciences
Programme : Master of Science Physiology
Title of the Thesis : Investigation of Effects of Repeated Hyperthermia on Skeletal Muscle and Motor Function in Mice
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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated and numbered

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Director of Institute of Health Sciences

DECLARATION

I hereby declare that this research has never exceeded ethical limits, and this thesis is my own work, and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material that has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Cansu Başış



DEDICATION

Dedicated to My Beloved Father, Nural Yakın.

ACKNOWLEDGEMENTS

“Educating the mind without educating the heart is no education at all.” -Aristotle

How devastating it is, there are few words to divulge the exact scope of the mind’s view. Nevertheless, I will try my best to express my gratitude. First of all, I’d like to thank you for his inexhaustible patience and a real role model for every student who is under his wings; dear Prof. Dr. Bayram Yılmaz. It was a great opportunity to be under his education which put little sparks in my mind and always devoted and thoughtful approach to solving our problems like a father. I could not have undertaken this journey without him. Furthermore, I’d like to express my major thanks to Dr. Cihan Süleyman Erdoğan during the experimental period; even though he was bombarded with questions, never gave up and supported me with endless patience. And without a doubt, every time he covered my back, even he never talked about it. And gave me a new target of being better than him in a manner of personality or his great wiz. Also, I’d like to thank Dr. Buğra Özgün who generously opens the doors of his vast knowledge with a warming smile under any circumstances. Just meeting him gives many students a reason to continue. I’d like to be honored by being their audience for further meticulous works. It was a splendid experience to learn from the best and get better and better daily. Additional thanks to Biologist Yunus Kosif for sharing his knowledge and his attentive work during the experiments and all his support. I’m grateful to have the chance to meet with her and to be an observer of the spectacular works of MSc. Biologist Busenur Bolat with her multifaceted experiments. I would like to express my gratefulness to Dr. Engin Sümer for every support during the experimental period. My endless respect to them all.

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LIST OF SYMBOLS and ABBREVIATIONS

°C	Centigrade Degree
Ca ⁺²	Calcium Ion
H ⁺	Hydrogen Ion
H ₂ O ₂	Hydrogen Peroxide
P ⁺	Phosphate Ion
p-	Phosphorylated
pH	Potential of Hydrogen
AA	Amino Acid
ACC	Acetyl-CoA Carboxylase
ADP	Adenosine Diphosphate
Akt	Protein Kinase B
Arc	Arcuate Nucleus
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ATF4/6	Activating Transcription Factor 4,6
ATG1-16L1	Autophagy Related Genes 1,5,12,13,14,16L1
AHA1	Activator of HSP90 ATPase
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BCL2, BCL-XL	B-cell Lymphoma 2, Extra Large
BDNF	Brain-Derived Neurotrophic Factor
BNIP3, NIX	B-cell Lymphoma 2 Interacting Protein 3, NIP3-like protein X
cAMP	Cyclic AMP
CaM	Calmodulin

CaMK/ II/IV	Calmodulin Kinase II/IV
CaMKK	Calmodulin Kinase Kinase
CaN	Calcineurin
cGMP	cyclic Guanosine Monophosphate
CGRP	Calcitonin Gene Related Protein
CSQ	Calsequestrine
CHIP	Hsc70-interacting protein
CHOP	C/EBP Homologous Protein
COX	Cytochrome Oxidase
CPu	Caudate Putamen
CRF	Corticotrophin Releasing Factor
CREB	Cyclic AMP Response Element Binding Protein
DMH	Dorsomedial Hypothalamus
DRN	Dorsal Raphe Nucleus
Drp1	Dynamin related protein 1
eIF2 α	Eukaryotic Initiation Factor 2 Alpha
EMG	Electromyography
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ERSE	ER Stress Response Element
ERR (α , β , γ)	Estrogen-related Receptor alpha, beta, gamma
FA	Fatty acid
FAO	FA oxidation
FAD/ $+$ /H/H ₂	Flavin Adenine Dinucleotide
FC	Frontal Cortex
FIP200	FAK-family Interacting Protein of 200 kDa

FOXO1/3a	Forkhead Box1/O3a
GABA	Gamma-Aminobutyric Acid
GCs	Glucocorticoids
GCR	Glucocorticoid Receptor
GLUT 1/4	Glucose Transporter 1,4
GOT 1/2	Glutamic-oxaloacetic Transaminase 1,2
GRP78/94	Glucose-Regulated Proteins 78,94
GSH	Glutathione
GS	Glutathione Synthase
HIF-1 α	Hypoxia-Inducible Factor 1 Alpha
HO-1	Heme Oxygenase 1
HOP	HSC70/HSP90-Organizing Protein
HSF-1	Heat Shock Factor 1
HSP27/60/70/90	Heat Shock Protein 27/60/70/90
IGF-1	Insulin-like Growth Factor 1
IRE1 α	Inositol-requiring Enzyme 1alpha
LC3	Microtubule-associated Protein 1A/1B-light Chain 3
LHb	Lateral Habenula
MEF2, MEF2A	Myocyte Enhancer Factor-2, 2A
MFN 1/2	Mitofusin 1,2
MS	Medial Septum
MTOR, MTORC1	Mechanistic Target of Rapamycin, Complex 1
MURF1	Muscle Ring-finger protein 1
MYOD	Myogenic Differentiation 1
NO	Nitric Oxide
NOS, nNOS, eNOS	Nitric Oxide Synthase, Neuronal, Endothelial

NRF1, NRF2	Nuclear Respiratory Factor 1, 2
PAX7	Paired Box 7
PERK	PRKR-like ER Kinase
PGC-1 α , PGC-1 β	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha, beta
PI3P	Phosphatidylinositol 3-Phosphate
PI3KC3-CI	Phosphatidylinositol 3-Kinase Complex I
POA	Preoptic Area
ROS	Reactive Oxygen Species
SREBP1	Sterol Related Element Binding Protein 1
T _c	Core Temperature
T _{end}	End Temperature
TFAM, TFBM	Mitochondrial Transcription Factor A
T _m	Muscle Temperature
TNF- α	Tumor Necrosis Factor Alpha
TRAF2	Tumor Necrosis Factor Receptor-Associated Factor 2
TRP, TRPV1-4	Transient Receptor Potential, Vanilloid 1,2, 3, 4
UCP1-3	Uncoupling Proteins 2, 3
ULK1	UNC51-like kinase 1
VEGF	Vascular Endothelial Growth Factor
WBH	Whole Body Hyperthermia

ABSTRACT

Başı, C. (2023). Investigation of Effects of Repeated Hyperthermia on Skeletal Muscle and Motor Function in Mice. Yeditepe University, Institute of Health Sciences, Department of Physiology, MSc thesis, İstanbul.

This study explored the impact of repeated hyperthermia on mitochondrial function, proteostasis in mice, and consequent effects on motor and anxiety-like behaviors. Forty Swiss male mice were divided into five groups: Control Exposure (con-E), 39°C/15 minutes/7 days (39E), 41°C/15 minutes/7 days (41E), Control Exposure and Recovery (con-ER), 39°C/15 minutes/7 days with 7-days recovery (39ER), and 41°C/15 minutes/7 days with 7-days recovery (41ER). Post-exposure, the 41E group showed muscle weakness ($p<0.05$), and both 39E and 41E exhibited anxiety-like behavior on the EPM test ($p<0.05$). Gene analysis from gastrocnemius tissues demonstrated altered mRNA expression. Both 39E and 41E had reduced Pgc-1 α levels ($p<0.05$). Pgc-1 β expression notably differed, between 39E and 41ER ($p<0.01$), 39ER and 41ER ($p<0.01$), and 41ER ($p<0.01$). There was a significant difference in TFAM expression between 39E and 41ER ($p<0.01$). The 39ER group had increased NRF1 ($p<0.05$). PINK1, affected by temperature, differed between 39E and 41E ($p<0.05$). PARKIN showcased recovery effects between 41E and 41ER ($p<0.01$). Ucp2 levels contrasted between 39ER and 41ER ($p<0.05$). In summary, mild temperatures (39°C) suppressed autophagy, maintaining strength, and initiating the Nrf1-Tfam-Ucp2 pathway post-recovery whereas muscle strength decreased. Severe temperatures (41°C) caused mitochondrial dysfunction and muscle strength decline. Recovery induced increase in Pgc-1 β modulated mitochondrial biogenesis and in Parkin indicates mitophagy. Moderate hyperthermia induced apparent anxiety-like behaviors in the acute phase, which subsided after the recovery period.

Key words: Repeated Hyperthermia, Mitochondria, Skeletal Muscle, Motor Function

ÖZET

Başış, C (2023). Tekrarlı Hiperterminin Farelerde İskelet Kası ve Motor Fonksiyon Üzerine Etkilerinin Araştırılması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Fizyoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Çalışmamızda, tekrarlanan hipertermi uygulamasının farelerdeki mitokondriyal fonksiyon ve proteostaz üzerindeki etkilerini ve sonuç olarak motor ve anksiyete benzeri davranışlar üzerindeki etkilerini incelemiştir. Kırk Swiss erkek fare beş gruba ayrılmıştır: Kontrol Maruziyet (con-E), 39°C/15 dakika/7 gün (39E), 41°C/15 dakika/7 gün (41E), Kontrol Maruziyet ve İyileşme (con-ER), 39°C/15 dakika/7 gün ile 7 günlük iyileşme (39ER), ve 41°C/15 dakika/7 gün ile 7 günlük iyileşme (41ER). Maruziyetten sonra, 41E grubu kas zayıflığı göstermiştir ($p<0.05$). 39E ve 41E grupları, EPM testinde anksiyete benzeri davranışlar sergilemiştir ($p<0.05$). Gastrokinemius dokularından yapılan mRNA ifadesi analizi anlamlı değişiklikler göstermiştir. Pgc-1 α seviyeleri 39E VE 41E gruplarında azalmıştır ($p<0.05$). Pgc-1 β ifadesi önemli ölçüde farklılık sıcaklık ve iyileşme etkenleri ile artış göstermiştir (39E ile 41ER ($p<0.01$), 39ER ile 41ER ($p<0.01$) ve 41ER ($p<0.01$)). Tfam ifadesi 39ER grubunda anlamlılık göstermiştir ($p<0.01$). 39ER grubunda NRF1 ifadesi artmıştır ($p<0.05$). Pink1 39E ile 41E arasında farklı yönelim göstermiştir ($p<0.05$). PARKIN, 41E ile 41ER arasında iyileşme etkeni ile değişmiştir ($p<0.01$). Ucp2 ifadeleri 39ER ile 41ER arasında anlamlı derecede farklıdır ($p<0.05$). Özetle, orta dereceli hipertermi (39°C) otofajiyi baskılanmış, kas gücünü akut dönemde korumuştur. İyileşme sonrası Nrf1-Tfam-Ucp2 aracılı mitokondriyal program aktive olurken, kas gücü gerilemiştir. Yüksek dereceli hipertermi ise (41°C) akut dönemde mitokondriyal disfonksiyona ve kas gücü düşüşüne neden olmuştur. İyileşme sürecinde Pgc-1 β 'nin mitokondriyal biyogenezi ve mitofaji ilişkili Parkin ifadesi artmıştır. Orta dereceli hipertermi, akut dönemde belirgin anksiyete benzeri davranışlar göstermiş, iyileşme sonrası bu durum gerilemiştir.

Anahtar kelimeler: Tekrarlı Hipertermi, Mitokondri, İskelet Kası, Motor Fonksiyon

1. INTRODUCTION and PURPOSE

Hyperthermia is a pathological condition in which the body's internal or core temperature (T_c) rises to levels higher than the hypothalamic set point, and thermoregulatory mechanisms fail to regulate body temperature ¹. Those who work in high-performance professions (e.g., athletes, soldiers, construction workers) can frequently be exposed to intermittent heat stress ^{2,3}. Temperature extremes and seasonal heat waves due to global warming pose a threat to large populations, particularly vulnerable individuals such as those using medication, the elderly, middle-aged, and with chronic illnesses ^{4,5}. Besides that, hyperthermia is a widely used therapeutic agent ⁶.

Passive heat stress significantly differs from the heating induced by exercise. When the whole body undergoes heating, the antioxidant response ⁷, as well as the availability of nutrients and oxygen differs from during exercise ^{8,9}. Hyperthermia involving the entire body elevates T_c and simultaneously affects the Central Nervous System (CNS). The body's central regulatory mechanisms, in response to high heat loads, manifest an intensified stress response.

Studies on whole body passive heat exposure show different methodological constructs. Whole body immersion baths with the head excluded are a therapeutic modality that is often being tried in cancer treatment ^{10,11}. Infrared lamps are used for dry heat with humidity and simulate the conversion and radiation of sun and ambient temperature, making it possible for gaining heat to be from the whole skin, including the scalp.

The *in vitro* effects of passive heat on cancer cells have been particularly important for therapeutical approaches and ablation. Experiments with infrared or radiofrequency are carried out to determine different doses and frequencies to prevent the heat resistance of tissues to applications and provide useful resources to the literature to reveal signaling during the adaptational process ¹²⁻¹⁴. Studies on the apoptosis of cancer cells and the effects of surrounding tissues, provided a detailed perspective on dose, frequency, and modality.

Although *in vitro* observations of cellular responses are useful, the impact of regional and central responses on cellular adaptations is undeniable. Even between *in vivo* models such as whole-body and local hyperthermia, heat tolerance and survival of cells differ ¹⁵.

Skeletal muscle is a major determinant of metabolic state, protein homeostasis and motor function. Also, it is the main target of therapeutic and acclimatization protocols with heat modalities. Our study aims to understand the effects of passively induced hyperthermic temperatures on the cellular and adaptational processes in a temperature-dependent manner. We believe that it will contribute to a clear perspective of acute and later consequences of passive heat exposure.



2. LITERATURE REVIEW

2.1. Thermoregulation in Passive Heat Exposure

As endotherms, birds and mammals regulate their body temperature across to environmental and metabolic challenges. In humans, blood or T_c (Core Temperature) are maintained at 37 ± 0.5 degree Centigrade ($^{\circ}\text{C}$) (98.6 ± 0.9 degree Fahrenheit) via homeostatic mechanisms ¹⁶. This relatively high T_c preserves protection from pathogens and optimal enzymatic activity ¹⁷.

In endothermic species, heating is managed by similar responses with few possible differences. Early stages of thermal challenges, the proactive phase involves avoidance from the heat source and looking for a cooler environment. The corrective phase of the thermoregulation occurs with autonomic responses depending on the intensity of exposure ¹⁸. When considering passive exposure to heat, it is beneficial to scrutinize the receptors and effectors involved in the sensory perception and subsequent modulation of thermoregulatory responses.

2.2. Perception and Transmission of Heat

2.2.1. Perception of Heat

The heat that reaches the body through radiation and convection is primarily detected by the skin. The Transient Receptor Potential (TRP) family plays a significant role in sensing changes in environmental temperature. Five polymodal receptors known in TRP Vanilloid (TRPV) channels respond to different thermal and chemical stimuli, facilitating the perception of thermal sensation. TRPV1 ion channels open at $40-43^{\circ}\text{C}$ and above ¹⁹. It is also activated by capsaicin, intracellular lipids ^{20,21}, low or high power of hydrogen (pH) ²² and contributes to both warmth and burning sensations. For the perception of peripheral heat, C and A delta fibers, which house these receptors are present in the epidermis ¹⁹.

TRPV2 modulates cellular responses in high temperatures as $45-52^{\circ}\text{C}$. It is localized in the brain nuclei regulate osmoregulation and autonomic regulation; supraoptic nucleus, Arcuate Nucleus (Arc), Paraventricular Nucleus (PVN), nucleus of the solitary tract, nucleus ambiguous and hypoglossal nucleus ²³, additionally, expressed in Purkinje cells of the cerebellum ²⁴.

TRPV3 is predominantly abundant in skin keratinocytes and opens at the temperature range of 34-39°C which is indicated as warm temperatures and chemical activators while TRPV4 responds to a temperature range of 27-35°C as well as osmotic pressure and cell swelling suggesting its involvement in hypoosmotic stress ^{25,26}. Compared to TRPV1 and TRPV2, TRPV3 and TRPV4 show activation in a lower range closer to body temperature. Transient Vanilloid Potential Melastatin 2 (TRPM2), is another known player of thermoregulatory responses in central and peripheral nervous system. TRPM2 is suggested as innocuous temperature regulation and induce hypothermia due to its activation and heat sensitivity in thermoregulatory centers ^{27,28}. However, TRPM2 is also expressed broadly in CNS and participate modulatory functions. TRP arrangement facilitates the quick reflexive response to high temperatures, besides its role on sensory transduction, regulates intracellular Calcium (Ca^{+2}) dependent dynamics.

2.2.2. Central Neural Network Involved in Heat Transmission

The initial response to a hot ambient temperature involves the body's dispersion of heat, preventing the region exposed to the heat from reaching excessively high temperatures. The sensation of temperature perceived from the skin can be conveyed through sensory and pain fibers, depending on the temperature range. If we consider the warming that starts from the skin on a hot day, innocuous temperatures activate TRPV3 channels in keratinocytes trigger Nitric Oxide (NO) activation, initiating a local response ^{25,29}. The increased oxygen-dependent metabolic activity and enzyme-reaction rate with temperature boost the demand for oxygen and glucose in the region ³⁰. Further innocuous temperature is transduced via TRPV1 channels and concurrently transmitted through sensory fibers from the trigeminal and dorsal root ganglion to second-order dorsal root thermal sensory neurons ³¹. Local response is proceeded by sensory neurons, primarily C-fiber afferents that, when stimulated, cause local vasodilation via release of calcitonin gene-related peptide (CGRP), Substance P (SP), and Neurokinin A and noxious sensations ³². This heat sensorial transmission is relayed from the dorsal subnuclei of the lateral parabrachial nucleus (LPBN) to the thalamus and cortex, facilitating perception and localization ³³. Hence LPBN, not thalamic projections is also shown to mediate avoidance responses during high ambient temperatures³⁴. Concurrently, the glutamatergic stimulus arriving at median preoptic (MnPO) interneurons from LPBN activates warm-sensitive neurons in the nucleus. It inhibits premotor sympathetic neurons in the ventral

rostromedial medulla (rmVM) and preganglionic sympathetic neurons in the intermediolateral nucleus via the rostral raphe pallidus (rRPN). As a result, sympathetic vasoconstrictor tone decreases, and blood flow in the region increases ³⁵. Therefore, central temperature is controlled by Preoptic Area (POA) intensely. Beside the relayed information from periphery, central temperature increase activates POA and triggers strong and coordinated responses to counteract heat, including behaviors like panting, sweating, widening of blood vessels, and seeking cooler environments ^{36,37}. 25%–50% of the POA neurons that are activated by local brain warming are also activated by warming of the skin or spinal cord ³⁸. TRPV1 and TRPM2, have been proposed to function as warm sensors in the MnPO, which is suggested to cationic properties of channels lead to membrane depolarization or inactivated potassium current ^{27,39}. In turn, similar channel properties modulates sensory neuronal transduction ⁴⁰.

2.3. Autonomic Responses

2.3.1. Distribution of Circulation

The 4-6-fold increased blood flow in the skin and decreased vascular resistance shift away from internal organs, muscles and brain that have not yet been exposed to heat ⁴¹. In humans, heat loss is facilitated from the surface through the cutaneous vasodilation mechanism ⁴². To compensate for changes in blood pressure corresponding to increased skin blood flow, to accelerate heat loss, and to ensure heat dispersion, cardiac output and skin blood flow are further increase.

However, this mechanism is limited in furry animals due to the low vascularity in the skin and the absence of a neurogenic vasodilator system. Heat exchange is present in acral regions such as ears, paws, and tails, and blood flow in these areas significantly increases ⁴³. The tail serves an important role for heat loss, and the vasodilation in this region is preserved even at high T_c . In rats, passive heating increased vascular resistance of superior mesenteric artery, iliac artery whereas external caudal artery resistance is decreased. Blood volume is increased via splenic and hepatic vasoconstriction, and the average arterial pressure is maintained ⁴⁴. Similar to humans, blood flow is distributed until systemic insufficiency arises. In order to inefficient dissipation, the heat will increase the temperature of central regions via circulation.

2.3.2. Cardiovascular Regulation

The cardiovascular stress induced by passive whole-body hyperthermia (WBH) in humans, leads to an increase in heart rate to 100 beats per minute and cardiac output to 13 L/min to compensate skin vasodilation and related blood pressure decrement.

Temperature is measured centrally in the POA. Temperature information obtained from the viscera, skin, and blood creates a synergistic effect in hypothalamic neurons, thus generating marked stress responses⁴⁵. The rise of T_c above normative values appears to be a precursor to the body's stress response. At T_c of 40 °C and above, plasma catecholamine levels show an increase with similar tendency with cardiac responses⁴⁶.

Peripheral temperature reaches the CNS via circulation and initiates central regulatory responses. An immediate impact of increased temperature on the chemoreceptors can lead to the onset of heat-induced hyperventilation⁴⁷. The activity of the carotid body intensifies with rising temperatures, and its thermal sensitivity is heightened during hyperthermia, in contrast to the sensitivity of baroreceptors⁴⁸. Temperature can also directly influence the functioning of the respiratory neural network situated in the medulla, specifically in the area of the pre-Bötzinger complex⁴⁹. Sympathetic activation of sweat glands results in evaporative cooling. Sympathetic activation of the kidneys can lead to diuresis, which can also contribute to thermoregulatory process. Salivation can also contribute to heat loss through evaporative cooling⁵⁰.

Heart rate and blood pressure gradually increase and increased respiration may cause metabolic alkalosis, hypocapnia and cerebral hypoperfusion⁵¹. Particularly in passive heat exposure, vasoconstriction in the brain may lead to a loss of consciousness and possibly marking the onset of systemic insufficiency⁵². Similarly, the baroreflex response to increasing blood pressure may end up loss of splenic sympathetic tone and hypotension⁴⁴. Due to hyperthermic temperatures, POA neurons may be harmed, or it is discussed that when the restriction of blood flow in turn into lethal threat for internal organs, systemic failure may occur, and vasoconstrictor tone may be lost⁵³. Another perspective is extravascular fluid escape seen with venous pooling and vasodilation can reduce plasma volume to avoid overheating of the skin and may cause shock⁵⁴. This concept of knowledge suggests that exposure to heat is controlled by peripheral and

central coordinated regulation of sympathetic surge. When heat begins to pose a vital threat directly or exhausts thermoregulatory centers lead to systemic failure ⁵⁵.

2.4. Skeletal Muscle Physiology and Comprehensive Thermal Response

2.4.1. Structural and Functional Overview of Skeletal Muscle

Muscle physiology encompasses a range of dynamic processes responsible for muscle development, function and adaptation. The intricacy starts with the two main types of muscle fibers: Type I, known as slow-twitch fibers, and Type II, referred to as fast-twitch fibers. Each type has distinct characteristics that determine their specific roles and responses ⁵⁶.

Type I fibers, with their high mitochondrial abundance and capacity for oxidative phosphorylation are more efficient at utilizing oxygen for prolonged, less intense activity. Conversely, Type II fibers are more suited for short, intense bouts of exercise due to their reliance on glycolytic metabolism ⁵⁷. The presence of myogenic regulatory factors like myogenic differentiation 1 (MyoD) and myogenin is crucial for the differentiation and growth of these fibers ⁵⁸.

Muscle functionality includes contraction and relaxation phases, processed by the movement of Ca^{+2} ions across channels in the sarcoplasmic reticulum (SR), an organelle specialized in Ca^{+2} storage and release. When a motor nerve fiber depolarizes, acetylcholine is released at the neuromuscular junction. Contraction of fibers is proceeded via depolarization of the muscle fiber membrane (the sarcolemma). Depolarization of the sarcolemma leads to a conformational change in dihydropyridine receptors (DHPRs) triggering Ca^{+2} leak from Ryanodine Receptor (RYR) via mechanical linkage ⁵⁹.

The release of Ca^{+2} from the RyR1 channel on the surface of the SR leads to a significant and quick increase in cytoplasmic Ca^{+2} . This binds to troponin C (TnC), permitting the formation of cross-bridges between myosin and actin filaments, which causes the sarcomere to shorten. This process culminates in muscle contraction and the generation of force. ^{60,61}. The extent of Ca^{+2} released from the SR into the cytosol is a key factor in determining the level of force produced during muscle contraction. ⁶².

2.4.2. Calcium Homeostasis

After contraction, Ca^{+2} disassociates from fibers with an energy cost, the sarcoplasmic reticulum Ca^{+2} -ATPases (SERCA), the plasma membrane Ca^{+2} -ATPases

and $\text{Na}^+/\text{Ca}^{+2}$ exchanger has the highest affinity for Ca^{+2} removal from the cytoplasm and together, set resting cytoplasmic Ca^{+2} concentrations, relaxation occurs. The primary routes for Ca^{+2} to enter the cytosol are through voltage-gated channels, receptor operated channels (ROC), and store operated entry channels (SOC) ⁶³.

Sarcolemma has low Ca^{+2} permeability from the extracellular area, leading to a high concentration difference with the extracellular area. Excess Ca^{+2} binds to buffers and effectors to reach a coupled energy state. Duration and gradient of intracellular Ca^{+2} is also the signal for insufficient energetic elements of muscle to handle essential energy needs. TnC, Adenosine Triphosphate (ATP), parvalbumin and Calmodulin (CaM) Ca^{+2} -binding regulate contractile function. CaM is a cytosolic Ca^{+2} sensor, also conducive to the activation of Ca^{+2} -dependent signaling pathways involved in muscle gene expression. SR related Ca^{+2} buffers such as Calsequestrin (CSQ), Sarcalumenin, Triadin and Histidine-rich Ca^{+2} binding protein (HRC), regulate Ca^{+2} release per excitation ⁶².

CaM is composed of two canonical EF-hand lobes that bind four Ca^{+2} ions and go conformational changes, expose hydrophobic side chains to the surface. Ca^{+2} -CaM complex interacts with enzymes and ion channels and activates cellular processes. Ca^{+2} dependent Protein Kinase C (PKC), TRPV channels, RYR, SERCA and Ca^{+2} -dependent phosphatase Calcineurin (CaN), Calpastatin, Calmodulin Kinase (CaMK) are only a limited portion of CaM modulated activity.

Amplitude and duration of Ca^{+2} determines CaN activation via CaM binding. It is shown that only a distinct high stimulatory pattern may induce CaN-Nuclear Factor of Activated T-cells (NFAT) interaction at continuous stimulation of 10 Hertz (Hz) or 50 Hz stimulation for 5 second (s) in every 50s, similar to slow-fiber recruitment ⁶⁴. NFATc1 activity is found to increase Myosin heavy chain (MHC) slow fiber transcripts in regenerating muscles, while MHC fast fiber decreased ⁶⁵. CaN is also mentioned as a nerve activity sensor that refers its pattern-dependent activity ⁶⁶. Myocyte Enhancer Factor-2 (MEF2) gene expression is contraction dependent and leads to fiber switching. CaN activation conduct Histone Deacetylase (HDAC) nuclear dislocation and activate MEF2 gene expression ^{67,68}. A novel protein nuclear receptor interaction protein is clustered Z line of sarcomere can bind CaM and CaN to induce NFAT- MEF2 nuclear translocation ⁶⁹. Therefore, fiber type switching is on influence of multiple intracellular or systemic signal, varying nutritional status to stressful events.

CaMK is another remarkable Ca^{+2} dependent enzyme that is regulated by Ca^{+2} - CaM binding. Ca^{+2} -CaM induces autophosphorylation of Calmodulin Kinase 2 (CamKII). Phosphorylated CAMKII (p-CAMKII) proceeds Ca^{+2} -independent activity after autophosphorylation. Seven days of stretch overload and 2 weeks of voluntary wheel running increased latissimus dorsi protein content with increased Ca^{+2} -independent activity of CAMKII with no changes in Ca^{+2} dependent and total activity ⁷⁰. CamKK (Calmodulin Kinase Kinase) and CamKIV (Calmodulin Kinase IV) are another CaMK members of skeletal muscle. CamKK is activated via CaM binding and phosphorylated by Nicotinamide Adenine Dinucleotide Hydrogen Oxygenase 2 (NOX2) or direct hydrogen peroxide (H_2O_2), recruited with CamKIV to induce nuclear translocation of several genes ⁷¹, which are initiated with mitochondrial biogenesis ⁷².

2.4.2.1. Fiber Type Specific Ca^{+2} Dynamics

As mentioned before, slow and fast skeletal muscle fibers have distinct characteristics in contraction force, velocity, maintenance of contractility and relaxation processes. These type-specific functional properties are highly dependent on Ca^{+2} dynamics. Different contraction and relaxation surges between muscle fibers are aligned with SR Ca^{+2} regulatory components.

Both fast and slow skeletal muscle fibers mainly possess the RyR1 isoform of the RyR, but fast fibers have a greater density of RyR. Type I fibers contract slowly, produce less force, and have high fatigue resistance. In type II fibers, the release of Ca^{+2} can be up to four times greater than in type I fibers, with a more rapid decrease in the Ca^{+2} transient. ⁷³. The proportion of Ca^{+2} that binds TnC to start contraction is substantially smaller in fast fibers, due to high cycling rate of Ca^{+2} saturation of MHC-fast ^{74,75}. TnC and SERCA pumps had more Ca^{+2} binding sites in fast fibers. SR buffer CSQ1 is higher in fast fiber luminal area leads to efficient Ca^{+2} uptake via SR. SERCA1 density is determined higher in fast-fibers and SERCA2A type (with higher Ca^{+2} affinity) is dominant in type I slow fibers. The higher content of luminal buffers supported the idea of fast-fibers high Ca^{+2} maximal capacity. Although resting SR Ca^{+2} content is found much higher in type I fibers, possibly due to different regulation of energetic conditions SERCA2A and SERCA1 ⁷⁶.

Phospholamban is cytosolic modulator of SERCA2A, abundant in type-I fibers and increase SERCA2A activity due to ATP and cAMP accumulation of intracellular area⁷⁷. In contrast, Sarcolipin (SLN) couples with SERCA1, abundant in type-II fibers

and decrease SERCA1 affinity to Ca^{+2} . When Ca^{+2} uptake is decreased, cytosolic Ca^{+2} increases and facilitates SR Ca^{+2} depletion ⁷⁸.

2.4.3. Metabolic Process in Skeletal Muscle

2.4.3.1. Mitochondrial Structure and Energy Metabolism

Mitochondria are the primer generators of energy metabolism in the cell and they play an elementary role in cell homeostasis ⁷⁹. Mitochondria play a vital role in several essential functions within animal cells, including oxidative phosphorylation (OXPHOS), the tricarboxylic acid (TCA) cycle, as well as in glycolysis and Fatty Acid (FA) oxidation (FAO) processes ⁸⁰, calcium homeostasis via connections with endoplasmic reticulum (ER) ⁸¹, amino acid (AA) metabolism ⁸², and apoptosis ⁸³.

Mitochondria, with their double membrane structure, are found in most cells of eukaryotic organisms. Often referred to as the cell's power generators, they produce ATP via oxidative phosphorylation, which is crucial for various metabolic activities. These organelles possess their unique genetic material, mitochondrial DNA (mtDNA), comprising 37 genes critical for their proper functioning in mammalian cells. In humans, mtDNA is a circular, double-stranded DNA molecule. It encodes 13 proteins that are vital hydrophobic components of the electron transport chain (ETC) complexes I, III, IV, and ATP synthase. Additionally, it includes 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs), essential for mitochondrial operations ⁸⁴. Structurally, mitochondria are unique due to their dual membrane system that delineates four different sections: the outer membrane (OMM), the intermembrane space (IMS), the inner membrane (IMM), and the matrix. The inner membrane folds inward to create structures known as cristae. Cristae are the location for the large complexes of the electron transport chain and ATP synthase, which are pivotal in regulating the fundamental rates of cellular metabolism ⁸⁵.

Muscle cells have distinct functional and structural properties to produce ATP, without oxygen need, in cytosolic area through glycolysis, phosphocreatine shuttle and/or in mitochondria via oxidative phosphorylation. Skeletal muscle stores glucose as glycogen in resting conditions. In order to high energy demand, muscle cells have ability to consume 80% of insulin-mediated glucose uptake and FA ⁸⁶. In other words, muscle cells increase energy need 20-30 fold during intense exercise by stimulating insulin-independent glucose uptake and by switching to higher FA uptake and oxidation and

determinant of basal metabolic rate ⁸⁷. The stimulation of motor functions and the rise in intracellular calcium levels enhance the conversion of glycogen to glucose. This process is facilitated by the activity of the enzyme glycogen phosphorylase ⁸⁸. Concurrent adrenergic stimuli precipitate an augmented accumulation of cyclic Adenosine Monophosphate (cAMP) as a consequence of β -adrenergic receptor engagement, which subsequently phosphorylates protein kinase A (PKA). In the context of vigorous and rapid muscular contractions, there is an escalation in Adenosine Diphosphate (ADP) concentrations and activation of Adenosine Monophosphate Kinase (AMPK) or factors associated with hypoxia. These changes facilitate the uptake of glucose and augment the vascular perfusion to the actively contracting musculature ⁸⁹.

Ca^{+2} is pivotal in aligning cellular energy requirements with ATP synthesis. Mitochondria possess a significant capacity for Ca^{+2} buffering. Besides contributing to cellular Ca^{+2} homeostasis, Ca^{+2} also modulates mitochondrial functions. Upon stimulation through motor fibers, adrenergic triggers, mechanical stretching, or mild thermal elevation, Ca^{+2} is released into the intracellular milieu. The Outer Mitochondrial Membrane's Voltage-dependent anion-selective channel 1 detects Ca^{+2} through its EF-hand domains or is activated by Ca^{+2} CaM binding, facilitating Ca^{+2} translocation into the IMS. The influx of Ca^{+2} through the Outer Mitochondrial Membrane (OMM) induces mitochondrial membrane polarization, subsequently activating the Inner Mitochondrial Membrane's Mitochondrial Calcium Uniporter Complex (MICU). In non-neuronal cells, the uniport mechanism is predominantly governed by a MICU1-MICU2 heterodimer complex. The internalization of Ca^{+2} by MICU into the mitochondrial matrix plays a crucial role in regulating OXPHOS coupling ⁹⁰. Matrix Ca^{+2} influx activates TCA cycle activity, increases oxygen uptake, induces oxidative phosphorylation and ATP synthesis ⁹¹.

Mitochondria has unique contacts with ER which is a key modulation for cellular health and cellular fate ⁹².

2.4.3.2.Substrate Utilization

Glycolysis is a ten-step enzymatic process that breaks down glucose to pyruvate as end product. Pyruvate is catalyzed to lactate in mitochondria or cytoplasm in an anaerobic manner. Hexokinase activity, phosphofructokinase and aldolase have rate limiting influence on glycolysis ⁹³. Substrate molecules like pyruvate and fatty acids

undergo metabolic transformations through the TCA cycle and β -oxidation pathways respectively. The end products of these metabolic pathways, namely flavin adenine dinucleotide (FADH₂) and nicotinamide adenine dinucleotide (NADH), play a crucial role in the ETC located in the mitochondria for ATP generation. The ETC is composed of a series of protein complexes embedded in the IMM. NADH and FADH₂ donate electrons to Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) within these complexes. Subsequently, these electrons are conveyed via coenzyme Q to Complex III (cytochrome bc1) and ultimately to Complex IV (cytochrome c oxidase), where they combine with oxygen molecules. This electron transfer process is crucial for establishing a proton gradient across the IMM, which is essential for the operation of F₀-F₁ ATP synthase, leading to ATP production. Therefore, any impairment in mitochondrial function affecting these essential processes can significantly disrupt ATP synthesis, adversely affecting cellular health and function ⁹⁴.

Muscle energy metabolism depends on cellular and systemic influence as autonomic nervous system, motor nerve stimulation and neurotransmitters, hormones, cellular Ca²⁺ dynamics ⁹⁵, allosteric elements (AMPK, ADP, P⁺, H⁺, heat, cold) ⁹⁶⁻⁹⁹, substrate availability ¹⁰⁰, stressful events like unfolded protein accumulation, lysosomal or endoplasmic reticulum stress and Reactive Oxygen Species (ROS) accumulation ^{101,102}. During cellular stress or homeostatic challenges, mitochondrial function is regulated via intracellular factors. Under conditions of normal oxygen concentration, known as normoxia, pyruvate is produced as a result of glycolysis. This pyruvate is then translocated across the IMM via the mitochondrial pyruvate carrier ¹⁰³. In a state of hypoxia, characterized by reduced oxygen availability, mitochondrial respiration becomes inhibited. This leads to a cellular adaptive response where electrons are preferentially directed to pyruvate through the action of lactate dehydrogenase. As a result, lactate is produced in the cytoplasm ¹⁰⁴. The research conducted by Otto Warburg highlights a distinctive metabolic characteristic of cancer cells, where they preferentially convert glucose to lactate even under normal oxygen levels, a phenomenon now referred to as the Warburg effect ¹⁰⁵. Recently, Warburg effect is observed with Uncoupling Protein 2 (UPC2) carrier activation, which will be mentioned ¹⁰⁶.

Palmitate, which is a fatty acid containing 16 carbon atoms, has an energy storage capacity of 39 KJ per gram, a significantly higher value compared to the 16 KJ per gram stored by glucose ¹⁰⁷. Consequently, fatty acids become a principal energy source during

periods of limited nutrient availability. The import of fatty acids into mitochondria is a crucial controlling step for FAO, highlighting the role of metabolic compartmentalization in response to the cellular environment. Since long-chain fatty acids cannot directly traverse mitochondrial membranes, a series of reactions and transport mechanisms facilitate their entry into mitochondrial β -oxidation. An enzyme on the outer mitochondrial membrane, carnitine palmitoyl transferase 1 (CPT1), is involved in converting fatty acyl-CoA into acylcarnitine¹⁰⁸. CPT2 plays a role in releasing fatty acids from carnitine, setting the stage for FAO¹⁰⁹. The activity of CPT1 is intricately regulated by the cellular nutrient state. Malonyl CoA, produced by acetyl CoA carboxylase (ACC), acts as an inhibitor of CPT1, thereby limiting the import of acylcarnitine. As the initial substrate for fatty acid synthesis, the concentration of malonyl CoA influences whether a cell engages in fat synthesis or oxidation¹¹⁰. During conditions of low energy, AMPK phosphorylates ACC, which leads to a reduction in malonyl CoA production and an increase in CPT1 activity¹¹¹. Additionally, in states of insulin resistance, FAO is upregulated to serve as an alternative energy source when glucose uptake is diminished¹¹¹. Mitochondria are also involved in the biosynthesis of various molecules including nucleotides, fatty acids, cholesterol, and AA¹¹². These biosynthetic routes are activated as part of the cellular response to stress. Besides producing electron carriers for the ETC, intermediates from the TCA cycle, like citrate, play a regulatory role in anabolic processes.

In the cytosol, citrate significantly influences the activity of ACC by acting as an allosteric enhancer, promoting its polymerization and function.¹¹³ The control of citrate export from mitochondria might represent a key physiological mechanism for conveying signals about lipid equilibrium to these organelles. The malate-citrate antiporter, specifically Solute Carrier Family 25 Member 1, responds to changes in membrane composition, such as increased levels of cholesterol or acidic phospholipids in the IMM, by reducing mitochondrial citrate export, a process influenced by lipid concentrations¹¹⁴.

Mitochondria serve as critical sites for the synthesis of amino acids including glutamine, glutamate, alanine, proline, and aspartate. Glutamine synthetase (GS) synthesizes glutamine by combining glutamate with ammonia¹¹⁵. Glutamate, a key nitrogen source, is also generated and utilized in this process. Several transaminases, like glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), exist in dual forms, one in the cytosol and the other in the mitochondria¹¹⁶. Different

requirements for Nicotinamide Adenosine Dinucleotide (NAD^+) exist between the cytosol and mitochondria, underscoring the importance of compartmentalized redox balances for cellular homeostasis and response to stress. The cytosol tends to be more oxidizing, while mitochondrial metabolism is more reductive, with a higher NAD^+/NADH ratio 7–8 ¹¹⁷. To maintain this NAD^+ distribution, mammalian cells utilize indirect pathways like the malate–aspartate shuttle, which also contributes to amino acid compartmentalization. Under conditions where oxidative phosphorylation is inhibited, cells can reverse the flux of GOT1 to produce cytosolic aspartate ¹¹⁸. Furthermore, UCP2 aids in exporting aspartate from the mitochondrial matrix ¹¹⁹. The malate-aspartate shuttle also plays a role in increasing the $\text{NADPH}/\text{NADP}^+$ ratio for glutathione synthesis through glutamine flux ¹²⁰.

Another unique pathway for redox balance is the α -glycerophosphate shuttle, closely associated with glycolysis and particularly active in brown adipose tissue (BAT). This shuttle helps regenerate cytosolic NAD^+ while facilitating electron transport into the ETC for thermogenesis ¹²¹.

2.4.3.3. Oxidative Stress and Reactive Oxygen Species

Mitochondrial oxygen is used for the last enzymatic stage of OXPHOS, oxygen may be reduced by electrons to superoxide anion, due to increased electrochemical gradient and increased enzymatic activity. Derivatives of reduced oxygen are defined as ROS; free radical (nitric oxide, superoxide, hydroxyl radicals) or non-radical form (hydrogen peroxide, peroxynitrate, ozone, hydroxide) ¹²². A slight elevation in ROS levels can stimulate cellular proliferation and differentiation ¹²³, while an overabundance of ROS may lead to oxidative harm to lipids, proteins, and DNA ¹²⁴. Skeletal muscle cell mitochondria have uncoupled proteins that regulate mitochondrial membrane polarization to maintain respiratory balance. Uncoupling Protein 3 (UCP3) is the main form of uncoupled protein in skeletal muscle, UCP2 is abundant as well. Therefore, cytosolic antioxidant molecules protect against cellular damage. Enzymatic and molecular agents such as Superoxide dismutase (SOD), Glutathione peroxidase, thioredoxin, glutaredoxin, peroxiredoxin, Glutathione (GSH), and Heme oxygenase-1 (HO-1) function to transform radical entities into more stable, less reactive forms.

During prolonged or severe stress, antioxidant elements may be inefficient to protect against ROS. High levels of ROS exposure can alter myofilament structure ¹²⁵,

exogenous ROS diminish calcium sensitivity of myofilaments, and impairs SERCA Ca^{+2} uptake ¹²⁶. Prolonged ROS exposure of myosin chain leads skeletal muscle dysfunction and decreased force production ¹²⁷. Muscle fibers have been reported to activate adaptive changes in gene expression of HO-1, muscle atrophy without cellular loss, when exposed to 25-100 micromolar (μM) extracellular hydrogen peroxide and muscle fibers lose viability when exposed to millimolar (mM) doses ^{128,129}.

ROS may alter cellular signaling process, may enhance gene expression. These findings are often construed to indicate that cellular mechanisms have evolved to employ ROS as signals in biological processes ¹³⁰. Although the exact processes of how redox regulation affects transcription factors and signal transduction remain unclear, it is understood that these effects are likely mediated by changes in the redox state of protein sulfhydryl groups, which result in conformational alteration ^{131,132}. The phosphoinositide 3-kinase (PI3K)/ a serine/threonine protein kinase (Akt or Protein Kinase B) pathway, p53, Mitogen Activated Protein Kinase (MAPK) and Nuclear Factor $\kappa\beta$ ($\text{NF}\kappa\beta$) signaling pathways are ROS sensitive and extensively studied. AlphaB-crystallin, a member of the Heat Shock Protein 27 (HSP27) family, is phosphorylated after treatment with H_2O_2 via activation of p38 and Extracellular Signal-regulated Kinase (ERK) MAPKs ¹³³. Upon activation, this protein lowers the production of ROS and enhances the synthesis of glutathione (GSH). Consequently, this leads to a reduced activation of $\text{NF}\kappa\beta$ by oxidizing agents ¹³⁴. $\text{NF}\kappa\beta$ and Activator Protein (AP-1) are regulated by cellular redox state. $\text{NF}\kappa\beta$ transcription factor is present in cell conjugated with inhibitory Inhibitor of $\kappa\beta$ (I κ B) proteins. Once cellular H_2O_2 increases (or external stimulants (Interleukin-1 (IL-1), Interleukin-6 (IL-6), lipopolysaccharide (LPS), Tumor Necrosis Factor- α ($\text{TNF}\alpha$) proinflammatory factors) I κ B's are proteolyzed by 26S proteasome and $\text{NF}\kappa\beta$ translocate to nuclei, and induce proinflammatory and antioxidant factors transcription with consensus of complexes (SOD2, GS, inducible NOS (iNOS), cyclooxygenase-2 (COX-2) and cytokines) ¹³⁵. Cytosolic Kelch-like ECH Associated Protein (KEAP)/ Nuclear Factor Erythroid 2-related Factor (NRF2) complex disassociates due to ROS, NO oxidation. NRF2 transcription factor translocate to nuclei, promotes catalase, SOD 1/2 antioxidants production and mitochondrial biogenesis gene expression Peroxisome Proliferator-Activated Receptor (PPAR) Gamma Coactivator (PGC) 1 α , mitochondrial transcription factor A (TFAM) ¹³⁶. Mitochondrial ROS production increase AMPK

phosphorylation independent of ADP/ATP ratio. Non-radical ROS H_2O_2 is mainly used as a biological signal during energy-dependent process.

Exogenous H_2O_2 induces redox changes in key cysteine residues of AMPK α 1 catalytic subunit, while Liver Kinase B1 (LKB1) and CamKK β phosphorylate tyrosine residues ¹³⁷. Excessive or blunted ROS production impairs expected AMPK phosphorylation during endurance exercise. Radical forms may inhibit cellular energy maintenance. P38 MAPK activated through increased Mitogen Activated Kinase Kinase (MAPKK) via phosphorylation due to endurance exercise in ROS accumulation and increase Glucose Transporter 1 (GLUT1) expression to regulate basal glucose uptake with insulin-independent manner ^{138,139}. PI3K/AKT/ Mechanistic Target of Rapamycin (mTOR) pathway activation increases protein synthesis. PI3K activated during H_2O_2 inhibition Phosphatase and TENSin homolog (PTEN) and leads disinhibition of Phosphatidylinositol 3,4,5-trisphosphate (PIP3) formation. PIP3 phosphorylates Akt and mTOR induces protein synthesis via activation of 70-kDA ribosomal protein s6 Kinase (p70S6K) phosphorylation ¹⁴⁰. Therefore, H_2O_2 phosphorylates cysteine residues of Akt causes direct phosphorylation of AMPK and prevents AMPK phosphorylation via LKB1. mTOR activation is highly dependent on AA abundance and AMPK activation inhibits mTOR Complex 1 (mTORC1) phosphorylation ¹⁴¹.

2.4.3.4. Mitochondrial dynamics

Mitochondria is regulated by mtDNA and nuclear DNA transcription factors in a highly dynamic manner. Due to energetic or structural disturbances mitochondria goes under morphological changes under control of large Guanosine Triphosphatases (GTPase) with fusion and fission events ¹⁴². Mitochondrial fission constriction and scission of mitochondria to divide two daughter mitochondria and regulated via dynamin related protein-1 (Drp1), dynamin 2. Mitochondrial fusion is union of two mitochondria with membrane tethering via Optic Atrophy -1 (OPA1) and Mitofusin-1/2 (MFN1/2) ¹⁴³. Overexpression or loss of fusion or fission activity may induce spherical hyperfused elongated or fragmented oval shaped increased number of mitochondria ¹⁴⁴. The process of mitochondrial fusion involves three distinct phases: initially, the trans tethering of two mitochondria, followed by the docking of their membranes which expands the contact area and narrows the gap between them. The final step is the fusion of the OMMs, a process driven by conformational shifts prompted by the hydrolysis of Guanosine Triphosphate (GTP).

During the process of mitochondrial division, the protein Drp1 is drawn to the OMM, where it assembles into a ring-like formation encircling the mitochondrion. This assembly results in the constriction of the mitochondrial membrane ¹⁴⁵. Then, GTP hydrolysis marks a potential future site of mitochondrial scission. Drp1 is not able to bind directly to phospholipid membrane. Beside that mitochondrial fission factor (MFF) ¹⁴⁶ and mitochondrial dynamics proteins 49 and 51 (MiD49 and MiD51) ¹⁴⁷ act as receptors for Drp1 in mammals. Recent research indicates that the constriction of the IMM is dependent on Ca^{2+} and takes place at the contact sites between mitochondria and the ER ¹⁴⁸. Upon stimulation, ER facilitates a pronounced calcium release to the mitochondria. This results in the constriction of the IMM compartment. Interestingly, this process can potentially initiate IMM division prior to the recruitment of the Drp1, suggesting a mechanism that is independent of OMM constriction. Concomitantly, calcium influx into the mitochondria correlates with a decline in the mitochondrial membrane potential. This subsequently activates Metalloendopeptidase 1 and initiates the processing of OPA1 into its short form (S-OPA1). The accumulation of S-OPA1 perturbs the functionality of the Mitochondrial Contact Site and Cristae Organizing System (MICOS) complex, impairing its capacity to maintain OMM-IMM tethering. This leads to a pronounced detachment of the IMM ¹⁴⁹. Drp1 and its adaptors MFF and MiD49/51 are also specifically recruited to these mitochondria–ER contact sites and led to fission ¹⁴⁹.

Drp1 can also be phosphorylated by other kinases during stress and inflammation by PKC ¹⁵⁰ and the Ca^{+2} / CaMKII / Akt dependent manner ¹⁵¹. Therefore, CaN may dephosphorylate Drp1 on site of Serine 656, leads to mitochondrial fragmentation ¹⁵². Post-translational modifications play a role in regulating Drp1 receptors. For instance, MFF acts as a substrate for the AMPK. When mitochondrial function is compromised, leading to a lowered ATP/ Adenosine Monophosphate (AMP) ratio, MFF becomes phosphorylated, which increases Drp1 recruitment and promotes mitochondrial fission and the degradation of damaged mitochondria ¹⁵³. During periods of starvation, HDAC6 interacts with and deacetylates MFN1, enhancing mitochondrial fusion. Additionally, the phosphorylation of MFN1 by ERK in its Heptad Repeat Domain 1 suppresses mitochondrial fusion and facilitates apoptosis ¹⁵⁴. It has been observed that fused mitochondria show higher ATP production efficiency and improved matrix content exchange, while fragmented mitochondria tend to produce more ROS and are more effectively targeted for removal through mitophagy ¹⁵⁵.

Difference between fiber type composition of mitochondrial content involves mitochondrial dynamics. In oxidative type IIA muscle fibers, both MFN1 and MFN2 are necessary to form longitudinally interconnected mitochondria. However, the presence of either MFN1 or MFN2 alone is adequate to sustain the overall morphology of these organelles. Whereas Type IIB/X fibers are shown fragmented and disconnected spheric mitochondria ¹⁵⁶.

2.4.3.5. Endoplasmic Reticulum and Cellular Proteostasis

Proteostasis, which refers to the equilibrium between the synthesis and breakdown of proteins, is a critical aspect of cellular functionality and health. Proteostasis network is known under regulation of 2,000 unique proteins in order to folding, trafficking or degradation processes. Besides regulation of Ca^{+2} dynamics, ER is the main hub for proteostasis, membrane and cellular structural maintenance via cellular communication. Nascent proteins translated via ribosomes are trafficked to the ER and Golgi apparatus to properly fold and to gain functionality. ER has a distinct Protein Quality Control system of chaperons and enzymes ¹⁵⁷. The proteins or newly synthesized polypeptide chains are translocated from ER membrane to luminal side. Proteins destined for the ER often possess N-terminal hydrophobic signal peptides or transmembrane domains. These sequences are identified co-translationally by the Signal Recognition Particle (SRP), which temporarily halts protein synthesis and prevents aggregation of the hydrophobic regions. Once these proteins with signal peptides reach their destination, the signal peptidase cleaves them to free the N-terminus ¹⁵⁸. Proteins that have C-terminal hydrophobic transmembrane anchors, known as tail-anchored proteins, are post-translationally directed to the ER. Regarding protein quality control within the cell, there are three main types of mechanisms: 1) Classical chaperone systems, which assist in proper protein folding, 2) Glycan-dependent molecular chaperone systems, which use sugar molecules in their chaperoning activity, and 3) Thiol-dependent oxidoreductases, enzymes that facilitate the formation of correct disulfide bonds in proteins ¹⁵⁹.

Heat shock protein (HSP) family of molecular chaperones binds hydrophobic sides for proper folding and prevent aggregation. The ER contains members from the 40-kDa HSPs (HSP40), 70-kDa HSPs (HSP70); Binding Immunoglobulin Protein (BiP) Glucose-regulated Protein 78 (GRP78) and 90-kDa HSPs (HSP90); Glucose-regulated Protein 94 (GRP94) which are coupled with ATPases ¹⁵⁸. Chaperones identify immature, aberrant, or aggregation-prone proteins by the presence of exposed hydrophobic

segments. BiP is the most abundant chaperone assisting protein folding, protein translocation and ER-associated Degradation (ERAD) of misfolded substrates¹⁶⁰. BiP interacts with Sec61 complex which is translocon for protein transport into ER lumen. Substrate binds with Substrate Binding Domain (SBD) and phosphorylation occurs on Nucleotide Binding Domain (NBD). When NBD phosphorylated, SBD undergoes conformational change for substrate affinity. Substrate release occurs when Nucleotide Exchange Factors (NEFs) dissociate ADP with ATP and chaperone turns back to the low affinity state¹⁶¹. Two mammalian homolog of NEFs interacts with BiP and Bap (BiP-associated Protein) and GRP170^{162,163}. Prolonged substrate binding, increased ER load may cause misfolded proteins retrotranslocated to the cytoplasm to degraded by ERAD¹⁶⁴.

At the center of the glycan-dependent protein quality control system in the ER are the lectin chaperones calnexin and calreticulin. These chaperones are crucial in the folding and quality control of glycosylated proteins within the ER. N-linked glycans, which are composed of glucose, mannose, and N-acetylglucosamine, are attached to the asparagine side chains of substrates in the ER lumen by oligosaccharide transferase. After glycosylation, two glucoses are trimmed by α -glucosidase I and II and binds with calnexin or calreticulin. The last glucose bond is trimmed by α -glucosidase II to a non-glycosylated state, disassociate with lectin chaperones¹⁶⁵. The retention of misfolded glycoproteins in the ER is facilitated through a process involving their cyclic binding to calnexin and calreticulin. This cycle includes the trimming of these glycoproteins by α -glucosidase II and their subsequent reglycosylation by glycoprotein glucosyltransferase I. Mechanism ensures that improperly folded glycoproteins are kept within the ER for corrective process¹⁶⁶.

The ER provides an oxidizing environment conducive to the formation of disulfide bonds between adjacent cysteine residues on maturing proteins, a critical aspect for the structure and function of many proteins within the secretory pathway¹⁶⁷. Typically, cysteine residues are bound in disulfides before leaving the ER, making free thiols a sign of non-native protein conformation. The ER possesses a quality control system that identifies proteins with unpaired thiols. When there is an accumulation of unfolded or misfolded proteins in the ER lumen, due to proteome stress, the UPR is activated through various signaling pathways¹⁶⁸.

2.4.3.6. Unfolded Protein Response

The UPR is initiated by the activation of three key ER membrane sensor proteins: 1) Protein Kinase R (PKR)-like ER Kinase (PERK), 2) Inositol-Requiring Enzyme 1 α (IRE1 α), and 3) Activating Transcription Factor 6 (ATF6)¹⁶⁹. During ER stress, an excess of unfolded proteins triggers IRE1 α and ATF6 to jointly activate genes for ER chaperones such as Grp94, GRP78/BiP, and Cyclophilin B, enhancing the ER's protein-folding capacity¹⁷⁰. When luminal chaperons bind to unfolded proteins and disassociate from ATF6 membrane bound form, ATF6 spliced to cytosolic soluble form to translocate to the nuclei. ATF6 soluble form, binds ER Stress Response Element (ERSE), and induces ER-chaperone transcription. IRE α is regulated by BiP allosterically and leads X-box Binding Protein 1 (XBP1) splicing and nuclei translocation¹⁷¹. XBP1 leads to transcription of Sterol Related Element Binding Protein 1 (SREBP1) and increase lipogenic enzymes and FA synthesis¹⁷². Prolonged activation of IRE1 α interacts with tumor necrosis factor receptor-associated factor 2 (TRAF2), initiates an apoptotic stimuli¹⁷³. When misfolded proteins accumulate in the ER, it triggers the activation of PERK, which then phosphorylates the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2 α). The phosphorylation of eIF2 α decreases the global protein synthesis¹⁷⁴. Besides global protein synthesis inhibition, ATF4 selectively increases¹⁷⁵. ATF4 binds AA Response Elements (AARE) to increase autophagy related genes transcription¹⁷⁶. Prolonged activation of PERK results in increased expression of C/EBP-homologous protein (CHOP), a transcription factor that plays a significant role in controlling apoptosis¹⁷⁷. Indeed, CHOP and ATF4 co-expression modulate autophagic influx. During ROS accumulation and related ER stress induces PERK-ATF4 phosphorylation. ATF4 is able to induce antioxidant response. It binds antioxidant response element, leading to transcription of NRF2. Increased NRF2, induces antioxidant gene transcription and inhibits ROS accumulation and related ER stress¹⁷⁸.

2.4.3.7. Heat Shock Response

During cytosolic unfolded protein accumulation, another co-activating system Heat Shock Response (HSR) is accompanied. In response to cellular stress that causes protein denaturation and aggregation, there is an increased production of heat shock proteins in the cytosolic compartment. These proteins, functioning as molecular chaperones, possess cytoprotective and anti-apoptotic characteristics¹⁷⁹. Stress-inducible transcription factor, Heat Shock Factor 1 (HSF-1), during healthy equilibrium, abundant

in cytosol as bonded with HSP90, HSP70 and T-complex Protein-1 Ring Complex. In course of ROS, heat, cold, mechanical damage or cytotoxicity, HSF-1 disassociates from HSP90. HSF-1 is translocated to the nuclei and interacts with Heat Shock Element which induces HSP chaperone translation. Cytosolic HSPs work as complexes such as ER chaperones and collaborate to cytosolic unfolded protein folding, trafficking, degradation. HSF-1 is widely present and crucial for safeguarding against damage from heat-induced protein stress and developing thermotolerance ^{180,181}. Heat Shock Factor 2 is more prevalent during mild thermal stress ¹⁸². While Heat Shock Factor 3 in humans is a pseudogene, it still contributes to heat shock resistance ¹⁸³. Heat Shock Factor 4 is predominantly found in embryonic and adult eye tissues, influencing lens development and fiber cell differentiation ¹⁸⁴.

Prominent heat HSPs with significant molecular weights, such as HSP90 and HSP70, possess ATPase activity. This activity is modulated by accessory proteins that are not client molecules, referred to as co-chaperones. The ATP hydrolysis mediated by co-chaperones leads to conformational changes in HSPs. Consequently, this enhances their affinity towards their target proteins, optimizing their protein-folding functionality ^{185,186}. Protein substrates are referred to “clients” are remodeled by HSPs. Clients of HSP90 include transcription factors, protein kinases, receptors and E3 ubiquitin ligases. The baseline expression of HSP90, even in the absence of stress factors, is notably elevated, making it one of the most prevalent proteins within the cell ¹⁸⁷. Various co-chaperones play distinct roles in relation to HSP90: some serve as regulators of its chaperone cycle, while others function as adaptor proteins, facilitating the recruitment of client proteins. HSPs tend to work as complex machinery with adaptors and work for protein folding, trafficking, prevent aggregation and modulates degradation. AHA1, an activator of the HSP90 ATPase, acts as a co-chaperone to HSP90 by attaching it to its N-terminal domain. Unlike p23, AHA1 enhances the ATPase activity of HSP90 in a positive manner ¹⁸⁸. HSP90 and HSP70 recruits with Heat Shock Cognate 70/HSP90-organizing protein (HOP) and conduct protein maturation and preventing aggregation ¹⁸⁹. HSC70-interacting protein (CHIP) is another ER-derived ubiquitin protein as CHOP and they assemble for proteasomal degradation with 26S proteasomes ¹⁹⁰. Therefore, HSP27 and AlphaB-crystallin has a distinct role in actin folding and recruitment ^{191,192}, UNC45 recruits for myosin fiber maintenance ¹⁹³. HSP70/HSP90 machinery is also able to bind Translocase of Outer Membrane 70 (TOM70) complex for mitochondrial protein

transport and communication ¹⁹⁴. Although mitochondria inner membrane and matrix host mitochondrial HSP70 and mitochondrial HSP60 to proteome stability and protein folding and transport. Indeed, HSP60 is more abundant in mitochondria than cytosol under cellular stress ¹⁹⁵.

Interestingly HSP90 and HSP70 bind with Glucocorticoid (GC) Receptors (GCR) and modulate their binding affinity. It has recently been shown that HSP90 disassociates from GCRs during cellular stress and prevents hormone-binding. In the same manner, HSP70 recruitment with HSP90-GCR complex decreases GCR affinity and leads to GCR proteasomal degradation ¹⁹⁶. This overlooked modulation may be a novel way to create a cellular stress state specific interaction with GCs during systemic stress response to prevent excessive protein loss.

2.4.3.8. Autophagy

Skeletal muscle cells are claimed to be protein deposits of the body. Due to cellular or systemic stress, ROS, toxins, heat, or mechanical damage to the protein structures of cytoskeleton or sarcomeres move onto an aggregate-prone state. To prevent toxicity, starvation or apoptosis, aggregates breakdown in lysosomes terms as autophagy. Autophagy is conducted as microautophagy (in lysosome), macroautophagy (autophagosome formation) or chaperon mediated autophagy. In the process of microautophagy, lysosomes expand their membrane to encapsulate components found in the cytosol directly, subsequently breaking them down with lysosomal proteases ¹⁹⁷. In the intricate process of Chaperone-mediated Autophagy (CMA), specific cytosolic proteins possessing a Characteristic Pentapeptide Sequence Motif Q are selectively recognized by the molecular chaperone HSPA8/ HSC70. Upon recognition by HSPA8, these proteins subsequently engage with the lysosomal receptor lysosomal-associated membrane protein 2A (LAMP2A). This interaction triggers the unfolding of the target proteins, which then translocate into the lysosomal lumen. Once inside, these proteins are subjected to degradation ¹⁹⁸.

Macroautophagy is the elimination of aggregates and tagged organelles, via trapping in isolated membrane called phagosome. Phagosome fuses with lysosome and eliminates inner material ¹⁹⁹. UNC51-like kinase 1 (ULK1) collaborates with Autophagy Related Gene (ATG) 13 and FAK-family interacting protein of 200 kDa (FIP200) to establish the ULK1 complex. This activated complex initiates the recruitment of the

transmembrane protein ATG9 and the complex responsible for producing phosphatidylinositol 3-phosphate (PI3P), known as PI3KC3-CI²⁰⁰. The formation of PI3KC3-CI at the phagophore is set in motion by the phosphorylation of BECLIN1, a process mediated by ULK1. As identified by Amoretti and others in 2002, ATG14 plays a crucial role in bridging BECLIN1 and Class-III PI 3 Kinase VPS34, leading to PI3P synthesis. This reaction also facilitates the gathering of various PI3P-binding proteins at the phagophore.²⁰¹ The ATG12–ATG5–ATG16L1 complex, interacting with these PI3P-binding proteins, forms an E3-like complex and catalyzes the lipidation of Microtubule-associated Protein 1A/1B-light Chain 3 (LC3/MAP1LC3A/B) on the membrane²⁰². LC3 conjugation system has been called LC3-lipidation. Preform of LC cleavage proceeded by ATG4b to LC-1 as the cytosolic form. LC3-I undergoes activation through Atg7, is subsequently relayed to Atg3, and ultimately becomes conjugated with the phospholipid PE, forming LC3-II²⁰³. Current developments have elucidated that the closure of the phagophore is driven by the endosomal sorting complexes required for transport system, which aids in the convergence of the phagophore's extremities, thereby promoting the abscission process²⁰⁴. LC3 complex can directly manage lysosomal fusion or facilitate Soluble N-ethylmaleimide-sensitive Factor Activating Protein Receptor (SNARE) recruitment²⁰⁵.

Macroautophagy shares the main characteristics of phagocytosis in macrophages. Due to bacterial invasion, receptor binding leads to recruitment of LC3 and autophagosome formation and NOX2 NADPH oxidase to produce ROS and eliminate invader²⁰⁶. Therefore autophagy may be induced by mitochondrial ROS production due to impaired energetic balance²⁰⁷. The swift initiation of autophagy following the generation of reactive oxygen species (ROS) from mitochondria suggests a rapid binary (ON/OFF) response, potentially governed by redox-responsive proteins. In this context, AMPK emerges as a plausible candidate for mediating this process²⁰⁸.

Glucose and AA nutrition status is controlled via the mTORC1. mTORC1 and AMPK interplay regulates cellular nutrient state²⁰⁹. Increased energy demand may enhance substrate availability via cytosolic energy sensor AMPK. During disrupted nutrient availability Hexokinase 2, aldolase and Glucose-6 Phosphate bind V-ATPases on lysosomes induce phosphorylation of AMPK modulates glucose and FA uptake, substrate switching, lysosome activity, autophagosome formation and autophagy²¹⁰. The downstream effectors of mTOR, namely ELF4-p70S6K, are recognized as central to

precise and effective protein synthesis. They orchestrate the interactions among the mRNA 5' cap, the poly(A) tail, and the 40S and 60S ribosomal subunits, thus playing a key role in the regulation of this process ²¹¹. Active mTORC1 prevents autophagy by phospho-inhibiting the ULK1 at Serine 757 and its interacting partner, ATG13. Upon AMPK phosphorylation, Tuberous Sclerosis Proteins 1 and 2 complex, Raptor and mTORC1 are inhibited, thus leading to the activation of ULK1 complex. Also at least four sites on ULK1 are phosphorylated by AMPK. ULK complex induces the following activation of the PI3KC3-CI complex via the activating molecule in BECLIN1-regulated Autophagy 1 ²¹².

2.4.3.9. Mitophagy

It has been suggested that AMPK becomes activated in response to H₂O₂ exposure. When there is a prolonged dysfunction in mitochondrial activity, ROS are produced in large quantities. This shift transforms their function from general autophagy triggers to specific signals that prompt the self-elimination of mitochondria through a selective mechanism known as mitophagy. Therefore, cellular stress may damage mitochondria and leads to uncontrollable ROS production. Loss of mitochondrial membrane voltage, transduced to catabolic signal via PTEN-induced Kinase 1 (PINK1) / PARKIN (E3 ubiquitin ligase). PARKIN ubiquitinates mitochondrial substrate channels to inhibit activity, recruits Sequestosome 1 (p62/SQSTM1) complex as autophagic adaptor ²¹³. Although p62 facilitates mitochondrial perinuclear clustering rather than mitophagy ²¹⁴. Also, B-cell Lymphoma 2 (BCL2) Interacting Protein 3 (BNIP3) and NIX, members of the BCL2 family possessing the BH3-only domain, are localized on the OMM. These proteins primarily function in detecting hypoxic stress and initiating cell death under cellular stress conditions. They have a significant role in facilitating macroautophagy and mitophagy during hypoxic conditions. An elevation in BNIP3 levels leads to the separation of Beclin1 from BCL2 and/or the engagement of B-cell lymphoma Extra Large in the initiation of mitophagy. This process is crucial for curbing ROS production and preventing cell death ²¹⁵.

2.4.3.10. Mitochondrial Biogenesis

Mitochondria play a multifaceted role in cellular processes, including intracellular signaling, ROS production, fatty acid β -oxidation, amino acid metabolism, pyridine synthesis, phospholipid modifications, and regulation of calcium, cell survival,

senescence, and death. Maintaining a balance in mitochondrial mass and function is crucial for meeting the energy requirements essential for cellular activities ²¹⁶. mtDNA, though compact, encodes 13 crucial polypeptides for the ETC complexes, along with 2 rRNAs and 22 tRNAs for the mitochondrial translation apparatus ²¹⁷. However, 70% to 95% of the mitochondrial proteome is transcribed from nuclear-encoded genes ^{218,219}. Key components of mitochondrial gene expression include TFAM, RNA polymerase γ , and mitochondrial transcription factors B1/2 (TFB1M, TFB2M) ²²⁰. Mitochondrial biogenesis is regulated by nuclear factors, with NRF1 and NRF2 being the initial nuclear transcription factors identified. They bind to a specific site in the cytochrome c promoter and are essential for the expression of nuclear genes related to the mitochondrial respiratory chain. This interaction leads to an increase in cytochrome c mRNA and protein levels, driven by both NRF1 and cyclic AMP Response Element Binding Proteins (CREB) through serine phosphorylation. CREB's phosphorylation is swift and brief, while NRF1's phosphorylation is more prolonged and stable. ²²¹. NRF1/2 also promote the expression of genes for the TOM70-20 complex, essential for mitochondrial protein import ^{222,223}. MEF2 and/or E-box elements are crucial for the tissue-specific expression of muscle-specific Cytochrome Oxidase (COX) genes, though NRF1 regulates MEF2A expression ²²⁴.

ERR α , β , and γ (ERRs) are significant regulators of genes involved in oxidative metabolism ²²⁵. ERR α , highly expressed in oxidative tissues, promotes β -oxidation and overlaps with PPAR targets ²²⁶. ERRs can amplify PPAR effects through cross-regulatory pathways ²²⁷. The PGC-1 coactivator family, including PGC-1 α , PGC-1 β , and PGC-1-related coactivator, PRC, interacts with various hormonal and nutrient signaling pathways. Various of hormonal and nutrient signaling pathways associated with PGC-1 α . Therefore, increased UCP2, and increased TFAM expression via directly binding NRF1 promoter leads to increase mtDNA replication and transcription ²²⁸. The precise mechanism by which PGC-1 β operates is still not fully understood. However, it is known that the heightened expression of PGC-1 β activates a range of its target genes, including cytochrome c, ATP synthase- β , and 5-Aminolevulinate synthase 1. This stimulation is achieved through PGC-1 β 's interaction with two transcription factors, NRF1 and ERR α ²²⁹.

Mitochondrial OXPHOS varies in its efficiency: it can either efficiently produce ATP from a minimal number of calories (coupled respiration) or may be less effective,

necessitating the burning of more calories for the same ATP yield (partially uncoupled respiration). This variation in efficiency results in the dissipation of excess energy as heat. Consequently, in endothermic organisms like humans, the efficiency of mitochondrial coupling influences how calories are allocated between generating ATP for cellular activities and producing heat for body temperature regulation ²³⁰.

In response to unexpected cellular stress, mitochondria generate ROS that act as a signaling mechanism to the nucleus. However, excessive production of mitochondrial ROS can lead to damage to both the mitochondria and mtDNA, leading to a decrease in mitochondrial energy production. To counteract this harmful state, cells deficient in mitochondrial function are eliminated through apoptosis. This process is triggered by the activation of the mitochondrial permeability transition pore (mtPTP), which responds to increased oxidative stress, diminished electrochemical potential, decreased levels of high-energy phosphates, and excessive calcium uptake by mitochondria ²³¹. In such scenarios, the depletion of mitochondrial energy renders the cell incapable of performing its normal functions.

2.4.4. Influence of Heat Stress on Skeletal Muscle

2.4.4.1. Skeletal Muscle as a Thermal Effector

Thermoregulation is one of the key evolutionary abilities for surveillance across the ages during habituation. As a consequences of fluctuating environments, as endothermic organisms, birds and mammals are able to maintain the T_c ²³². The majority of organisms are unable to operate at a core temperature (T_c) below 0°C, as water solidifies at this point, leading to a substantial disruption of cellular functions and often, though not invariably, resulting in cell death. Similarly, sustaining life becomes exceedingly difficult at a T_c exceeding 45°C, due to the denaturation of enzymes essential for biochemical reactions at these higher temperatures. Even a minor adjustment in T_c , as little as 0.5°C, can have significant impacts on the lifespan of organisms such as mice ²³³; thermal injury was observed in a healthy monkey brain following exposure to a temperature of 44°C for a duration of one hour. ²³⁴. To sustain optimal metabolism and survive, T_c is regulated strictly via thermoregulatory mechanisms about 37°C in humans ²³⁵.

However, powerful regulatory systems come with higher energy expenditure. In a comparison between reptiles and mammals of similar size, it has been observed that the

skeletal muscle mass in homeothermic mammals is approximately 30% greater than in reptiles. Birds and mammals can produce heat with repetitive contraction of skeletal muscle shivering. Because of the energetic requirements of thermogenesis, mitochondrial respiratory capacity is increased in mammals. The surface area of mitochondrial membrane is %220 greater in mammals and has twice the enzymatic activity than in reptiles ^{236,237}.

Skeletal muscle is known as the primitive non shivering heater for all homeotherms ²³². The evolutionary pathway of birds is shaped by aerial life with feathers, subcutaneous white adipose tissue, high muscle metabolism with shivering and non-shivering thermogenesis. Recruitment with these effectors, birds have the ability of preserving 44°C T_c in below 0°C ambient temperature, during the flight ²³⁸. In mammals, BAT has evolved to lose myofibers and become enriched with mitochondria and a proton carrier protein that uncouples with ATPases. The mitochondria in BAT contain UCP1, which uses the proton gradient to generate heat without muscle contraction. In larger mammals, including species like rabbits, ruminants, and humans, the activity of BAT is significantly reduced following the neonatal stage and remains minimally active during adulthood ²³⁹⁻²⁴¹. Regardless of BAT presence, oxidative skeletal muscle has the ability of changing to uncoupled state for higher heat generation after cold exposure ²⁴². Another heat generator, UCP3 recruits skeletal muscle mitochondria during acute exercise and cold stimuli. Due to activation of skeletal muscle, UCP3 prevents excessive proton accumulation and maintains mitochondrial membrane potential. The uncoupling of the mitochondrial ETC from ATP production is physiological and optimizes efficiency, fine tunes the coupling of oxidative phosphorylation, and prevents generation of ROS ²⁴³. UCP2 is also abundant in skeletal muscle mitochondria with high content of glycolytic fibers. It is shown that UCP2 has a major role in glucose metabolism and increased expression is responsible for aerobic and anaerobic glycolysis. UCP2 is also known as a player of uncoupled respiration and maintenance of mitochondrial membrane potential. However, it is also under research for specialized role in energy metabolism ²⁴⁴.

2.4.4.2. Thermal Acclimation

Animals experiencing prolonged exposure to elevated temperatures undergo physiological modifications to adapt to their surroundings, a process referred to as heat acclimation ²⁴⁵. Thermal acclimation acquisition occurs as short term as 5-7 days repeated thermal exposure. During adaptational process, stress response is rescheduled during

stressful conditions, which is tertiary for energy conservation. After 5 days of acclimation, onset T_c of salivation decreases, after 28 days, locomotion increases, and heart rate decreases during heat stress. Seasonal adjustment to increased environmental temperatures results in a shift of the thermal neutrality zone along with a corresponding alteration in the absolute temperature threshold for the heat dissipation response. Concurrently, the POA raises its neutral temperature by up to 1°C , while the T_c adaptively decreases ²⁴⁶. In order to high temperature challenges, acclimation is shown to increase temperature sensitivity and ability to maintain metabolism in higher temperatures. Besides that, inflammatory responses due to high hyperthermic challenges, Blood-Brain Barrier (BBB) disruption, increased gut permeability induces septic challenges and may enhance metabolic responses ^{247,248}. Increased stress responses and GCs suppress inflammatory pathways. Hence it is important to follow the surge end of the stress exposure. It may induce high inflammatory accumulation during recovery phase ²⁴⁹.

2.4.4.3.Motor Function

Experimental setup including different humidity, regional or gained with activity, given passively could awaken differential responses during research. Human studies on heat stress can reach 40°C only regionally or after warming. Regional heat could not simulate systemic response. Thomas et al. indicated that voluntary activation was impaired by an elevated T_c rather than by the increased muscle temperature ²⁵⁰. Lately, it is emphasized that systemic metaboreceptor function, Muscle Sympathetic Nerve Activity (MSNA) feedback alteration, central fatigue and metabolite clearance has major effect on muscle motor function during heat challenges ²⁵¹. Furthermore, in higher temperatures as 42°C for 15 min, both muscle types are shown decrement at 5-30 min twitch tension in vitro experiments ²⁵² (**Table 1**).

Table 1. Single or Repeated Heat Stress and Effects on Skeletal Muscle

Muscle/Tissue Type	Heat Load	Muscle Strength and Protein Level Changes	Mitochondrial Markers
C2C12 myotubes ²⁵³	Single / 1 hour (40°C)	PL ↑ (24 h), HSP72 ↑ (24 h), HSP1a-b ↑ (1 h)	NRF1, NRF2 ↑, PGC-1β ↑, PGC-1α ↑, TFAM ↑, mtDNA ↑
Mouse EDL and Soleus ²⁵²	Single regional bath / 15 min (T _m 42°C)	Peak tension ↓ (in vitro, after 24 hour)	HSP72 ↑
Rats EDL and Soleus muscle (in vitro) ²⁵⁴	Single / 1 hour incubation (40°C)	Peak tension ↓	Oxygen diffusion ↓
L6 rat myoblasts ²⁵⁵	Single / 1 hour (41°C)	PL ↑ (after 96 h), HSP72 ↑ (after 96 h)	-
Human vastus lateralis ²⁵⁶	Single Regional/ 2 hour Repeated / 2 hour / 6 days (T _m 39°C)	-	Repeated Exposure: p-ERK1/2 ↑, p-AMPK ↑, PGC-1α ↑, HSP72, HSP90 ↑, HSP27, HSP60 –, CS activity ↑, cI ↑, cIV ↑
Rat EDL and Soleus ²⁵⁷	Single WHB / 1 hour (T _c end 41°C)	Soleus muscle dry weight to total body weight ↑ (7 days)	Soleus+ EDL: HSP72 ↑ (1,7,14 days), Calcineurin ↑ (7 days)
Mouse EDL and Soleus ²⁵⁸	Single / 30 min (40°C) Repeated 30 min / 5 days (T _c end 39°C)	-	Soleus+EDL: Single: Akt ↑, p-p70S6 ↑, p-AMPK ↑, p-ACC ↑, p38MAPK ↑, Corticosterone ↑, Repeated: HSP72 ↑, HSP60 ↑, CS activity ↑, COXs ↑, Soleus: β-HAD ↑
Rat Soleus ²⁵⁹	Single WHB / 1 hour (T _c end 41°C)	Proliferative markers (1 day), Muscle dry weight to total body weight (7 days)	p-p70S6 ↑, HSP72 ↑ (1 day)

PL: Protein Level; HSP72: Heat Shock Protein 72; HSP1a-b: Heat Shock Protein 1a-b; NRF1, NRF2: Nuclear Respiratory Factor 1 and 2; PGC-1β, PGC-1α: Peroxisome Proliferator-Activated Receptor

Gamma Coactivator 1-beta, 1-alpha; TFAM: Mitochondrial Transcription Factor A; mtDNA: Mitochondrial DNA; EDL: Extensor Digitorum Longus; p-ERK1/2: Phosphorylated Extracellular Signal-Regulated Kinases 1/2; p-AMPK: Phosphorylated AMP-Activated Protein Kinase; HSP90, HSP27, HSP60: Heat Shock Proteins 90, 27, 60; CS activity: Citrate Synthase Activity; cI, cIV: Complex I, Complex IV; Akt, p-p70S6, p-ACC, p38MAPK: Protein Kinase B, Phosphorylated p70 S6 Kinase, Phosphorylated Acetyl-CoA Carboxylase, p38 Mitogen-Activated Protein Kinases; β -HAD: Beta-Hydroxyacyl-CoA Dehydrogenase; p-p70S6: Phosphorylated p70 S6 Kinase; COXs: Cytochrome c Oxidases.

In order to warm up via bicycle training for 20 min, increased muscle temperature to 39°C and improved in Type II muscle power²⁶⁰. In 30 min sauna, endurance and voluntary strength is decreased in human, whereas vertical jump (power) increased²⁶¹. In repeated local application of heat with 39°C T_m increased strength whereas higher T_m is shown no change^{262,263}. Local heating muscle to 38°C increase blood flow and muscle fluidity which support cross bridge formation and muscle torque production, with higher Ca^{+2} release from SR²⁶⁴. To better understand that acute application of heat in vivo experiment on humans, 10 Hz stimulation showed decreased peak force due to 39-40°C muscle temperature. 10 Hz stimulation targets Type I muscle fiber and reduction in its maximal force in motor nerve fibers. Therefore repeated application improved muscle torque and contractility without Electromyography changes²⁶⁵. Nevertheless, with higher temperatures and repeated applications, limited data is available.

To prevent muscle atrophy heat treatment with repeated lower temperatures or single high temperatures are under research. Induction of HSPs is shown to prevent muscle atrophy. Decreased atrophy in soleus muscle via single 1 hour heat session with end 41.5°C T_c , is shown after 8 days of unweighting of hindlimb²⁶⁶. The single heating session with similar heat load increased proliferative capacity of muscle satellite cells in rats in Type I muscle²⁶⁷. Although the most accurate muscular enhancement with WBH occurs when applied before or after atrophic signals and most of the gain in mass from Type I fibers. Hence increased muscle protein content, protein synthesis or proliferative or regenerative markers were not the main contributors of muscle contractility or voluntary strength which are induced during stressful conditions. Another gap in the literature is increased proliferation or protein content related function or signal changes after repeated heat stress and changes in HSP induction.

In response to heat exposure, Type II muscle fibers do not exhibit optimal adaptability. Elevated muscle metabolism might be better tolerated by oxidative fibers, leading to the perception of Type II fibers as merely protein reservoirs during systemic

perturbations. Depletion of ATP, increased ROS content, proteome, and membrane structure instability, constricted vascular bed and increased metabolite challenge may cause high cellular stress and cytotoxicity. Although mitochondrial content and respiration is higher in Type I muscles, stressful conditions lead 3-fold higher H2O2 production in Type II fibers ²⁶⁸. Also Type II muscles express more GCR which inhibits inflammatory signaling and protein synthesis and leads to degradation ²⁶⁹. In a study examining hypertrophy through overload in the plantaris muscle, it was observed that a single session of heat exposure prior to overloading led to a reduced mass gain ²⁷⁰. This outcome suggests that hypertrophy may be inhibited by a singular high load episode. While at first glance, these findings appear contradictory, they provide complementary insights. Heat stress may not promote an increase in Type II muscle mass. Instead, a single exposure to heat appears to induce proteome stress, trigger HSP induction and enhance proteome stability. Consequently, when the muscle undergoes overload, its protein structure appears to be better maintained compared to muscles that have not been subjected to heat stress. Therefore, even its protective effects of muscle proteome, HSP70 has no shown protective influence on muscle force ²⁷¹.

To better understand the mechanism under that it is important to emphasize muscle fatigue progression during stressful events.

2.4.4.4. Muscle Fatigue

Muscle fatigue refers to a decrease in the capacity to generate force or power in muscles ²⁷². Fatigue progression may occur due to central regulatory responses which are fed by peripheral information intensely. During systemic stress as exercise, the buildup of metabolites in peripheral blood, which are ROS, inorganic phosphates, calcium ions, lactate, ADP, and magnesium, combined with the depletion of glycogen reserves, disrupts homeostasis. This disturbance impacts the formation of cross-bridges within the actin-myosin complex ^{273,274}. Failure of energy metabolism leads to disturbances of resynthesis of ATP and increased phosphates and hydrogen ions induce acidosis. In turn, loss of affinity to binding Ca^{+2} in filaments decreases contractile force.

Hyperthermia is also proposed to be the limiting factor for fatigue during exercise ²⁷⁵. Single heat stress and repeated exposure influence on skeletal muscle protein levels, proliferation and hypertrophy is a field of research where opposing views and results are revealed. Temperature significantly influences the contractile characteristics of muscle

fibers. When core muscle fibers are exposed to higher temperatures, they tend to depend more on anaerobic glycolysis. This increased reliance is probably because the peripheral fibers act as an oxygen sink, hindering adequate oxygen diffusion to the central fibers. As a consequence of this enhanced anaerobic glycolysis, there is an increased production of lactate and hydrogen ions, leading to a quicker onset of muscle fatigue²⁷⁶. Which may be a part of oxidative and glycolytic fiber contractility responses differ among hyperthermic challenges.

NO, another factor contributing to fatigue, is an uncharged, diatomic, gaseous molecule. It is highly lipophilic and easily diffusible, characterized as a free radical due to its unpaired electron²⁷⁷. NO is generated from L-arginine AA by NO synthase enzyme (NOS). Three isoforms of NOS; endothelial isoform (eNOS) and neuronal form (nNOS) of NOS are expressed in skeletal muscle. A specific form of nNOS, tailored to skeletal muscles, has been identified as a constituent of the dystrophin complex²⁷⁸. Three different fractions of nNOS in skeletal muscle: nNOS β of Golgi apparatus, sarcolemmal nNOS μ and cytoplasmic nNOS μ regulate cellular dynamics and force production. nNOS β is implicated as critical regulator of fatigue resistance and postexercise force output, and nNOS μ signaling maintains blood delivery to the muscle during physical exercise. A splice variant of nNOS, situated in the Golgi complex, plays a role in maintaining the integrity of the microtubule cytoskeleton within skeletal muscle. Furthermore, nNOS β affects mitochondrial function. Long-term exposure to NO enhances mitochondrial biogenesis in mammalian cells via generation of cyclic guanosine monophosphate (cGMP)²⁷⁹. During metabolic challenges AMPK and NO are shown to contribute synergistic action. Pretreatment with NOS inhibitor L-NG-monomethyl arginine (L-NMMA, 1 mM), completely prevented AMPK α phosphorylation which is a major inducer of PGC-1 α ²⁸⁰. A clear distinction between exercise condition and passive hyperthermia is shown by Tamura et al. via protein analysis in their detailed work. After a single bout of endurance exercise (30 min, treadmill) phosphorylated AMPK is increased, whereas in single passive heating or endurance exercise with passive heating led to significant decrease. In same manner, Akt phosphorylation is increased in passive heat groups and P38 MAPK phosphorylation was higher significantly in Type II muscles due to higher temperatures²⁵⁸ (**Table 1**). Single heating group showed marked decrement in AMPK phosphorylation, may be interpreted as endurance exercise partly saved NO induction²⁸¹. As mentioned before, central hyperthermia has sympatholytic effect on muscle arterial

bed. In another perspective, central regulation during hyperthermia increased ROS may induce TRPM2 related POA inhibition and lowers arousal state to generate hypothermia²⁸².

Some instances of muscle fatigue are attributed to a gradual, exercise-induced decrease in the voluntary activation of muscles, a phenomenon known as 'central fatigue',²⁸³. Nielson and colleagues posited that central fatigue might be more pronounced under conditions of hyperthermia. In their research, participants engaged in exhaustive cycling in a heated environment, raising their core temperature (T_c) to approximately 40°C. It was observed that after cycling under hyperthermic conditions, there was a more significant occurrence of central fatigue during prolonged Maximum Voluntary Contractions (MVCs). However, this increase in fatigue was not noted during short-duration MVCs, especially in comparison to performance after cycling at a similar intensity but with a T_c of 38°C²⁸⁴. In the absence of physical exertion, when the T_c was passively raised to approximately 39.5°C. Morrison et al. in 2004 found a reduced voluntary activation in the knee extensor muscles during 10-second MVCs²⁸⁵. The altered contractile properties or muscle transmission might not be the sole contributors to the heightened central fatigue experienced during hyperthermia. Numerous studies have also documented the impact of hyperthermia on brain function. For example, prolonged exercise in the heat can lead to a reduced blood velocity in the middle cerebral artery and brain metabolism^{286,287}. Recent studies suggest that it is in the brain where central fatigue originates, which is overwhelmed by increased monoaminergic substances for regulatory purposes²⁸⁸. To support this idea, “serotonin hypothesis” of central fatigue has been suggested as part of the fatigue development. Increased tryptophan levels in blood and permeability of BBB are observed during hyperthermic conditions and leads to lethargy and fatigue symptoms²⁸⁹. Another element contributing to the onset of central fatigue involves alterations at the spinal cord level, specifically pertaining to the afferent input from neuromuscular spindles and tendon organs. This includes changes in the Golgi structures and the circuitry of nerve fiber types III and IV. Central fatigue has important effect on muscle force production, which is shown via additional decrease in MVC due to T_c changes when compared to local heating²⁹⁰.

2.4.4.5. Skeletal Muscle Cellular Responses to Hyperthermic Challenge

Passive heat stress significantly differs from the heating induced by exercise. When the whole body undergoes heating, the antioxidant response⁷, as well as the

availability of nutrients and oxygen differs from during exercise ^{8,9}. Hyperthermia involving the entire body elevates T_c and simultaneously affects the central nervous system. The body's central regulatory mechanisms, in response to high heat loads, manifest an intensified stress response ²⁹¹.

Intracellular signaling mechanisms regulate the availability of oxygen and nutrients in skeletal muscles. Recent research underscores the fact that glycolytic fibers, or Type IIb fibers, rely more on the enzymatic activity of NOS1, which is abundantly expressed in the sarcolemma ²⁹². Additionally, NOS3 is present on the IMM ²⁹³. Glycolytic fibers exhibit elevated calcium levels, coupled with a disorganized placement of mitochondria. In contrast, oxidative or Type I fibers demonstrate a highly organized structure with an abundance of elongated mitochondria. The strategic internal positioning and enhanced vascularization of Type I fibers are essential for maintaining oxygen and nutrient supply during tonic contractions, which are associated with slow calcium accumulation ²⁹⁴.

Moreover, glycolytic fibers facilitate NO synthesis through contractions mediated by the sarcoglycan complex in association with NOS ²⁹⁵. In application of passive heating, the absence of contraction-mediated NO may lead to impaired cellular heat dissipation, nutrient availability, oxygen availability or hindered metabolite clearance. Concurrently, the slow calcium current through TRPV3 channels amplifies calcium entry into the mitochondria. Calcium accumulation in the IMS is modulated by the action of UCP2 during excitation-contraction in glycolytic fibers ²⁹⁶. An increase in calcium within the IMS enhances membrane polarity and triggers mitochondrial NOS due to heightened ETC activity. The presence of NO in the mitochondrial matrix inhibits cytochrome c oxidase, resulting in uncoupled respiration. This nuanced regulation is pivotal during rapid contraction-relaxation cycles ²⁹⁷.

In scenarios where there is slow and prolonged calcium accumulation in the IMS of Type II fibers, cellular temperature rises, leading to the activation of TRPV1 channels. Activation of TRPV1 results in the depletion of SR calcium and a subsequent increase in cytoplasmic calcium levels. This cytoplasmic calcium interacts with proteins such as CaM and CaN leading to the dephosphorylation of the NFAT in the nucleus, which further drives fiber type switching ^{65,298}. TRPV1 channel activation also alters UCP2 expression in mitochondria with fiber type dependent manner. In Type I muscles, UCP2 level increases whereas Type II muscle UCP2 level decreases. UCP2 levels may conduct

aerobic glycolysis and proliferative accumulation through inflammatory pathways^{299,300}. The activity of cytoplasmic NOS is amplified by CaN, which propels the phosphorylation of AMPK. AMPK phosphorylation induces ULK1 complex recruitment and leads to autophagic influx. Furthermore, Ca⁺² depletion from SR activates UPR and increases SREBP1 via XBP1 induction. XBP1 increases ER chaperon transcription and UCP2 expression. SREBP1 modulates intracellular lipid accumulation. After heat stress, ER stress induced intracellular lipid droplets tend to prevent further mitochondrial damage^{301,302}. A novel role for ER membrane bound NRF1 is described as cellular cholesterol sensor which regulates intracellular lipid transport to prevent excess lipid accumulation³⁰³. Interestingly, another modulator of intracellular and membrane cholesterol content is TRPV1 channels. Intracellular lipids bind C terminal of TRPV1 channel and decrease sensitivity. In order to agonist accumulation or lack of intracellular lipid concentration, channel activates. Also, TRPV1 activation with capsaicin is observed for decreased vascular FFA accumulation. TRPV1 may induce intracellular FFA uptake during heat challenges.

It is not known if AMPK dephosphorylation is proceeded by hyperoxia and NO via increased respiratory frequency or Insulin-like Growth Factor 1 (IGF-1) and insulin dependent way. However, increased oxygen levels in blood after 30 min sauna bath session via hyperventilation, increased ROS in blood serum and diminished pro-oxidant/antioxidant balance^{304,305}. After the session, nitrogen oxide production and lipid peroxidation products increased. Furthermore, in hyperthermia or regional heat, increased glucose uptake is emphasized and actively researched recently.

Under conditions of heat stress, there is an elevation in lipolysis in adipocyte, resulting in an increased concentration of FFAs in the bloodstream and inhibited adipocyte differentiation. UCP3, activated by the H₂O₂ content in the matrix, may facilitate lipid accumulation in the cytoplasm. Single heat stress is also shown to decrease ACC phosphorylation, which is downstream of AMPK. Therefore, increased glucose metabolism in muscle cells is shown due to heat stress which is a consequence of increased insulin sensitivity which is an under regulation of HSP70³⁰⁶⁻³⁰⁸.

Elevated ROS phosphorylates p38 MAPK and culminating in the induction of Heat Shock Factor 1 (HSF-1). The phosphorylation of p38 MAPK suppresses the induction of HSP70. Once ROS levels are mitigated through autophagic mechanisms, the phosphorylation levels of p38 MAPK decline, and the induction of HSP70 ensues.

Delayed induction of HSP70 may be related to this action. S-nitrosylation of HSP90 inhibits the ATPase reaction and impairs the activating effect of HSP90 on the client protein eNOS. It has been demonstrated that overexpression of HSP70 can suppress the basal level of LC3-II protein and inhibit the activation of autophagy³⁰⁹. As a secondary response, induced HSP60 and its derivatives take on a carrier role in the activation of IGF-1 receptors³¹⁰. In turn IGF1 inhibits autophagy by activating the AKT/MTOR pathway³¹¹. On the other hand, knockdown or deletion of HSF-1 can increase the basal level of autophagy³⁰⁹, while constitutive activation of HSF-1 results in decreased macro autophagy³¹². It is shown that HSP70 inhibits Forkhead box (FOXO) 3a and NFκβ transcriptional activities and decreases Muscle Ring-finger protein 1 (MURF1) and ATG1 and prevents skeletal muscle atrophy³¹³. In heat stressed rat (42°C 1h) after one session, autophagic influx appeared as ATG1 and MURF1 expression. In order to second session after 24 h, HSP70 induction and autophagy related proteins downregulated which consequently decreased cell viability. When HSP70 expression is failed to handle proteome stress, then autophagy becomes the primary proteostasis mechanism, when both failed, inflammatory signaling and apoptosis occur³¹⁴. Coordination of two responses maintains protein homeostasis in cells during heat stress.

2.4.4.6. Mitochondrial Adaptations to Heat Stress

Endurance exercise has multiple beneficial effects on systemic and muscular oxidative capacity, including improvements in cardiovascular capacity and mitochondrial efficiency and content-related adaptations³¹⁵. Heat stress is being researched for its ability to induce mitochondrial biogenesis and protective effects on the muscle proteome. Heat stress-related mitochondrial adaptations involve energy, substrate, and protein homeostasis, as well as cell survival and apoptosis responses. Passive application of heat (2 hours at 40°C for 6 days, without muscle contraction) has been shown to stimulate increases in mitochondrial enzyme content and respiratory capacity in C2C12 myotubes via NRF1-TFAM and PGC-1α upregulation. An increase in GLUT4 indicates enhanced glucose utilization³¹⁶. Repeated heat stress via regional heating to about 40°C muscle temperature may have beneficial effects on vascularization, oxygen and nutrient availability, ROS scavenging, and oxidative capacity. A single episode of 2 h regional heating with diathermy induced AMPK phosphorylation and p38 MAPK induction. Repeated application for 30 minutes at 40°C in the end temperature T_m increased PGC-

indicated proteome stress and mitochondrial dysfunction³²³. Therefore, increased cellular stress and ROS accumulation led to DNA damage, proteotoxicity, and prolonged mitochondrial dysfunction. Besides its protective effects on proteome, HSF-1 also induces Cellular-myelocytomatosis (c-MYC) DNA binding during DNA damage and facilitates transcriptional programming³²⁴. Another vital compartment of cell, mitochondria is damaged in high heat loads and researchers observed mitochondrial swelling and energy depletion³²⁵. Cellular damage and proteome stress inhibits protein synthesis via the PERK-ATF4-EIF2a pathway and activates IRE1a-XBP1 to induce lipogenesis^{326,327}. Perturbations in ER folding capacity could activate mitochondrial UPR and increase HSP60 transcriptional activity, which induces prolonged activation of the NFκβ pathway. Increases in TNFα are related to rises in T_c. WBH caused an increase in gut membrane permeability and endotoxemia³²⁸, which further increased TNFα and IL-6 which induces thermogenesis in brain in inflammation. Additive hyperthermia may lead to fibrotic and atrophic changes^{329,330}. Also, TNF-α modulates HIF-1α degradation and increases susceptibility to heat-induced apoptosis via downregulation of the HSF-1 response^{331,332}. During inflammatory challenge Interferon-α (IFNα) is shown to induce PGC-1β to maintain efficient ROS production in host defense³³³. It is shown that ER response in CNS modulated by PGC-1β due to nutritional stress³³⁴. PGC-1β is related to lipid utilization and upregulated in hyperlipidemia²²⁹ (**Figure 2**).

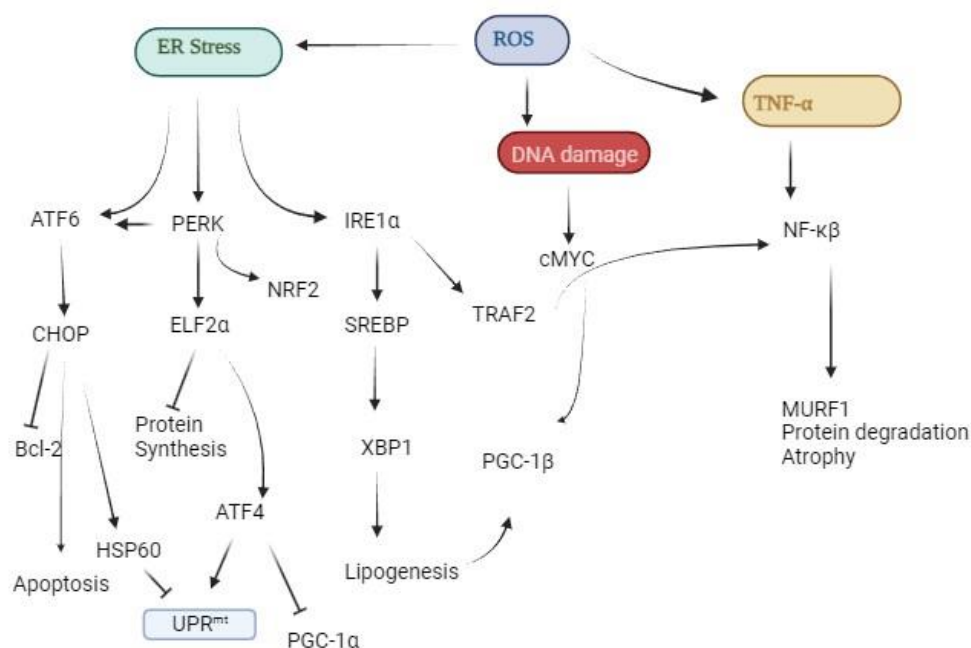


Figure 2. ER stress-related cellular responses.

2.4.4.7. Behavioral Responses and Adaptations

The existing research, shows a link between heat stress and a decline in mental health, evident through the emergence of depressive symptoms and post-traumatic stress disorder ³³⁵. Heat stress is associated with the onset of various mental health issues including anxiety disorders, behavior akin to anxiety, increased suicide rates, episodes of mania and depression, as well as schizophrenia ³³⁶. Experiments involving humans have demonstrated that heat stress can alter metabolic levels and CNS activity ³³⁷.

Aversive sensory induces unpleasant emotions, which cause avoidance behavior to move from subjected environment/context. The initial behavior of mice exposed to heat is looking for cooler places and avoiding direct radiance. Incidentally or relatively, mice maintain this behavior until Tc 38-39°C. Behavioral responses of superficial hyperthermia is observed in more active states, whereas deep hyperthermia induces passive laying down with extended posture ³³⁸. Postural corrections are necessary to regulate heat loss via heat radiance, convection and evaporation ³³⁹. Higher and lethal heat load conducts even more intense avoidance as jumping behavior ³⁴⁰. During repeated exposure, coping with stress and motivational process may be affected due to adaptational process, inflammatory surge, or Hypothalamus-Pituitary-Adrenal (HPA) axis induction.

Sensorial sensitivity is also a determinant of behavioral responses. TRP channels play a crucial role in cellular processes that are involved in the production of various inflammatory mediators linked to both neuroprotection and neurotoxicity. They are instrumental in the regulation and signaling of intracellular calcium and the transmission of painful stimuli. The expression of these channels varies, and their recurrent or extended activation can alter their responsiveness under conditions of stress. TRPV1 signaling within the peripheral nervous system acts to continuously inhibit overall locomotor activities, potentially aiding in the reduction of body temperature. Repeated activation triggers adaptational processes. TRPV1 desensitization depends on intracellular Ca²⁺ and CaM like TRPV4, intracellular ATP sensitizes TRPV1 ^{341,342}. Besides that, TRPV3 has a different nature. TRPV3 currents are increased by prolonged or repetitive stimulation and ATP prevents TRPV3 sensitization ³⁴³. Therefore, increased TRPV3 activity led to Prostaglandin 2 (PGE₂) and Nerve Growth Factor NGF release which increases TRPV1 sensitivity and may lead to earlier initiation of central regulatory behaviors ³⁴⁴. In the dorsal root ganglion, release of pain modulators as SP, CGRP. Amplificated sensory stimuli induce Corticotrophin releasing factor induction and

activate the HPA axis. Corticotrophin Releasing Factor (CRF) also has a modulatory role on Dorsal Raphe Nucleus (DRN) stress related locomotion.

The medial septum (MS) is implicated in facilitating aversion responses to both auditory and somatosensory stimuli, as identified in research by Zhang et al.³⁴⁵. Optogenetic activation of the two cell types of GABA-aminobutyric acid (GABA) and Glutamatergic, results in preference and place avoidance, respectively. The lateral habenula (LHb) plays a key role in triggering aversive responses. This effect can be counteracted by GABAergic projections from the MS to the LHb. These projections directly connect to midbrain GABAergic neurons and indirectly inhibit dopaminergic cells³⁴⁶. The sensory-induced place avoidance is facilitated by enhanced locomotion mediated by glutamatergic MS projections to the POA. Hence skin sensory transmission may enhance locomotion via glutamatergic pathway to POA and modulate initial cold seeking behavior and avoidance from heat³⁴⁵. Therefore, repeated heat stress and central temperature changes may demolish this effect by inhibiting POA related locomotor activity.

The caudate putamen (CPu) plays a crucial role in regulating behavior and locomotor activity, with its functioning being significantly affected by the activity of dopamine receptors³⁴⁷. After 7 days of 32°C 3h heat chamber, rats are shown decreased dopamine levels in CPu. Frontal Cortex (FC), POA and DMH 5-Hydroxytryptophane/serotonin (5-HT) levels are also decreased. Reduced 5-HT and enhanced dopaminergic system in the FC are related to anxiety-like behaviors. Repeated application of heat stress may induce anxiety-like behaviors and decreased locomotion during stressful environment whereas single heat stress increase activity response to novel environment³⁴⁸. During heat exposure, 5-HT neurotransmission plays a significant role. When subjected to a warm environment, there was an observed increase in body temperature and an elevated expression of c-Fos in spatially distinct groups of 5-HT within the DRN. Therefore, due to POA inhibition of raphe pallidus decreased 5-HTergic activity which modulates sympatholytic activity on peripheral arterial beds³⁴⁹.

Repeated exposure to heat stress may alter the brain's monoaminergic system. It is known that high dopaminergic activity is involved in hyper-locomotor activity in rodents³⁵⁰. In medaka fish, 32 °C ambient temperature in 30 minutes can increase locomotion and induce anxiety-like behaviors. Only repeated application decreased translation of serotonin rate limiting enzyme tryptophan hydroxylase 1 and 2 (tph1 and

2) and dopamine biosynthesis catalyzer tyrosine hydroxylase 2 (th2) in brain ³⁵¹. Which are highly initiated with anxiety-like phenotype and decreased locomotor activity. In human subjects, 1,5 h with T_{end} skin temperature 39°C led to increased cortisol and HSP70 levels in serum and decreased GABA and Brain-derived Neurotrophic Factor (BDNF). GABA and BDNF levels were negatively correlated with anxiety. Heat exposure additionally increased TNF- α levels with dose-dependent manner ³⁴⁸. Furthermore, ROS accumulation and inflammatory surge actively participate in the stress response ³⁵². TRPM2 has wide range temperature action, hence the major ligand is ADP-ribose. ADP-ribose is produced during low energy states with ROS accumulation, and DNA damage which is strongly related to arousal state. During high central temperatures, POA TRPM2 receptors, when activated, relay information to Paraventricular Hypothalamus neurons and induce Corticotrophin Releasing Hormone release but not Thyrotropin Releasing Hormone. The main effect also may be conducted by HPA-axis ³⁵³. When faced with a new, unexpected, or threatening situation, commonly referred to as a stressor, the HPA axis is stimulated, leading to the secretion of GCs ³⁵⁴. Similarly, exposure to heat stress elevates cortisol levels through HPA axis activation, which in turn can induce behaviors resembling anxiety ³⁵⁵. GCs play a role in the body's immediate adaptive response to perceived threats, which includes increasing blood glucose levels, enhancing cardiovascular tone, and suspending non-essential functions to prioritize short-term survival ³⁵⁶. Therefore, repeated stress and ROS related activation of HPA axis may induce anxiety-like behavior and decreased locomotion ³⁵⁷.

Extensive research has established that a variety of compounds possess potential antidepressant attributes, which may be attributed to their influence on ER stress ³⁵⁸. Persistent administration of luteolin has been observed to generate antidepressant-like impacts, primarily by counteracting apoptosis provoked by hippocampal ER stress ³⁵⁹. This action consequently amplifies the expression of genes responsible for producing GRP78 and GRP94. In a different mechanism, longistylone C demonstrates its antidepressant qualities by attenuating the concentrations of GRP78, CHOP, and XBP1, facilitating the alleviation of ER stress ³⁶⁰. Panax ginseng exhibits antidepressant properties in both acute and sustained stress-induced depression models. The foundational molecular mechanism for this effect appears to be rooted in the downregulation of specific ER stress-associated proteins, notably CHOP, GRP78, XBP1, and caspase-12 ³⁶¹.

3. MATERIALS and METHODS

3.1. Experimental Setup and Animal Husbandry

Forty Adult male Swiss mice (36,4±2,8 grams) were provided by Yeditepe University Medical School Experimental Research Center (YÜDETAM) Laboratories. The mice were randomized and allocated into five groups with the following distribution: Control (n=8, con-E: n=4; con-ER: n=4), 39°C for 15 minutes over 7 days (39E, n=8), 41°C for 15 minutes over 7 days (41E, n=8), 39°C for 15 minutes over 7 days followed by a 7-day recovery (39ER, n=8), and 41°C for 15 minutes over 7 days followed by a 7-day recovery (41ER, n=8). Prior to experiments, the mice were acclimatized for a week in a controlled environment (12-hour light/dark cycle, 22 ± 1°C, and 50-60% humidity). During this period, they were provided with ad libitum access to food and water (**Figure 3**).

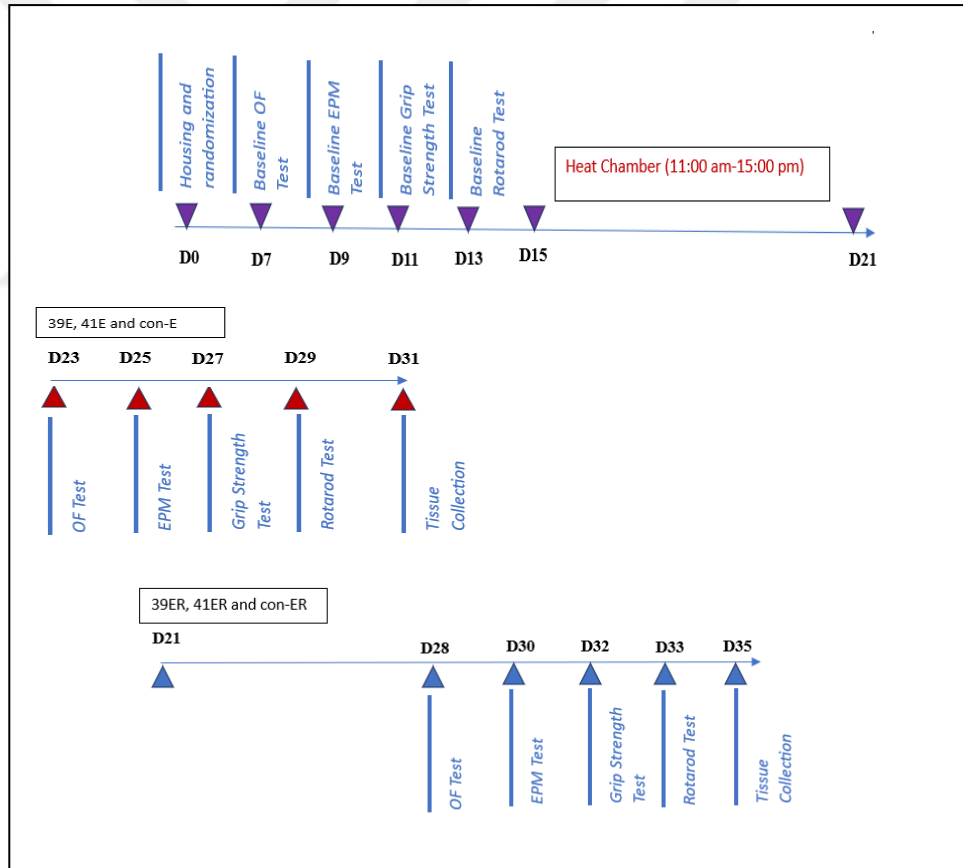


Figure 3. Timeline of Experimental Design.

3.2. Heat Chamber and Heat Exposure

Mice from the hyperthermia groups were placed daily in a plexiglass box surrounded by a heat insulator, under the direction of an infrared heat lamp. The inner

temperature of the box is monitored with a digital thermometer. T_c temperature is evaluated by rectal thermometer for every 5 min. Heat load and starting temperature determined based on previous studies on hyperthermia and heat shock models using heat chamber ³⁶².

Hyperthermia exposures occurred every day between 11:00-15:00, with temperatures set at either 39°C or 41°C, depending on the group. The control group was maintained at room temperature ($22\pm1^\circ\text{C}$).

To ensure the desired exposure temperature was reached and maintained, the temperature was monitored rectally using a thermometer every 5 minutes. This ensured that the hyperthermia groups were exposed to their target temperatures (39°C and 41°C) for the full 15 minutes (**Table 2**). Following the daily heat exposure, all mice were returned to a room temperature environment.

Table 2. Parameters for Achieving and Maintaining Target Temperatures in the Heat Chamber.

Target T_c Temperature (°C)	Inner box initial temperature (°C)	Time to Reach Target T_c Temperature (min)	Target Temperature Maintenance Time (min)
39°C	36°C	5 min	15 min
41°C	38°C	10 min	15 min

3.2.1 Post-Exposure Period

After the 7-day hyperthermia exposure, mice from the 39ER and 41ER and the con-ER groups were given a recovery period of 7 days at room temperature, with continued ad libitum access to food and water.

3.3. Motor Function and Behavioral Evaluation

All behavioral tests were conducted in YÜDETAM Laboratories. Before behavioral evaluation, mice acclimatized for seven days and were given handling treatment to minimize handling stress.

3.3.1. Open Field Test

The Open Field Test (OFT) evaluated the movements and anxiety-like behavior mice by measuring center activity to assess response to a new environment. 1) Frequency of central area entries and 2) duration are used for an indicative measurement of anxiety-related behavior which is explained as aversion to open spaces it is seen as the animal spending more time in the enclosed arms of the maze and 3) total distance traveled for locomotor activity³⁶³. Evaluations were carried out in a plexi box which is provide an arena 80×80 cm² and 40 cm walls to prevent escape and influence from external environment. The testing area is divided into 16 equal squares to create an outer and central zone. The central zone selected with software as 40x40 cm² (**Figure 4**). The equipment was wiped with a 70% alcohol and water solution before every trial. Movements were recorded and analyzed for 10 minutes by the Noldus Ethovision software.

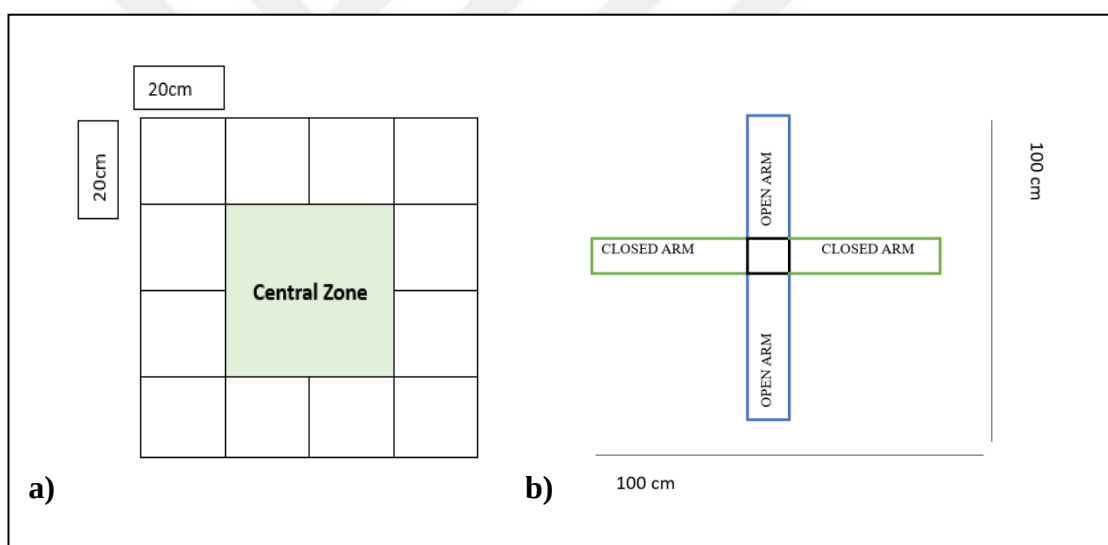


Figure 4. Zone distribution of Open Field (a) and Elevated Plus Maze Test (b)

Mice brought in their home cages from their housing room into the testing room and allowed to be acclimatized for 30 minutes before testing. Camera tracking started immediately after animal placement to the center of the box. Data among different groups were analyzed using two-way ANOVA with Tukey's post-hoc analysis. GraphPad Prism 8.0 is used for statistical evaluation. Comparisons were carried out with baseline and after tests and control groups data.

3.3.2. Elevated Plus Maze Test

The Elevated Plus Maze (EPM) has been described as a simple method for assessing anxiety-related behavior ³⁶⁴. EPM is constructed with four arms that extend from a central platform, giving it a plus-shaped appearance (as depicted in **Figure 4**). Two diagonally opposed arms are enclosed with walls, leaving only the entrance, exit, and ceiling open. The other two diagonally opposed arms are open, having no walls but just the base platform ³⁶⁵. The EPM test exploits the natural aversion of rodents to open and elevated areas, which is balanced against their intrinsic drive to explore unfamiliar territories. A less anxious rodent is expected to venture more into the open arms, while a more anxious individual will predominantly stay in the enclosed arms ³⁶⁶.

Zones are determined via Ethovision software to open arms, closed arms and center. 1) Open Arm Entries, 2) Time spent in Open Arm 3) Closed Arm Entries, 4) Time spent in Closed Arm data is recorded with camera tracking. The equipment was wiped with a 70% alcohol and water solution between every trial.

Mice brought in their home cages from their housing room into the testing room and allowed to be acclimatized for 30 minutes before testing. Camera tracking started immediately after animal placement to the center of the box and recorded for 5 min. Data among different groups were analyzed using two-way ANOVA with Tukey's post-hoc analysis. GraphPad Prism 8.0 is used for statistical evaluation. Comparisons were carried out with baseline and after tests and control groups data.

3.3.3 Grip Strength Test

The grip strength test is widely used in neurobiology and pharmacology to evaluate muscular strength and neuromuscular function in mice, particularly in the context of neuromuscular diseases, drug treatments, or genetic modifications ^{367,368}.

The apparatus was cleaned and sanitized to remove any scent that might deter the mice. The mouse was gently removed from its home cage by gripping the middle/base of its tail. For forelimb measurements, the mouse was lowered over the grid, ensuring its torso remained horizontal and allowing only its forepaws to touch the grid. The mouse was then pulled gently backward until it released its grip. The maximal force exerted by the mouse before releasing its grip was recorded. This procedure was repeated a total of five times with 10 minutes rest after 3 replies for each mouse ³⁶⁹. The average and maximum grip strength (from the five measurements) for each mouse was calculated and

normalized with body weights. Data among different groups were analyzed using two-way ANOVA with Tukey's post-hoc analysis. GraphPad Prism 8.0 is used for statistical evaluation. Comparisons were carried out with baseline and after tests and control groups data.

3.3.4. Rotarod Test

Mice brought in their home cages from their housing room into the testing room and allowed to be acclimatized for 30 minutes before testing. Harvard Apparatus Rotarod was checked to ensure cleanliness and proper function. Mice were placed on a non-moving rod for 1-2 minutes for familiarization. Rotation was introduced gradually at slow speeds to adapt mice to the motion.

3.3.4.1. Training Phase

Rotarod training and testing protocol is selected for motor coordination and balance evaluation ³⁷⁰. The rodent was placed on the rod that started at a slow rotation speed (4 rpm). The latency for the rodent to fall was recorded. The speed increased gradually. 4-10 rpm 1 min achieved in 2 days. Each rodent underwent 4 trials/day, with resting periods of 15 minutes between trials to prevent fatigue. Training was conducted over 3 consecutive days until consistent performance was achieved.

3.3.4.2. Test Procedure

After 48 hours of resting, mice brought in their home cages from their housing room into the testing room and allowed to be acclimatized for 30 minutes before testing. Rotarod is cleaned and dried between every trial with %70 ethanol and water solution. Each trial session was conducted with three mice, with intervals between rod placements. Care was taken to ensure that each mouse used the same rod throughout all experiments. The rods started at 4 rpm and mice were placed. Accelerating rod set for 4-40 rpm in 10 minutes. After placement of all mice, trial was started immediately. 1) Latency to fall and 2) Speed at fall were noted. The mouse that fell was promptly removed from the vicinity of the rod. Three trials were replied with 10-minute breaks.

Latencies to fall and speed among different groups were analyzed using two-way ANOVA with Tukey's post-hoc analysis. Comparisons were carried out with baseline and after tests and control groups data. GraphPad Prism 8.0 is used for statistical evaluation.

3.4. Isolation of mRNA from Muscle Tissue

3.4.1. Cardiac Exsanguination and Transcardial Perfusion

Adult mice were anesthetized with isoflurane via inhalation before and during the process. Mice were monitored for depth of anesthesia, ensuring no response to toe pinch before initiating the exsanguination procedure. Once the mice were confirmed to be deeply anesthetized, a midline thoracotomy was performed to expose the heart. An injector (26G, 1 ml) was inserted into the left ventricle, and blood was drawn until complete exsanguination was achieved. Collected blood transferred to 1.5 µL Eppendorf tube. Immediately following exsanguination, mice were perfused transcardially with ice-cold 0.9% saline solution (physiological saline) until clear fluid was observed exiting from the right atrium, indicating that the circulatory system was cleared of blood.

3.4.2. Tissue Collection

Gastrocnemius Muscle Collection: The gastrocnemius muscle from one leg was carefully dissected, quickly stored at -80°C for future biochemical assays.

Brain and Contralateral Gastrocnemius Muscle Collection: After the first muscle collection, mice were further perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS) solution, pH 7.4, for tissue fixation. Subsequently, the gastrocnemius muscle from the contralateral leg and the brain were dissected.

Tissue Fixation and Storage: The dissected brain and contralateral gastrocnemius muscle were post-fixed in 4% PFA at room temperature for 24 hours. After fixation, tissues were stored at 4°C until further analysis.

3.4.3. Blood Serum Collection

Collected blood samples are left at room temperature for 2 hours for clotting. Centrifuged 15 minutes at 2,000 x g. Supernatant collected with pipetting to sterile Eppendorf tube, stored at -80 °C until analysis.

3.5. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) Analysis

3.5.1 RNA Isolation from Muscle Tissue

Isolation protocol is conducted with manufacturer's protocol (Zymo Direct-zol™ RNA MiniPrep Plus Kits).

A 10 mg of muscle tissue are rinsed with saline solution to prevent contamination and placed in a 2 mL eppendorf tubes. 600 µL TRIzol solution with metal beads were added. Placed in homogenizator (Bullet Blender® Storm 24, NextAdvance) for 5 minutes in low speed after tubes are fixed with parafilm. 600 µL (equal volume) absolute (%100) ethanol was added to Cell lysate and transferred to Zymo-Spin™ IIICG Column2 in a Collection Tube and centrifuged at 10.000 x g for 30 seconds. Columns were transferred to a new tube washed with (400 µL) RNA wash buffer and treated with DNAase and Digestion Buffer mixture (80 µL). Incubated 15 min at room temperature. Columns were washed with Pre-Wash and RNA Wash buffer (x2), RNA is eluted with DNAase/RNAase free water. Isolated RNAs were assessed for quantity and quality with NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, ND-2000). Total concentrations were equalized with dilution.

Reverse transcription was performed on 60 ng / 15µL of total RNA with 20 µL reaction volume using iScript™ cDNA Synthesis Kit to synthesize complementary DNA (cDNA).

Table 3. List of primer sequences used for qPCR analysis.

Genes	Primer Sequences (5'-3')	Accession Number
β-actin	F: AGCCTCTTTGCCCAGATCTT R: GGCAATCCGTCTTCATCCAC	NM_177838.3
PGC-1α	F: AGCCTCTTTGCCCAGATCTT R: GGCAATCCGTCTTCATCCAC	NM_008904.3
PGC-1β	F: CCGTATTTGAGGACAGCAGC R: CCTCTGCTCTCACGTCTGAA	NM_133249.3

Tfam	F: CGGGGAATGTGGAGCGTG R: CTCCACAGGGCTGCAATTTT	NM_009360.4
Nrf1	F: GTCCGCACAGAAGAGCAAAA R: CTACTGTTGCCCCTGTACCA	BC005410.1
Mfn1	F: ACAAGCTTGCTGTCATTGGG R: TCGACACTCAGGAAGCAGTT	NM_024200.5
Mfn2	F: CCCTGCTCTTTTCTCGATGC R: AGGTACCCTTTGACGTCCAG	NM_001285922.1
Map1lc3a (LC3)	F: GTTGGTCAAGATCATCCGGC R: AAGGTTTCTTGGGAGGCGTA	NM_025735.3
SQSTM1 (P62)	F: GGACAGCCAGAGGAACAGAT R: GGGAGATGTGGGTATAGGGC	NM_011018.3
Pink1	F: GCTGGAGAGTATGGAGCAGT R: TAACGAGGAACAGTGTGCGA	NM_026880.2
Parkin	F: CCTCAGAGGGCTCCATATGG R: ACTGAACTCGGAGCTTTCCA	AF250294.1
Ucp2	F: TTGGCCTCTACGACTCTGTC R: TCCTCTCGTGCAATGGTCTT	BC012697.2
F: Forward, R: Reverse		

Primers for the target genes (*β-actin*, *PGC-1α*, *PGC-1β*, *Tfam*, *Nrf1*, *Mfn1*, *Mfn2*, *LC3*, *P62/SQSTM1*, *Pink1*, *Parkin*, *Ucp2*) were used for the qPCR reactions, a master mix was prepared using the reconstituted primers, SsoAdvanced™ Universal SYBR® Green Supermix and the cDNA template in 10 µl reaction volume. Primer sequences and access numbers are shown in **Table 3**. Experiment was run by using a InstaQ96 device (HiMedia, India). After the qPCR run, the cycle threshold (Ct) values for each sample

were determined using the instrument's software. Relative expression of the target genes was calculated using the $2^{-\Delta\Delta C_t}$ method, with housekeeping gene (β -actin) as references.

3.5.2. RT-qPCR Analysis

Customized temperatures and durations are specified for each gene group. The initial incubation stage is set at 95°C for 30 seconds for all gene groups. Stage 1 represents the denaturation phase for each cycle, while Stage2 and Stage3 represent the annealing and extension phases, respectively (**Table 4**).

Table 4. Thermal cycling conditions according to the qPCR amplification protocol.

GENES	Initial incubation	Stage1	Stage2	Stage3
		x 45 cycles		
<i>P62, Parkin, LC3</i>	95 °C 30 sec x1 cycle	95 °C / 15 sec	51°C /20 sec	60°C / 20 sec
<i>Pgc-1α, Pgc-1β, Nrf1, Tfam, Ucp2</i>	95 °C 30 sec x1 cycle	95 °C / 15 sec	56 °C /20 sec	60°C / 20 sec
<i>Mfn1, Mfn2, Pink1</i>	95 °C 30 sec x1 cycle	95 °C / 15 sec	50 °C /20 sec	60 °C / 20 sec

3.5.3 Data Analysis

The table specifies forward (F) and reverse (R) primer sequences for each gene of interest. All primer sequences are presented in the 5' to 3' orientations. β -actin is used as the reference gene for normalization purposes in the qPCR analysis.

qPCR data were analyzed using the $2^{-\Delta\Delta C_t}$ method, a standard approach for relative quantification of gene expression. The $2^{-\Delta\Delta C_t}$ method allows for the normalization of target gene expression to a reference gene and further normalization to a control sample or condition.

$$\Delta C_t = C_t (\text{target gene}) - C_t (\text{reference gene})$$

$$\Delta\Delta C_t = \Delta C_t (\text{experimental sample}) - \Delta C_t (\text{control sample})$$

By raising 2 to the power of the negative $\Delta\Delta C_t$ value, the relative fold change in gene expression between the experimental and control samples can be determined.

$$\text{Relative Expression} = 2^{-\Delta\Delta C_t}$$

3.6. Statistical Analysis

Statistical analyses conducted with a significance level set at $p < 0.05$. Data were initiated with the Shapiro-Wilk Test to assess data distribution and Bartlett's Test to determine heteroscedasticity. When deviations from normality or homogeneity of variance were detected, data were adjusted using the ROUT method. Based on the results from the Shapiro-Wilk and Bartlett's Tests, statistical evaluations were conducted using one-way ANOVA, Kruskal Wallis, Brown-Forsythe, or Welch's ANOVA. Post-hoc analyses for one-way ANOVA employed Tukey's test, while Games-Howell's test was utilized for both Welch's ANOVA and Brown-Forsythe. For Kruskal Wallis, Dunn's multiple comparisons analysis was chosen to align with data conditions. GraphPad Prism 8.0 was used for statistical evaluation.

4. RESULTS

4.1. Motor Function and Behavior

4.1.1. Open Field Test

A mixed effect ANOVA was performed to analyze within-group and between groups effects of heat treatment to determine heat and temperature impact on locomotion and anxiety-like behavior (**Figure 5**). In **Exposure** groups, Open Field center frequency (OPCF), there was no significant effect of either the heat treatment ($F(1, 7) = 1,054$, $p=0.3387$) or temperature ($F(2, 14) = 0,2278$, $p=0.7992$). Furthermore, their interaction was also not significant ($F(2, 10) = 1,396$, $p=0.2920$) (**Figure 5a**).

Regarding the Open Field center duration (OPCD), the heat treatment effect was not significant ($F(1,000, 7,000) = 0,1654$, $p=0.6964$), and neither was the temperature effect ($F(1,699, 11,89) = 1,170$, $p=0.3348$) (**Figure 5b**).

Open Field total distance moved (OPTDM) data analysis revealed a significant effect of heat treatment within groups ($F(1,000, 7,000) = 15,45$, $p=0.0057$). The between group variance differences showed a significant temperature effect ($F(1,030, 7,213) = 7,371$, $p=0.0286$). Importantly, the interaction of heat x temperature factors was significant ($F(1,063, 5,316) = 7,030$, $p=0.0417$). Tukey's post-hoc analysis for within groups indicated a significant decrease in 39E group ($p=0.0458$). Between-group comparisons revealed no significant difference 39E compared to control ($p=0.9304$). However, significant differences were found between 41E compared to control ($p=0.0228$) and between 39E and 41E groups ($p=0.0312$) (**Figure 5c**).

In the **Exposure+Recovery** groups, there was a significant interaction of heat x temperature on the OPCF ($F(1.243, 6.216) = 7.467$, $p= 0.0292$). However, neither the within group effect ($F(1.000, 7.000) = 0.9793$, $p=0.3553$) nor the between group ($F(1.533, 10.73) = 0.5905$, $p=0.5284$) had a significance on their own. Tukey's post hoc analysis within groups revealed 39ER decreased significantly ($p=0,0351$). For the between-groups comparisons, none of the groups showed any significant differences (**Figure 5d**).

There was a notable interaction between within groups and between groups in the OPCD ($F(1.703, 7.664) = 12.11$, $p= 0.0050$). Heat treatment was not significant on its own ($F(1.000, 7.000) = 2.461$, $p=0.1607$), while the temperature effect approached significance ($F(1.974, 13.82) = 3.560$, $p=0.0571$). Tukey's between-groups analysis

revealed that only the comparison between 39ER and 41ER was significant ($p=0.0136$), with no significant differences found between 39ER ($p=0.4398$) and 41ER ($p=0.8939$) compared to control (**Figure 5e**).

Furthermore, a significant interaction was also observed between the within x between group analysis ($F(1.792, 34.04) = 12.19, p=0.0002$) in OPDM data. Both the within groups effect ($F(1.000, 38.00) = 34.97, p<0.0001$) and the between groups effect ($F(1.648, 31.31) = 12.43, p=0.0002$) showed significant differences. In Tukey's post-hoc analysis within groups, a significant decrease was identified in 39ER group ($p=0.0048$). Between groups, significant differences were found between 39ER and 41ER ($p=0.0149$) and 41ER compared to control ($p=0.0213$). However, the comparison between 39ER vs. con-ER was not significant (Mean Diff = 764.6, $p=0.9838$) (**Figure 5f**).

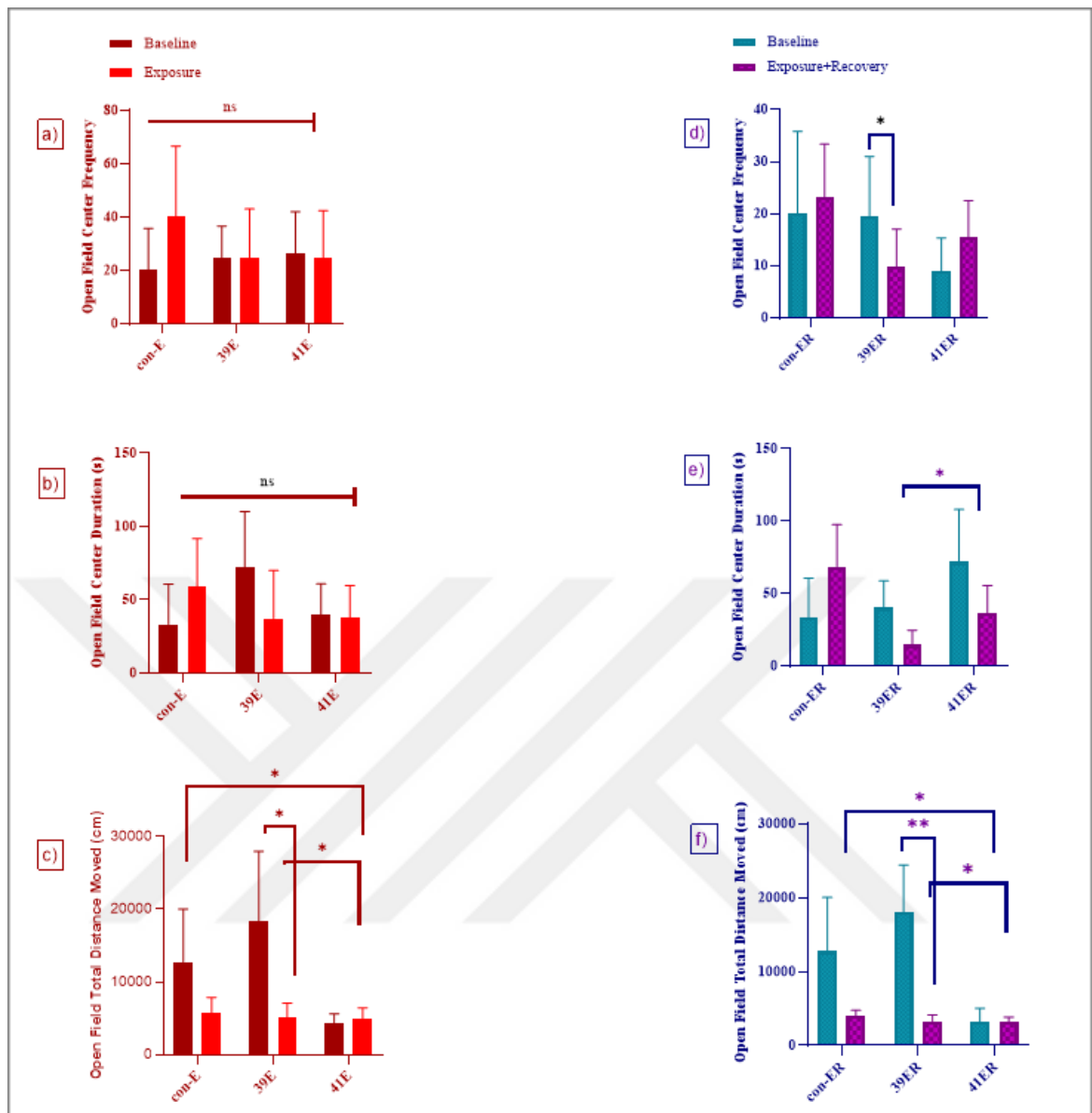


Figure 5. Open Field test total 5 minutes, **a, d)** Center Duration, **b, e)** Total Distance Moved, **c, f)** Center Frequency in Exposure (con-E, 39E,41E) and Exposure+Recovery (con-ER, 39ER, 41ER) Groups. Statistical evaluations were conducted using mixed-effect ANOVA with Geisser-Gaussian correction and post-hoc analysis was performed with Tukey's multiple comparisons test. n.s not significant, $p > 0.05$, * $p > 0.05$, ** $p < 0.01$. Data are represented as Mean+SD.

4.1.2. Elevated Plus Maze Test

A mixed effects ANOVA was performed to analyze between and within-group effects of heat treatment on EPM to reveal anxiety-like behavior. It is shown that there was a significant interaction between heat x temperature on EPM open arm frequency (OAF) ($F(1.073, 5,364) = [8.745]$, [$p = 0.5364$]) (**Figure 6a**) and whereas there were no

significant interaction or variable effect in EPM open arm duration ($F(1.823, 9.117) = 2.622$; $p=0.9117$) (**Figure 6b**) and EPM closed arm duration ($F(1.758, 8.792) = 2.461$; $p=0.8792$) (**Figure 6c**) in **Exposure groups**. Fixed effects analysis showed that heat treatment has a significant effect on EPM OAF ($p=0.0246$), Tukey's post-hoc between groups analysis revealed a significant difference in OAF on 39E group compared to the control ($p=0.0139$) and no significant effect on 41E compared to the control ($p=0.0645$) (**Figure 6d**).

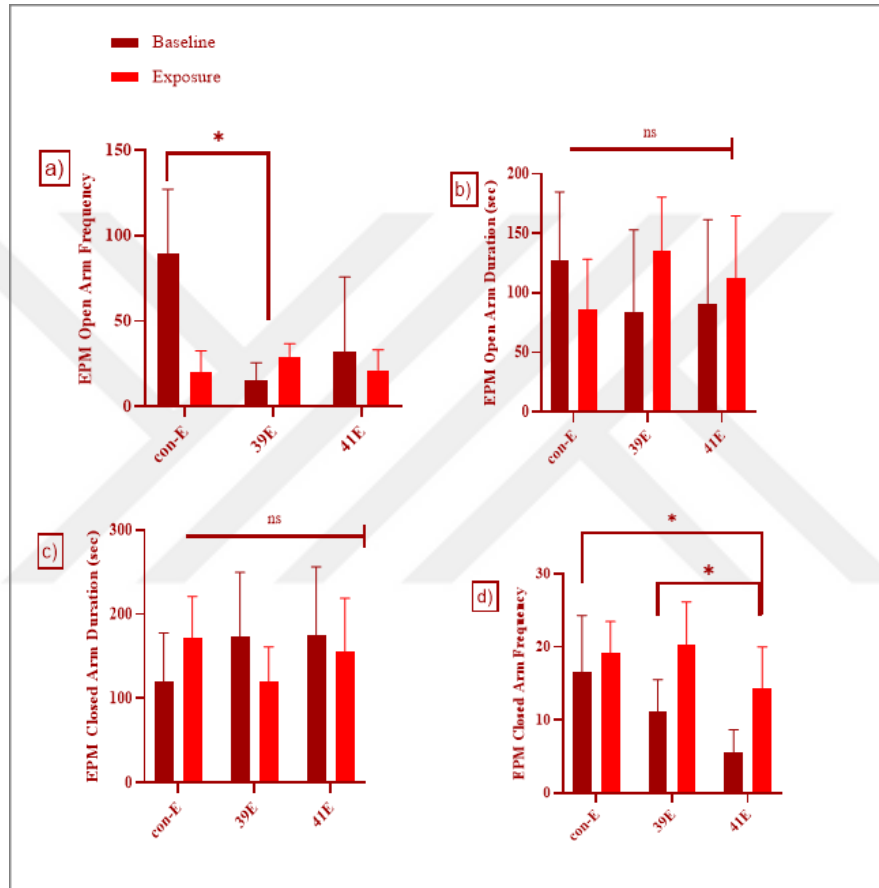


Figure 6. Elevated Plus Maze Test **a, e**) Open Arm Entries; **b, f**) Open Arm Duration; **c, g**) Closed Arm Entries; **d, h**) Closed Arm Entries in Exposure Groups. Statistical evaluations were conducted using mixed-effect ANOVA with Geisser-Gaussian correction and post-hoc analysis was performed with Tukey's multiple comparisons tests. n.s not significant, $p>0.05$, * $p>0.05$, ** $p<0.01$. Data are represented as Mean+SD.

A mixed effects ANOVA was performed to analyze between and within-group effects of heat treatment on EPM to reveal anxiety-like behavior. It is shown that there was a significant interaction between heat x temperature on EPM open arm frequency (OAF) ($F(1.073, 5,364) = [8.745]$, [$p= 0.5364$]) (**Figure 7a**) and whereas there were no

significant interaction or variable effect in EPM open arm duration ($F(1.823, 9.117) = 2.622$; $p=0.9117$) (**Figure 7b**) and EPM closed arm duration ($F(1.758, 8.792) = 2.461$; $p=0.8792$) (**Figure 7c**) in **Exposure groups**. Fixed effects analysis showed that heat treatment has a significant effect on EPM OAF ($p=0.0246$), Tukey's post-hoc between groups analysis revealed a significant difference in OAF on 39E group compared to the control ($p=0.0139$) and no significant effect on 41E compared to the control ($p=0.0645$) (**Figure 7a**).

Furthermore, there was no significant interaction between heat x temperature in EPM closed arm frequency (CAF) ($F(1,480, 7,402) = 1,273$; $p= 0,3192$) (in fixed effect analysis, a statistically significant heat factor ($F(1,000, 7,000) = 16.00$; $p= 0,0052$) and temperature factor ($F(1,329, 9,301) = 7,778$; $p= 0,0157$) are shown. Tukey's post-hoc analysis revealed 41E significantly differed from 39E and the control ($p=0,0283$, $p=0,0208$; respectively) (**Figure 7d**).

In **Exposure+Recovery** groups, it is shown that there was a significant between and within groups interaction ($F(1,415, 7,073) = 7,777$; $p= 0,0211$) in OAF (**Figure 7a**) and no significant interaction CAD (**Figure 7b**) or CAF (**Figure 7d**). OAF is significantly affected by heat+recovery ($F(1,000, 7,000) = 31,09$, $p=0,0246$) and temperature ($F(1,723, 12,06) = 10,42$, $p=0.0430$) factors. Tukey's post-hoc test revealed that the 39ER and 41ER groups significantly differs from control ($p= 0.0025$, $p= 0.0190$; respectively) (**Figure 7a**). OAD is also shown to be significantly affected by temperature factor ($F(2, 14) = 4,090$, $p= 0.0399$). Tukey's post-hoc analysis showed, 39ER significantly differed from control ($p=0,0329$) (**Figure 7c**).

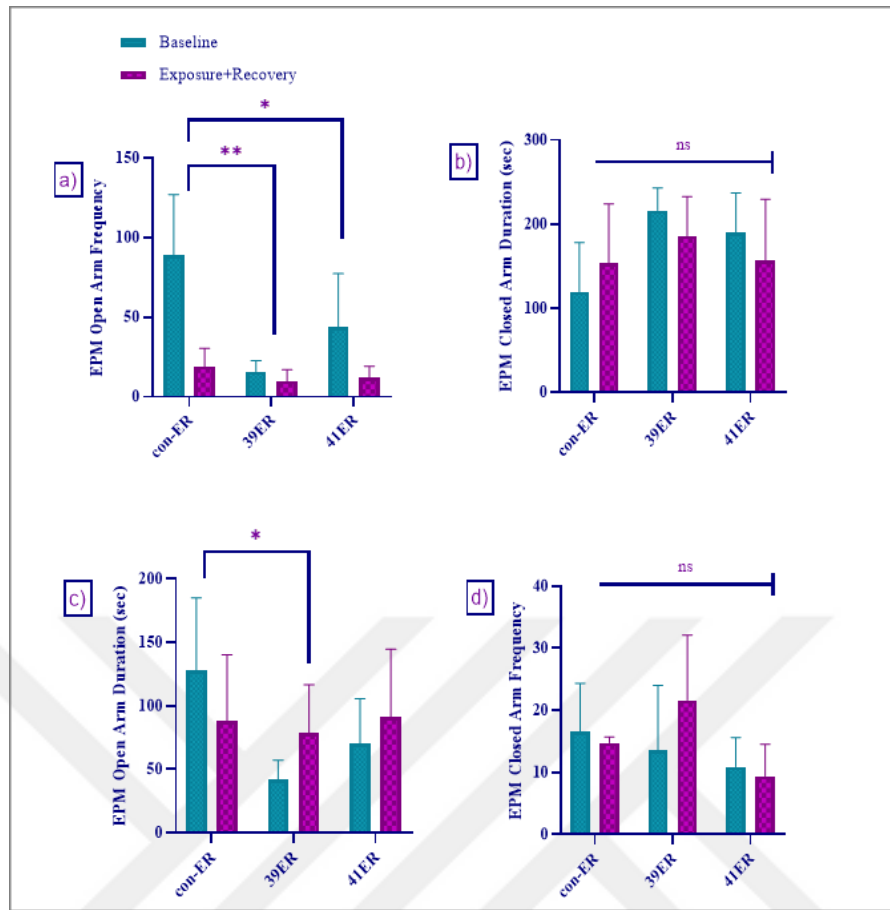


Figure 7. Elevated Plus Maze Test **e)** Open Arm Entries; **f)** Open Arm Duration; **g)** Closed Arm Entries; **h)** Closed Arm Entries in Exposure+Recovery Groups. Statistical evaluations were conducted using mixed-effect ANOVA with Geisser-Gaussian correction and post-hoc analysis was performed with Tukey's multiple comparisons tests. n.s not significant, $p > 0.05$, $*p > 0.05$, $**p < 0.01$. Data are represented as Mean+SD.

4.1.3. Rotarod test

A mixed effect ANOVA was performed to analyze the effect of heat treatment on Rotarod Test to reveal balance and endurance deficits. Speed in **Exposure** groups, there was no significant interaction between heat x temperature ($F(1,203, 3,608) = 1,002$, $p = 0,3994$) and no significant effect of heat ($F(1,000, 7,000) = 0,06319$, $p = 0,8087$) or temperature ($F(1,294, 9,060) = 0,251$, $p = 0,6883$) individually (**Figure 8a**). Neither in latency to fall, there was no significance in heat x temperature interaction ($F(1,167, 3,501) = 0,9268$, $p = 0,4158$), heat ($F(1,000, 7,000) = 0,05492$, $p = 0,8214$) or temperature ($F(1,232, 8,621) = 0,3016$, $p = 0,6434$) (**Figure 8b**).

In **Exposure+Recovery** groups, there were no significance in heat x temperature interaction in speed ($F(1,551, 4,652) = 2,143, p = 0,2162$), neither in heat $F(1,000, 7,000) = 1,542, p = 0,2544$ nor in temperature ($F(0,7027, 4,919) = 1,011, p = 0,3236$) factors (**Figure 8c**). Additionally, latency to fall data have no significance on heat x temperature interaction ($F(1,581, 4,743) = 0,02291, p = 0,9568$), heat ($F(1,000, 7,000) = 0,03226, p = 0,8625$), or temperature ($F(1,359, 9,511) = 0,5375, p = 0,5345$) factors (**Figure 8d**).

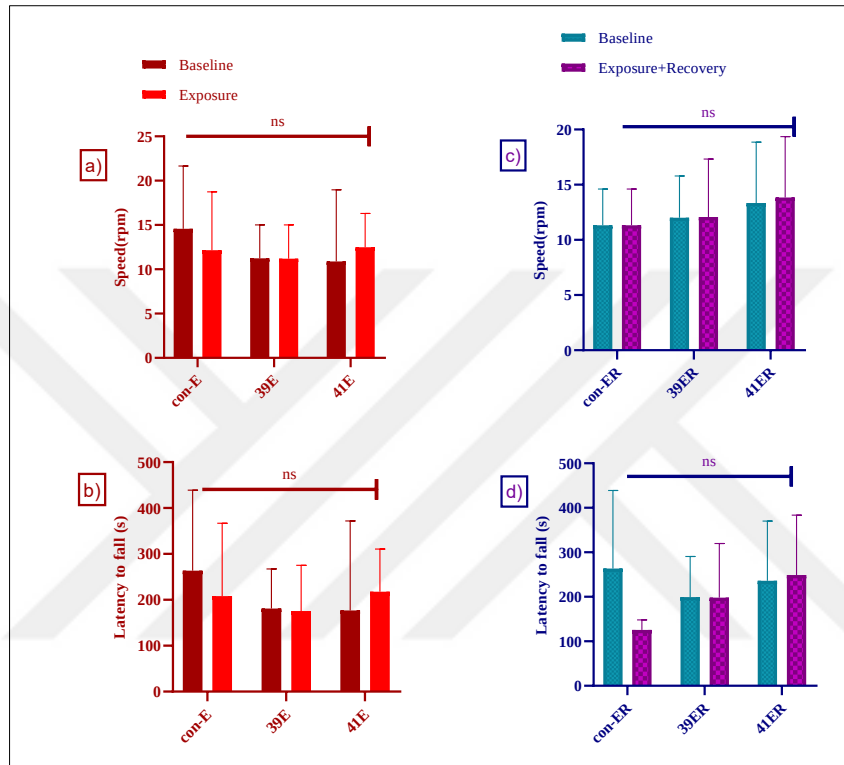


Figure 8. Rotarod Test **a, c)** Mean rotational velocity at the time of falling or speed (rotation per minute, rpm); **b, d)** Mean value of the time (seconds) that mice stayed on the rotarod when tested accelerating rod between 4 to 40 rpm in 10 min in Exposure and Exposure+Recovery groups. Statistical evaluations were conducted using mixed-effect ANOVA with Geisser-Gaussian correction and post-hoc analysis was performed with Tukey's multiple comparisons test. n.s not significant. Data are represented as Mean+SD.

4.1.4. Grip Strength Test

A mixed effect ANOVA was performed to analyze the effect of heat treatment on Grip Strength to reveal voluntary muscle force deficits. Statistics are conducted with

maximum value and mean value normalized with body weights. In **Exposure** groups, there was a significant interaction between heat x temperature effects on Grip Strength mean value/body weight (N/g) ($F(1,255, 6,274) = 5,598, p = 0,0494$). Hence there was no significant effect is determined in heat or temperature factor effects individually ($F(1,000, 7,000) = 1,010, p = 0,3483$; $F(1,376, 9,630) = 1,987, p = 0,1920$, respectively). Tukey's post hoc analysis within groups showed significant decrease in 41E group ($p = 0,0008$) (**Figure 9a**).

Additionally, there were no significant effects indicated in Grip Strength Max Force/body weight (N/g), within or between groups or interaction of effects ($F(1,000, 7,000) = 3,055, p = 0,1240$; $F(1,603, 11,22) = 2,288, p = 0,1521$; $F(1,583, 7,914) = 2,469, p = 0,1510$, respectively) (**Figure 9b**).

In **Exposure+Recovery** groups, there were significant effect is shown within groups ($F(1,000, 7,000) = 75,03, p = <0,0001$) and heat x temperature interaction ($F(1,726, 8,628) = 13,44, p = 0,0027$) in Grip Strength mean value/body weight (N/g). Tukey's post hoc analysis revealed a significant decrease in the 39ER ($p = 0,0148$) and 41ER ($p = <0,0001$) groups (**Figure 9c**).

Furthermore, Grip Strength Max/body weight (N/g) is significantly affected in within groups ($F(1,000, 7,000) = 80,59, p = <0,0001$) and heat x temperature interaction ($F(1,448, 7,239) = 15,47, p = 0,0035$). Tukey's post-hoc within groups analysis revealed 39ER ($p = 0,0064$) and 41ER ($p = <0,0001$) groups significantly decreased (**Figure 9d**).

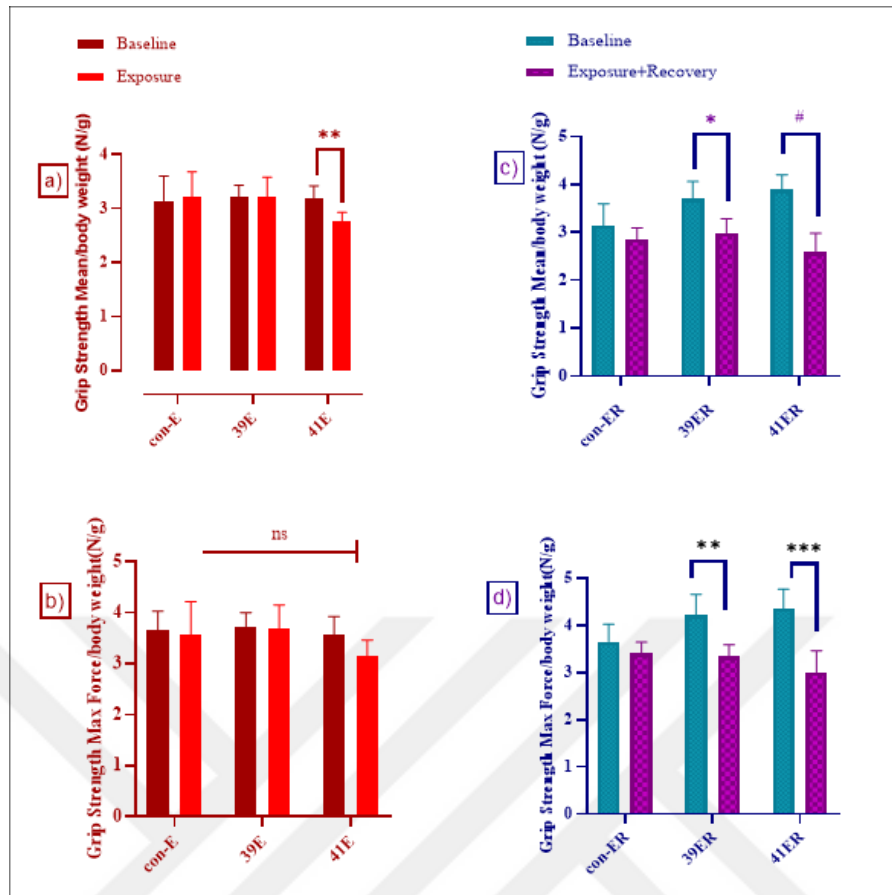


Figure 9. Grip Strength Test **a, c)** Mean value of Grip Strength normalized for body weight (N/g); **b, d)** Grip Strength maximum force normalized for body weight in Exposure and Exposure+Recovery Groups. Statistical evaluations were conducted using mixed-effect ANOVA with Geisser-Gaussian correction and post-hoc analysis was performed with Tukey's multiple comparisons test. n.s not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.0001$. Data are represented as Mean+SD.

4.2. RT-QPCR Analysis

The expression levels of *PGC1- α* , *PGC1- β* , *Tfam*, *Nrf1*, *Mfn1*, *Mfn2*, *LC3*, *Prkn*, *Pink1* and *Ucp2* genes were quantitatively assessed via RT-qPCR across different experimental groups, and normalization was conducted using β -Actin as reference gene as the internal control.

For the relative mRNA expression levels of *Pgc-1 α* , a one-way ANOVA was performed. The analysis revealed a statistically significant difference among the means ($F = 3.737$; $p = 0.0111$). Post-hoc analysis with Tukey's test in the overall groups, there was a significant difference in expression levels of 41E group compared to control ($p = 0.0297$). Within **Exposure** groups, ANOVA indicated significant differences ($F = 6.932$,

$p=0.0113$). Tukey's post-hoc analysis showed *Pgc-1 α* gene expression significantly decreased 39E ($p=0.0323$) and 41E ($p=0.0124$) compared to control. The temperature or recovery effect is not able to appear. Therefore, there were no significant differences among means within **Exposure+Recovery** groups ($F=2.915$; $p=0.0852$) (**Figure 10a**).

For the relative mRNA expression levels of *Pgc-1 β* , Welch's ANOVA test was performed. There was a significant difference among means ($W=11.86$ (5,000, 10,48), $p=0.0005$). Games Howell post-hoc test showed significant difference between 39E and 41ER ($p=0.0030$), 39ER and 41ER ($p=0.0027$) and 41ER compared to control ($p=0.0030$). The temperature effect during recovery and temperature x recovery interaction are interpreted from results. Within **Exposure** groups, Welch's ANOVA showed no significant differences (2,588 (2,000, 4,641), $p=0.1758$). Within **Exposure+Recovery** Groups Welch's ANOVA test indicated significant differences among means ($W=27.95$ (2,000, 6,633), $p=0.0006$). Games Howell's post-hoc analysis showed increased *Pgc-1 β* gene expression between 39ER and 41ER ($p=0.0008$) and 41ER compared to control ($p=0.0009$) (**Figure 10b**).

Tfam relative mRNA expression levels are evaluated using Kruskal Wallis test. There was a significant difference among medians ($H=16.06$; $P=0.0067$). Dunn's post-hoc analysis showed significant mean rank difference between 39E and 41ER ($p=0.0030$). Within group analysis conducted with the Kruskal Wallis test in **Exposure+Recovery** Groups or in Exposure Groups Welch's ANOVA did not significantly differ ($H=4.714$, $p=0.0897$; $W=2.419$ (2,000, 6,218), $p=0.1671$, respectively) (**Figure 10c**).

For the relative mRNA expression levels of *Nrf1*, Welch's ANOVA test was performed. There was a significant difference among means ($W=5.806$ (5,000, 13,15), $p=0.0048$). Games Howell's post-hoc analysis showed that 39E vs. 39ER ($p=0.0402$), 41E and 39ER ($p=0.0275$). Within **Exposure** groups one-way ANOVA was not significantly differed ($F=3.113$, $p=0.0705$). Within **Exposure+Recovery** groups, Welch's ANOVA test showed significant differences among means ($W=7.684$ (2,000, 8,866), $p=0.0116$). Games Howell's post-hoc analysis showed 39ER significantly increased compared to control ($p=0.0224$). The temperature increase showed a negative impact on *Nrf1* gene expression, whereas recovery facilitated it (**Figure 10d**).

Mfn1 relative mRNA expression levels are evaluated using Kruskal-Wallis test. There was no significant difference among medians in all groups ($H=7.752$, $p=0.1704$)

within **Exposure** ($H= 1,776$, $p= 0,4309$) or within **Exposure+Recovery** groups among means in Welch's ANOVA ($W= 3,035$ (2,000, 9,917), $p=0,0937$) (**Figure 10e**).

Mfn2 relative mRNA expression levels are evaluated using Kruskal-Wallis test. There was no significant difference among medians in all groups ($H= 7,568$, $p= 0,1817$) within **Exposure** ($H= 2,721$, $p= 0,2676$) or within **Exposure+Recovery** groups among means in one-way ANOVA ($F= 1,581$, $p=0,2383$) (**Figure 10f**).

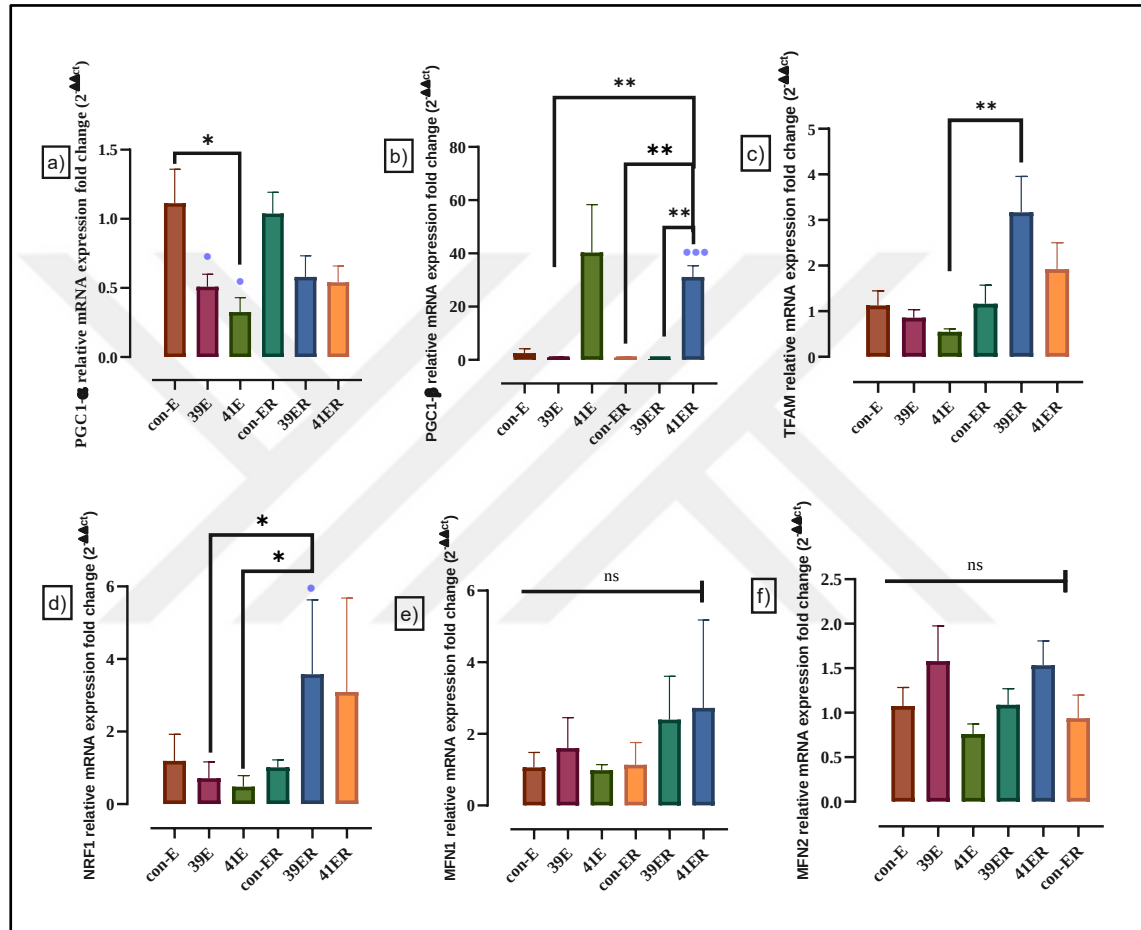


Figure 10. Relative mRNA Expression of Mitochondrial Biogenesis and Dynamics Genes; **a)** Pgc-1α, **b)** Pgc-1β, **c)** Tfam, **d)** Nrf1, **e)** Mfn1 and **f)** Mfn2 in Exposure and Exposure+Recovery Groups. Data are presented as fold change utilizing 2^{-ΔΔCt} method for gene expression normalization. One-way ANOVA was employed to statistically evaluate the gene expression across all groups, with the specific test (Welch's ANOVA, Kruskal Wallis, or ordinary one-way ANOVA) selected based on the data distribution and variance homogeneity. Post-hoc analysis was conducted using either Tukey's, Dunnet's or Games-Howell's multiple comparisons test to discern significant differences between individual groups. Data is shown as Mean+SEM. Within **Exposure** or

Exposure+Recovery groups, • $p<0.05$, •• $p<0.01$, ••• $p<0.001$; between all groups, n.s not significant $p>0.05$, * $p<0.05$, ** $p<0.01$.

For the relative mRNA expression levels of *LC3*, a Brown-Forsythe ANOVA test was performed. There was no significant difference in all groups among means ($F= 1,327$ (5,000, 14,28), $p= 0,3079$). Within **Exposure** groups one-way ANOVA revealed a significant difference ($F=3,839$, $p= 0,0450$). Tukey's post-hoc analysis indicated a significant decrease in the 39E group compared to control ($p= 0,0434$). Within **Exposure+Recovery** groups, one-way ANOVA showed no significant difference. ($F=0,05454$, $p= 0,9471$) (**Figure 11a**).

For the relative mRNA expression levels of *p62/SQSTM1*, Welch's ANOVA test is performed. There was no significant difference among means in all groups ($W= 2,293$ (5,000, 12,67), $p= 0,1074$). Neither within Exposure groups analysis conducted using Welch's ANOVA, nor within Exposure+Recovery groups analysis one-way ANOVA tests, achieved significance ($W=2,908$ (2,000, 6,348), $p=0,1269$; $F= 2,026$, $p= 0,1664$) (**Figure 11b**).

PINK1 relative mRNA expression levels are evaluated using Brown-Forsythe ANOVA test. There was a significant difference among means ($F = 3,795$ (5,000, 12,95), $p=0,0245$). Games Howell's post-hoc analysis revealed a difference between 39E and 41E groups. However, the p -value was just above the significance threshold of 0.05 ($p = 0,0575$). Further within group analysis of Exposure groups using Welch's ANOVA test ($W= 8,731$ (2,000, 6,405), $p= 0,0148$) revealed significant difference which is specified by Games Howell's post-hoc analysis. Significant difference between 39E and 41E groups indicated ($p=0,0182$). Temperature effect is observed. Within group analysis conducted in Exposure+Recovery Groups using Welch's ANOVA was not significantly different ($W= 0,1363$, $p= 0,1671$) (**Figure 11c**).

PARKIN relative mRNA expression levels are evaluated using Kruskal Wallis test. There was a significant difference among medians ($H = 9,064$, approximate $p= 0,0056$). Dunn's post-hoc analysis revealed a difference between 41E and 41ER groups ($p= 0,0015$), recovery effect appeared. Within group analysis of Exposure groups using Kruskal Wallis test ($H= 9,064$, exact $p= 0,0051$) revealed significant difference which is specified by Dunn's post-hoc analysis. Significant difference between 39E and 41E groups indicated ($p= 0,0205$). Temperature effect is observed. Within group analysis

conducted in Exposure+Recovery Groups using Welch's ANOVA was not significantly different ($W = 4,222$ (2,000, 8,414), $p = 0,0537$) (**Figure 11c**).

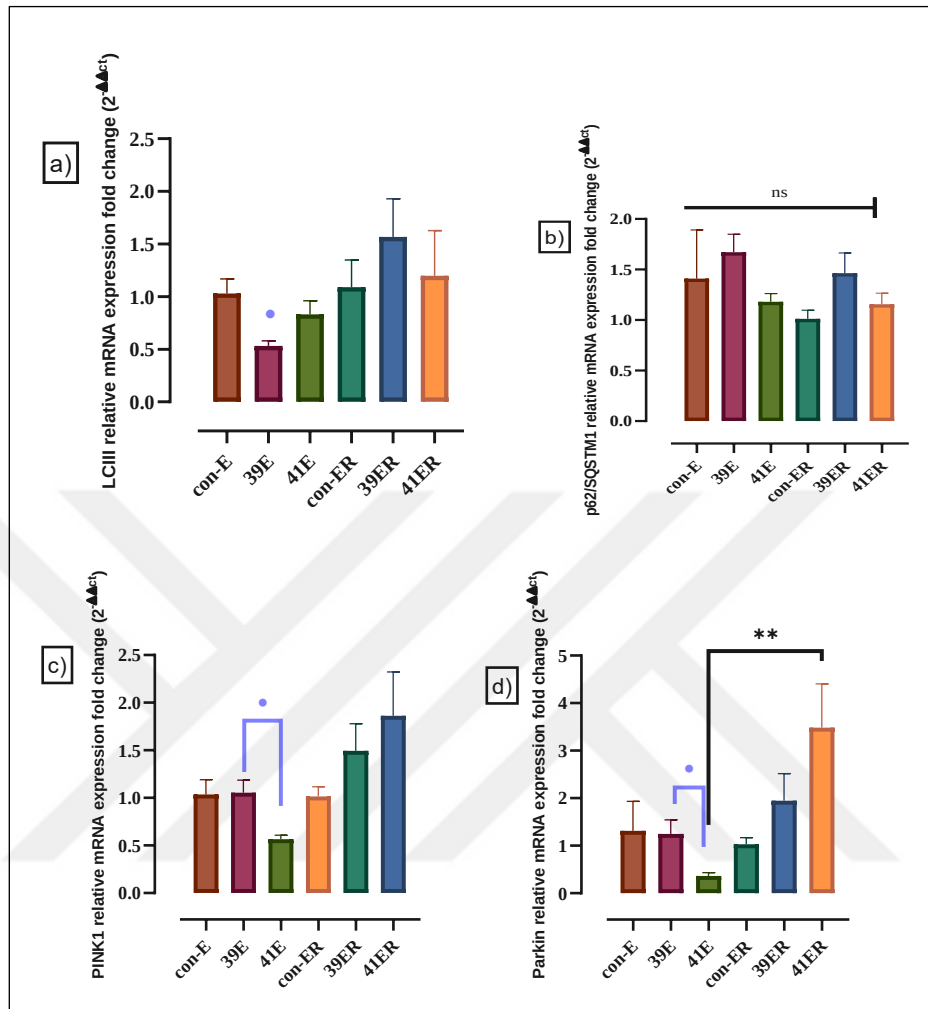


Figure 11. Relative mRNA Expression of Autophagy and Mitophagy Genes; of **a)** *p62*, **b)** *LC3*, **c)** *Pink1* and **d)** *Parkin* in Exposure and Exposure + Recovery groups in Groups. Data are presented as fold change utilizing the $2^{-\Delta\Delta C_t}$ method for gene expression normalization. Differences between the groups were evaluated using one-way ANOVA, with subsequent post-hoc analysis as needed to discern significant pairwise differences. Within Exposure or Exposure+Recovery groups; • $p < 0.05$; between all groups, n.s not significant $p > 0.05$, * $p < 0.05$, ** $p < 0.01$

UCP2 relative mRNA expression levels are evaluated using Brown-Forsythe ANOVA test. There was a significant difference among means ($F = 5,062$ (5,000, 18,69), $p = 0,0042$). Games Howell's post-hoc analysis revealed a significant difference between 39ER and 41ER groups ($p = 0,0223$). The exposed temperature changed the direction of expression during recovery phase. Further within group analysis of Exposure groups

using Welch's ANOVA test ($W = 4,662$ (2,000, 6,454), $p = 0,0559$)) showed no significance. Within group analysis conducted in Exposure+Recovery Groups using Brown-Forsythe ANOVA test was significantly different among means ($H = 11,73$ (2,000, 8,991), $p = 0,0031$), specified using Games Howell's post-hoc analysis. 39ER and 41ER have shown significant difference ($p = 0,0073$) (**Figure 12**).

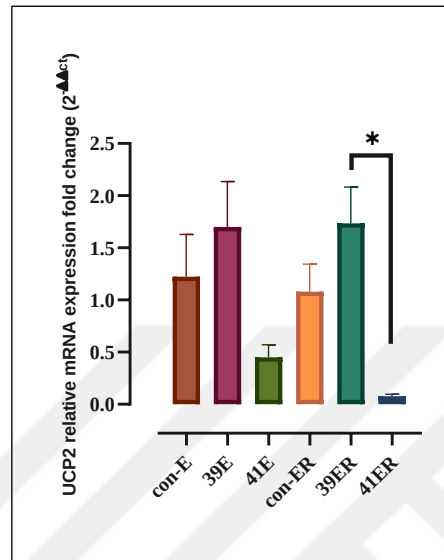


Figure 12. Relative mRNA expression levels of Ucp2 in Exposure + Recovery groups. Data are presented as fold change utilizing the $2^{-\Delta\Delta C_t}$ method for gene expression normalization. Differences between the groups were evaluated using one-way ANOVA, with subsequent post-hoc analysis as needed to discern significant pairwise differences. Within Exposure or Exposure+Recovery groups; • $p < 0.05$; between all groups, n.s not significant $p > 0.05$, * $p < 0.05$.

5. DISCUSSION

In deciphering the complex interplay between heat stress and cellular mechanisms, our study elucidates the multifaceted impact of temperature on mitochondrial dynamics and muscular function. Acute response to repeated application may maintain muscle strength in temperature dependent manner, hence it is not observed beneficial effects on motor function. Furthermore, higher temperatures impaired muscle strength progressively. Furthermore, the distinct regulatory roles of PGC-1 α and PGC-1 β in energy homeostasis and mitochondrial biogenesis, providing deeper insights into their contribution to different courses of cellular stresses. A duality is underscored by our data indicating a decrease in PGC-1 α expression and an intricate response of PGC-1 β to temperature variations and recovery phases.

After 1 week of exposure, *Pgc-1 α* expression decreased both in 39E and 41E. Recovery normalized expression levels compared to control. However, *Pgc-1 β* significantly affected temperature and recovery. PGC-1 β is central to the orchestration of mitochondrial biogenesis and functionality, in addition to managing FA metabolism. In contrast, PGC-1 α is primarily responsible for overseeing adaptations to physical exercise, whereas PGC-1 β is involved exclusively in the regulation of basic metabolic processes³⁷¹. It has been suggested that ER stress may be one of the mechanisms linking mitochondrial dysfunction. PGC-1 β induces MTOR-dependent ER stress response and increase lipogenesis via SREBP³³⁴. In an extensive and detailed work demonstrated by St-Pierre and colleagues, there are distinct structural and metabolic adaptational processes are reprogrammed due to expression of PGC-1 family. PGC-1 α potentially plays a role in establishing a more uncoupled state at rest, which might facilitate a system capable of rapid ATP turnover, particularly during exercise. On the other hand, PGC-1 β tends to induce a lower rate of proton leak at rest, thereby allowing for a greater ATP yield when required. However, PGC-1 β lacks the ability to offer metabolic flexibility and primarily relies on lipid metabolism for its function³⁷².

The induction of HSR and chaperones can potentially exert a detrimental effect on heat-induced macroautophagy. Elevated levels of HSPs observed after one week might aid in the functional recovery of cellular components that have been impaired by stress. As a result, this process could diminish the need to eliminate these stress-damaged components through macroautophagy. *LC3* is inhibited in 39E group after one week, hence 41E and recovery groups do not show significant differences. Although autophagy

may be inhibited through proper HSR response, whereas it is maintained via high ER stress during 41°C episodes, supports with *Ucp2* loss³⁷³. It is also observed decreased *Pink1*, and *Parkin* mRNA expression commonly interpreted as dysfunctional mitochondrial turnover and aggregates. Furthermore, *Parkin* expression increased after recovery phase in 41ER groups,

UCP2 modulation may be initiated with increased glycolytic surveillance and lipid oxidation, increased metabolic rate³⁰⁰. Therefore, adaptational gene expression is shown, mitochondria copes with heat stress with faster metabolic response in order to mild stress exposure³⁷⁴. Increased basal metabolic rate ensures efficient proliferation and reconstruction of cellular environment. Therefore 41°C shows impaired mitochondrial turnover and low *Ucp2* levels indicate different surge. Mild heat stress also leads to increased IGF-1 levels after 7 days in pigs. It is known that IGF-1 may enhance UCP2 expression and inhibit FOXO1 related atrophy in Akt dependent pathway³⁷⁵. Also, UCP2 deletion led to persistent NFκβ activation and inflammatory alleviation, promoted lipid synthesis and oxidation in colon tumor³⁷⁶. Unfortunately, UCP2 loss or downregulation and related cellular dynamics in skeletal muscle needs further investigations.

Interestingly, despite altered mitochondrial dynamics, Rotarod performance showed no significant difference between groups or compared to control. In human subjects, single heat stress with 39°C T_c is able to alter proprioception and balance which are indicated via Star Balance Test³⁷⁷ and running velocity³⁷⁸. Contradictory results are indicated in diabetic rats 41°C T_c for 30 min. Rotarod performance is improved after 3 and 6 weeks. Even though, diabetes may impair motor performance progressively, this protective effect may be interpreted as prevention modality for regression in motor performance³⁷⁹. In the present study, functional loss is not observed in balance, coordination, or endurance on rotarod speed, main or total duration, respectively. It is interpreted as lean muscles and cardiovascular capacity is maintained, whereas strength poorly affected.

However, Grip Strength Mean Value that normalized with body weight decreased with 41°C repeated exposure, which indicates altered mitochondrial function with susceptibility to fatigue during force production. Mitochondrial dysfunction triggers catabolic signaling pathways and promote the expression of muscle atrophy genes^{380,381}. Maximum Forces show no change between exposure groups, which may be maintained via osmotic modulation as mentioned before. Furthermore, predicted recruitment of

muscle force after recovery is not achieved. Mean value and maximum force are regressed in 39ER and 41ER. Former research has shown there was no significant effect of Hsp expression on strength. After one-week, mitochondrial quality control related protein *Pink1* and mitophagy ubiquitin *Parkin* increased in 41ER. 39ER activated *Nrf1-Tfam* expression and autophagic surge normalized. Maximum Force is mainly initiated with muscle fiber recruitment and Ca^{+2} handling and motor units. In order to atrophic changes due to increased GCR action and receptors which is supported with high *Pgc-1 β* expression in 41ER. It is speculated that uncoupled respiration may cause susceptibility to atrophy withdrawal of stress with low ROS content. Therefore, macroautophagy is not observed via *LC3* expression in this study.

Additionally, it is important to understand WHB effects on CNS during high and low temperatures. It is shown that, endogen hyperthermia has harmful effects on cerebellum, hypothalamus and cerebral cortex^{382,383}. Interestingly, further damage occurs after the recovery period. Exposing rat striatal neuron cultures to heat stress at 43°C for two hours did not result in immediate visible damage within the first 12 hours post-stress. However, over the subsequent three days, there was a significant loss of neurons, with over 50% of them perishing³⁸⁴. 43°C and 60±10% humidity for 15 min/1 week was sufficient to observe impaired motor activity in mice and 2 weeks to decreased cell viability on nigro-striatal dopaminergic neurons³⁸⁵. Progressive neuronal loss may present additional insight for effects of hyperthermia on motor function after recovery.

Interestingly, locomotor activity in OFT impaired in 39E group, whereas 41E did not differ compared to baseline levels. Temperature effected inversely, and this distinct surge maintained between 39ER and 41ER groups. In mild hyperthermic temperatures, behavioral adaptations to energy conservation induces decreased locomotion. Furthermore, repeated application shows decreased brain 5-HT and dopamine levels may alter exploratory behavior and may lead to anxiety-like behavior³⁸⁶. In severe temperatures, BBB disruption, neurogenic inflammation and corticosterone levels may induce cognitive dysfunction and hyperkinesis^{387,388}. Locomotor alterations may be initiated to increased or decreased thermal response. Mild temperatures induce shifted autonomic regulation with lower threshold³⁸⁹. Therefore, inflammatory surveillance may induce higher set temperatures^{390,391}. Initiation of thermoregulatory responses may influence locomotor activity.

Repeated hyperthermia also led to behavioral alterations. OFT center frequency or duration, there were no significant changes in 39E and 41E groups. Recovery phase, increased anxiety-like behavior in OFT. Single episode of WBH induces increased serotonin levels in brain and observed beneficial effects on depressive symptoms in humans. Therefore, additional Serotonin Reuptake inhibitor treatment diminished WBH effects. Repeated exposure to high levels of 5-HT, HPA-axis modulation in brain may alter serotonergic drive.

Loss of striatal dopaminergic neurons leads to increased risk-taking behavior as seen in Parkinson's Disease ³⁹². It is speculated that decreased center duration without changes in center frequency may be related to impaired dopaminergic drive. The EPM test showed increased closed arm frequency with temperature effect, which indicates anxiety-like behavior. Mild temperature and recovery in 39ER, induced increased open arm duration, while open arm entries decreased. It may partially interpret as regression in anxiety-like behavior, hence may indicate alterations in initiation of action. Nevertheless, EPM test is inadequate to evaluate motivational processes.

6. CONCLUSIONS

In view of information discussed, therapeutic and harmful doses of heat showed high dependence on temperature, velocity, frequency, and modality when compared to former investigations. Our research was conducted using Tc temperature with short time intervals. It is revealed that temperature has a differential effect on mitochondrial adaptational process with may be initiated with systemic perturbations and cellular proteome, further mitochondrial and DNA damage. Lower temperature changes can induce metabolic process without strength, endurance, or coordination loss for short term. Hence, our findings emphasize that it is important to being aware of later consequences. Furthermore, high Tc temperatures even for short time interval may induce progressive strength loss and delayed mitophagy response initiated to proteome stress when exposed repeatedly. To understand systemic and central modulation, further analysis will be conducted.

Due to mitochondrial dysfunction, it was observed strength loss after 41°C/1 week exposure and after one week in 39ER and 41ER. Our future plan is to support our data with histological changes on muscle tissue, serum Creatine Kinase levels, HSP70 and HSP60 expression to reveal HSR response and mitochondrial UPR. To understand contradictory results with the literature in coordination and balance, cerebellar HSR response and cellular viability will be assessed. To evaluate systemic stress response induction during high temperatures and different surges after 39°C and 41°C experiments, serum corticosterone levels will be analyzed.

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8. APPENDICES

8.1. Ethical Approval

	HAYVAN DENEYLERİ YEREL ETİK KURULU KARAR FORMU	Doküman No : FR83
		Yayın Tarihi : 06.12.2022
		Revizyon No : 00
		Revizyon Tarihi : 00.00.0000
		Sayfa : 1 / 1

ETİK KURUL KARARI				
Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2023-21	18.04.2023	2023/04	2023/04-05	Prof.Dr.Bayram Yılmaz
"Tekrarlı Hipertermiinin Farelerde İskelet Kası ve Motor Fonksiyon Üzerine Etkilerinin Araştırılması" isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				
Hayvan Türü / Irkı	Toplam Hayvan Sayısı	Hayvan Cinsiyeti		
Fare/C57	40	Erkek		

Görevi	Adı Soyadı	Onay/Ret Durumu	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	☐ Onay ☐ Ret	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	☐ Onay ☐ Ret	
Üye	Dr. Engin SÜMER	☒ Onay ☐ Ret	
Üye	Prof. Dr. M. Ece GENÇ	☒ Onay ☐ Ret	
Üye	Prof. Dr. Rukset ATTAR	☐ Onay ☐ Ret	
Üye	Prof. Dr. Gamze TORUN KÖSE	☐ Onay ☐ Ret	
Üye	Prof. Dr. Özlem MALKONDU	☒ Onay ☐ Ret	
Üye	Prof. Dr. Aylin YABA UÇAR	☒ Onay ☐ Ret	
Üye	Prof. Dr. Burcu GEMİCİ BAŞOL	☒ Onay ☐ Ret	
Üye	Atakan Mücahit YAVUZ	☒ Onay ☐ Ret	
Üye	Ahmet ŞENKARDEŞLER	☒ Onay ☐ Ret	

Hazırlayan YÜDETAM SORUMLU YÖNETİCİ	Sistem Onayı YÜDETAM MÜDÜRÜ	Yürürlük Onayı YÜDETAM MÜDÜRÜ
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8.2. Curriculum Vitae

Personal Informations

Name	Cansu	Surname	Başış
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Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate			
Master			
University	Physiotherapy and Rehabilitation	İstanbul University	2016
High school	Science	Samsun Atatürk Anadolu High School	2011
Languages		Grades (#)	
English (YDS)		67,5	
Almanca			

All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Work Experience (Sort from present to past)

Position	Institute	Duration (Year - Year)
Content creator	Sosyal Klinik	10/2021-02/2022
Physiotherapist	İstanbul Şerifali Özel Eğitim Merkezi	08/2016-10/2020
Intern Physiotherapist	Samsun Romatem Fizik Tedavi ve Rehabilitasyon Merkezi	07/2015 – 09/ 2015
Intern Physiotherapist	İstanbul Bakırköy Ruh ve Sinir Hastalıkları Hastanesi/Nöroloji	07/2014- 08/2014

Computer Skills

Position	Institute
Microsoft Office	Excellent
GraphPad Prism	Excellent

*Excellent, good, average or basic

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

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Articles published in other journals

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Proceedings presented in international scientific meetings and published in proceedings book.

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Journals in the proceedings book of the refereed conference / symposium

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Others (Projects / Certificates / Rewards)

Certificate of Animal Use in Experimental Research – January, 11 2021
