Effects of long term concurrent heat stress on performance and muscle molecular response to resistance exercise: *Is hot really hot or is it just smoke?*

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Abstract

Natural performance enhancement, muscle hypertrophy and muscle rehabilitation on an expedited timeframe is highly coveted in sports and fitness industries. Heat stress (HS) can enhance muscle hypertrophy and strength when used in combination with resistance exercise (RE), however, the additive effects of HS on power, speed and agility have not been investigated. Furthermore, the efficacy of previously tested heating methods in achieving muscle heating is highly variable and inconsistent, which probably contributes to the inconsistent effects of heating reported in the literature. Therefore, primarily, this thesis aimed to investigate the effects of HS on performance adaptations to RE. Secondarily, how the cellular signal transduction pathways regulating skeletal muscle adaptations to RE are altered with long term concurrent HS were also investigated. Thirdly, we aimed to develop a reliable localised heating method. We hypothesised that full body HS applied concurrently to heavy progressive RE may improve upon the phenotypic and molecular muscle adaptations to RE.

In the first study, we aimed to develop a localised heating method that is capable of raising core muscle temperature above 38-5-39°C, by testing three different models of localised heating. However, none of the models were able to raise core muscle temperature (CMT) to the desired levels. Therefore, in the second study, eighteen recreationally active males were assigned to two groups, HEAT (n=8, 40°C, 30% RH), and CON (n=10, 23°C, 20% RH). Each group undertook an identical, ten week, full body RE program three days a week. Peak core body temperature (HEAT 38.18 ± 0.27 °C; CON 37.97 ± 0.32 °C), as well as *vastus lateralis* muscle temperature 3.5 cm under the skin (HEAT 36.79 ± 1.55 °C; CON 35.94 ± 1.51 °C) were measured. Strength, peak force, speed, agility and body composition (DXA) were measured pre-, mid (week 5), and post-intervention. Muscle biopsies were obtained from the *vastus lateralis* pre-intervention at rest, one hour and 48 hours post the first resistance training session. An identical biopsy trial was performed ~72-96h after the last training session of the intervention. In study three, the muscle samples at each time point were analysed via western blots for key markers of muscle protein synthesis. Fibre cross sectional area (CSA), satellite cell (SC) and myonuclear density were quantified via immunofluorescence. In study four, muscle samples were analysed for the heat shock protein (HSP) response via western blots. Study five investigated the mitochondrial and angiogenic adaptations via western blots and capillarisation response via immunofluorescence.

Ten weeks of RE improved lower body strength and relative upper body strength, however had no effect on power, agility or speed. Lean muscle mass, fibre CSA, SC content and myonuclear

density improved in response to RE. Concurrent full body HS applied at 40°C failed to increase CMT in the *vastus lateralis*. Therefore, full body HS applied at 40°C did not improve upon performance, molecular or phenotypic adaptations to RE.

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Conference presentations

2021 - Federation of American Societies for Experimental biology (FASEB) – Poster

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Student declaration

I, Owala Arachchige Shavin Nauchal Chandrasiri, declare that the PhD thesis entitled: **Effects of long term concurrent heat stress on performance and muscle molecular response to resistance exercise: *Is hot really hot or is it just smoke?*** is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures. All research procedures reported in the thesis were approved by the Human ethics committee, Victoria University under approval numbers HRE 15-302 and HRE 18-229.

Signature: 

Date: 29/11/2021

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List of abbreviations

(1RM): 1 Repetition Maximum

(4E-BP1): 4E-binding protein 1

(AMPK): AMP activated protein kinase

(Ang1): Angiopoietin1

(BM): Body mass

(BMC): Total bone mineral content

(BrdU): 5-bromo-2′-deoxyuridin

(BSA): Bovine serum albumin

(CMT): Core muscle temperature

(CS): Citrate synthase

(CSA): Cross sectional area

(DTNB): 5,5-dithio-bis-(2-nitrobenzoic acid)

(DXA): Dual x-ray densitometry scanning

(eNOS): Endothelial nitric oxide synthase

(HS): Heat stress

(HSE): Heat shock element

(HSF-1): Heat shock factor -1

(HSP): Heat shock protein

(LMM): Lean muscle mass

(MCHI): Myosin Heavy Chain I

(MCHIla): Myosin Heavy Chain IIa

(MCHIlx): Myosin Heavy Chain IIx

(MPS): Muscle protein synthesis

(mTOR): Mechanistic target of rapamycin

(MVC): Maximal voluntary contractions

(NO): Nitric oxide

(OAA): Oxaloacetate

(OXPHOS): Mitochondrial complexes I-V

(P70S6K): Ribosomal protein S6 kinase

(Pax7): Paired box transcription factor

(PCNA): Proliferating cell nuclear antigen positive myonuclei

(PGC-1α): Proxisome proliferator activated receptor γ coactivator 1-alpha

(PI): Pixel intensity

(RE): Resistance exercise

(RH): Relative humidity

(Rheb): Ras homologous enriched in brain

(ROM): Range of motion

(RPE): Rating of Perceived Exertion

(rpS6); Ribosomal protein S6

(SWD): Shortwave diathermy

(TBST): Tris buffered saline-Tween

(TRP): Transient receptor potential

(TRPV1): Transient receptor potential vanilloid type

(TSC2): Tuberous sclerosis complex 2

(VEGF): Vascular endothelial growth factor

1 Chapter 1: Literature review part 1: Resistance exercise and heat stress

1.1 Introduction

The human body continuously strives to maintain homeostasis in order to ensure the optimal functionality of all critical systems. A number of foreign and internal physical inputs are capable of disrupting this balance by causing tangible changes in the chemical and physical homeostatic environment (Adolph, 1964). These inputs can be defined as stressors. The resulting changes to the system are identified as strains. Functional, morphological, chemical as well as genetic alterations that reduce the strain caused by the stressors are identified as adaptations. When subjected to these stressors and put under strain, the internal homeostasis moves to compensate via short and long term adaptations (Anderson, 1999, Adolph, 1955).

Overloading the muscles and external heat are two of the above defined stressors. They are capable of straining the internal balance, consequently driving muscle adaptations (Kraemer and Ratamess, 2005).

Overloading of the muscle defines the practice of subjecting a single muscle or muscle group to a greater resistive stress than it's accustomed to at a given stage. The overloading is accomplished via increasing loads, increasing frequency of work against the load, increasing time against the load and variety of overloading methods (ACSM, 2009, Ratamess et al., 2009).

Over the last century, overloading the muscles has been developed systematically into a controlled methodology where the stress response by the body has been harnessed and morphed into multiple modes of resistance exercise (RE) (Hamilton and Booth, 2000).

RE is used heavily by athletes as well as recreational enthusiasts to enhance strength, power, force and hypertrophy as well as various sport specific fitness attributes (Kraemer et al., 2002). Furthermore, RE is now being utilised as a health aid. A number of leading health organisations have recognised RE as an effective auxiliary treatment for cardiovascular and pulmonary rehabilitation, stroke recovery, neuromuscular disease, obesity as well as for general physical rehabilitation post moderate and serious physical injuries (Feigenbaum and Pollock, 1999, ACSM, 2009, Ratamess et al., 2009)

Methods and strategies by which muscle adaptations to RE can be augmented are highly beneficial for athletes looking to enhance their performance as well as for individuals with

impaired muscle function to improve their quality of life as well as muscle recovery after exercise. Combining exercise with heat stress (HS) in a variety of models is one such approach (Naito et al., 2012, Hyldahl and Peake, 2020). In some cases, HS alone has shown the potential to improve muscle function via an increase in protein synthesis leading to an increase in muscle mass as well as muscle contractility (Guo et al., 2016, Goto et al., 2011, Racinais et al., 2017, Racinais and Oksa, 2010). Over a number of years, the link between the ability of HS to induce muscle adaptations and its ability to improve upon adaptations to RE in the skeletal muscle has been studied and the evidence is encouragingly positive albeit limited.

The concurrent combination of HS with RE is one such approach. However, at the inception of this study, only three previous studies had tested this approach in humans. Goto and colleagues saw that ten weeks of HS to the *biceps brachii* combined with low intensity RE improved the cross sectional area (CSA) of the muscle and improved isometric torque in the non-dominant arm (Goto et al., 2007). In their seminal study, Kakigi and colleagues demonstrated that localised HS in combination with RE resulted in an enhanced muscle molecular response where the key responses to RE alone were surpassed by the combinatory application of both in the *vastus lateralis* (Kakigi et al., 2011). HS was found to augment RE induced increases in muscle hypertrophy pathways, compared to RE alone (Kakigi et al., 2011). However, Stadnyk and colleagues saw no improvements in muscle mass or performance after 12 weeks of localised HS to the *vastus lateralis* (Stadnyk et al., 2017). Moreover, it has been indicated that the adaptive skeletal muscle response to HS maybe predicated upon a certain temperature threshold, possibly in a dose response manner (Guo et al., 2016, Yoshihara et al., 2013). This notion has not been investigated in humans. However, predicated upon muscle temperature, studies have shown large spectrum results from no improvements to clear improvements in the human skeletal muscle in response to HS (Labidi et al., 2020, Kakigi et al., 2011, Stadnyk et al., 2017, Hafen et al., 2019a, Morton et al., 2007).

1.2 Adaptive phenotypic muscle response to resistance exercise

RE is defined as skeletal muscles working against high external loads over brief periods of time (Wackerhage, 2014). It has been observed that a strong correlation exists between RE and increasing muscle size as well as the activation of novel neural connections (Wackerhage, 2014). Muscle hypertrophy is driven by triggering cellular pathways that regulate muscle protein synthesis, degradation, transcription and in some instances satellite cell movement. The mechanical load is received by the target cells and converted in to a chemical signal that initiates

a growth response. The two phenotypic growth responses to RE are hypertrophy and hyperplasia (Wackerhage, 2014). Hyperplasia is the increase in fibre number in response to RE (Antonio and Gonyea, 1993). MacDougall and colleagues compared the fibre count between five elite body builders and seven intermediate caliber body builders to 13 age matched untrained controls and reported that in *biceps brachii*, there was no difference between highly trained and untrained individuals (MacDougall et al., 1984). Implying that hyperplasia is not a dominant contributor to size gain. Hypertrophy is more dominant in contribution to size gains and the muscle growth is achieved via a positive balance between protein synthesis and protein breakdown (Endo, 2015). The phenotypic outcomes of hypertrophy are an increase in the CSA of existing single muscle fibers which leads to an overall increase in the CSA of the trained muscle. It has been established that both fibre type I and type II respond to long term RT. Halkjaer-Kristensen measured a 27% and 33% increase in volume (hypertrophy) in fibre types I and II respectively in the *triceps brachii* after six months of training (Halkjaer-Kristensen and Ingemann-Hansen, 1981). Furthermore, in a tightly controlled longitudinal study, Narici and colleagues reported that after six months of heavy RE, a linear hypertrophy response coincided with the training input of maximal isokinetic knee extensions (Narici et al., 1989). However, the growth rate of skeletal muscle will decrease and plateau following chronic exposure to RE, as investigated by Alway and colleagues in a cohort of experienced bodybuilders (Alway et al., 1992).

It has been reported previously that upper body muscles show a greater hypertrophic response compared to lower body in untrained individuals (Cureton et al., 1988). Furthermore, the growth response to standard RE over 12 weeks, where muscle thickness was measured by ultrasound, was greater in the upper body compared to lower body (Abe et al., 2000). It has been proposed that this difference could be due to the fact that lower limbs are more constantly activated and loaded at a higher level during day to day activities and therefore require a higher level of loading compared to the upper body to generate a similar response. It has also been suggested that the difference in androgen receptor content between upper body and lower body could contribute to this differential response (Folland and Williams, 2007). Interestingly, individual muscles within a muscle group have been found to respond differently, in percentage growth, to the same load. For instance, Housh and colleagues reported that, following an eight week intervention of isokinetic training, the *rectus femoris* increased by 23.3% while the *vastus lateralis* only increased by 7.5%. Beyond this preferential hypertrophy, they further reported that within the same muscle, different fibers displayed varying levels of hypertrophy (Housh et al., 1992). However, it is crucial to note that the same levels of activation cannot be assumed during isolated

movements. Narici and colleagues reported similar findings in terms of differential hypertrophy between trained (dominant leg) and untrained legs after sixty days (four days a week) of isokinetic knee extensions. They observed CSA increase in the trained leg but no significant changes in the untrained leg, confirming the training effect on hypertrophy (Narici et al., 1989). Interestingly, type II fibres have been observed to display preferential or differential hypertrophy compared to type I fibres. A report by Häkkinen and others indicated a higher gain in plasticity in type II fibres during long term (21 weeks) high intensity leg extensions in recreationally active males (Häkkinen and Keskinen, 1989). This corroborates with the evidence from more short term (14 weeks) interventions where only type II fibres displayed significant hypertrophy (Aagaard et al., 2000).There has been speculation that type II fibres may have higher specific tension, that contributes to the preferential hypertrophy, however, it had been suggested earlier that there exists no discernable difference between specific tensions between the two fibre types (Fitts et al., 1991). The later evidence shows however, that there is preferential hypertrophy in type II over type I (D'Antona et al., 2006, Pansarasa et al., 2009) This alludes directly to neuro-muscular response and adaptations and preferential recruitment and activation in response to RE. Based on Hennemen's size principal, there is a graded order of recruitment of motorneurons and higher intensity of exercise will recruit type II fibres at a higher rate compared to type I (Henneman, 1985).

Neuromuscular adaptions occur via the activation of prime mover motor neurons (Sale, 1988). These adaptations are task specific in nature. However, in contrast to the morphological adaptations, the neurological adaptations and responses are not as well established. It is recognised that neural adaptations drive improvements or changes in muscular coordination and learning which in turn increase the recruitment and activation of muscles during specific tasks (Folland and Williams, 2007). The early onset exponential strength in response to RE has been attributed to neurogenic factors. Ozmun and others conducted an eight week long RE program (three sets of bicep curls, 7-11 repetitions, three times a week) with a cohort of prepubescent children (mean age of 10.3 years) and reported a 22.6% rise in isotonic strength and 27.8% rise in isokinetic strength gains. However, the pre and post intervention anthropometrics did not indicate any significant changes in muscle size. Therefore, the observed strength gains were attributed to increased muscular activation (Ozmun et al., 1994). However, the phenotypical changes also have the capacity to contribute the same changes in strength as well. The contribution made by each aspect has not been clarified (Folland and Williams, 2007).

1.3 Adaptive phenotypic muscle response to heat stress

While the phenotypic muscle response to RE has been widely investigated, the phenotypic response to HS alone as an intervention is limited. A number of *in vitro,* animal (rodent) and human studies provide insight into the muscle response to HS. In an early *in vitro* study, Kobayashi reported that HS induces muscle hypertrophy in rat myoblasts (Kobayashi, 2003). Uehera and others reported that, after exposing a group of 24 rats to environmental HS in a heat chamber (41°C for 60 minutes) for 14 days, the relative wet and dry weights of the extracted soleus muscle and total body weights of the HS groups were significantly higher than the control group on day seven (10.1% and 17.5% respectively), demonstrating a heightened growth rate in response to HS (Uehara et al., 2004). Furthermore, Goto and colleagues reported that HS facilitated the recovery of atrophied muscle in rats. Following the atrophying of the rat hind legs for five days (rats were housed in controlled 23°C enclosed spaces during this time), the treatment group was subjected to 60 minutes of incubation at 41°C (Goto et al., 2004). At day three of the heat treatment, they reported a relative mean muscle weight increment of 22% (12% higher compared to the non-heat treated group). At the concluding point of treatment on day 5, the heat group maintained the gained relative muscle weight compared to the control (Goto et al., 2004). These reports are further reinforced by the findings by Naito and others that HS (applied at 41°C for 60 minutes) prior to eight days of hind leg unweighting, was able to significantly attenuate disuse muscle atrophy in rats (Naito et al., 2000). More recently, Takeuchi and colleagues demonstrated that HS applied in the form of hot water packs set at 42°C, to crush injuries in the extensor digitorum longus muscle of rats expedited the rate of regeneration. This observation was supported by the fact that the rate of proliferation in the muscle satellite cells along with facilitating the maturation of regenerating muscle fibres and the cross sectional area of the muscle fibres in the heat group was significantly higher compared to the non-heat group at measurement points of 14 and 28 days (Takeuchi et al., 2014). These findings align well with the reports by Tamura and colleagues that environmental heat applied at 40°C for 30 minutes for seven days rescued the muscle from atrophy and arrested mitochondrial loss and maintained overall mitochondrial health overriding the clearance response triggered by muscle disuse. A distinction to be made at this juncture is that, they did not use anaesthesia prior to the heat treatment compared to the investigations above as they cited a report by Leon and others which concluded that anaesthesia is a confounding factor that effects normal thermoregulation (Tamura et al., 2015, Leon et al., 2005). They posited, based on previous reports by Støen as well as Washington and colleagues, that the concentration of anaesthetic (Isoflurane) is inversely

proportional to the thermoregulatory response due to the effect of the anaesthetic on the vasoconstriction and increased sweat threshold (Støen and Sessler, 1990, Washington et al., 1993).The other studies cited above did not take this factor into account. Whether the use of anaesthetics has an effect on muscle adaptations in response to HS has not been investigated in isolation. Cumulatively however, these findings indicate that HS alone has the ability to drive expedited muscle growth, proliferation and maintenance of muscle quality (Takeuchi et al., 2014, Kojima et al., 2007, Naito et al., 2012, Tamura et al., 2015).

Human studies investigating the standalone effects of HS on phenotypic muscle adaptations are extremely limited. After ten weeks of eight hours a day (four times a week) application of HS in the form steam/heat sheet to one leg while leaving the other leg untreated, Goto and colleagues reported a mean CSA increase by 6.1% ± 1.7% in the *vastus lateralis* and 2.7% ± 0.7% in the *rectus femoris* in all heat-treated legs. They further reported a 1.53% ± 0.49% ($p < 0.05$) increase in the entire quadriceps while there was no significant increase in the non-heated legs (Goto et al., 2011). Similar to the rodent-based studies, they reported that the corresponding mean CSA of muscle fibres along with the number of nuclei also increased significantly compared to the non-heat treated leg (Goto et al., 2011). In a more recent study, Hafen and colleagues reported that HS has the ability to attenuate muscle atrophy. The cohort was mixed with 12 males and 11 females. They immobilized the left legs of the participants at a 60°C flexion, using a knee brace. The legs were immobilised and locally treated with HS for two hours a day for ten consecutive days. In a randomized manner, they either received two hours of diathermy or a sham heat treatment (Hafen et al., 2019). They reported a mean intramuscular temperature increase of 4.2 ± 0.29°C at depths of 3.5cm. At the termination of the intervention, they reported that the cross sectional areas of the heated *vastus lateralis* had decreased to a lesser extent compared to the placebo treated legs. Myofibre CSA decreased by 10.8% from immobilisation however only decreased 5.8% in the heat treated legs during the same time period (Hafen et al., 2019).

1.4 Phenotypic muscle responses to resistance exercise and heat stress in combination

A number of studies have investigated the combinatory effect of heat and RE in skeletal muscle. The modalities can be divided to HS prior to RE, concurrent application of HS and RE and HS following RE as well as HS before/after and during RE. The most common among these is heat treatment prior to RE. Acute and chronic, localised and full body, has been utilised as heating modalities.

Jayaraman and colleagues compared the effects of HS, static stretching, and HS combined with static stretching on muscle damage post a multi set bout of acute single leg knee extensions to failure (six to eight sets of five to ten repetitions) in a cohort (n = 32) of untrained males. Three test groups (n=8 in each) received one the three mentioned conditions. All treatments began 36 hours post the RE bout. Localised HS was applied for two hours via heat pads set to 41°C, the stretch treatment involved supine leg lifts, standing and prone hamstring and quadriceps stretch, and the combined treatment incorporated HS followed by the stretch routine. The termination point for the treatments was subjective as they instructed the participants to stop once the muscle soreness subsided completely. Attenuation of muscle damage was verified via a test battery of three maximal voluntary contractions (MVC) of the *quadriceps femoris* (knee extensions) with three minutes rests in between at seven time points (days 2, 3, 4, 6, 8 and 15 post RE bout). They reported no significant differences between any of the treatment groups compared to the untreated control group in attaining pre RE bout MVC levels (Jayaraman et al., 2004). Therefore, the authors concluded that localised HS applied post-acute RE does not improve the attenuation of muscle damage. However, they did not measure core muscle temperature (CMT) and the total duration of HS application was not standardised given that fact that participants were given the control in when to terminate the treatment.

In a more defined study, Nosaka and colleagues tested the ability of HS to attenuate muscle damage by localised heat treatment (via microwave diathermy) on the *biceps brachii* of 15 male volunteers for 20 minutes, 16-20 hours (18.9 ± 0.4h) prior to them performing 24 maximal eccentric elbow flexions with a contralateral control (dominant non-dominant randomised) (Nosaka et al., 2007). Indirect markers of muscle damage in MVC, range of motion (ROM), and muscle soreness were used to assess recovery at one through to four days post intervention. They reported a significant difference MVC in the heat group, recovering to 79.7 ± 4.4 % of pre-exercise value compared to 61.2 ± 4.9%, in the control group at four days. However, they did not observe a similar trend in ROM or muscle soreness at the same time point (Nosaka et al., 2007). They indicated that muscle temperatures >40°C were reached, however the data was not reported. In another recovery focused study, Iguchi and Shields recruited a mixed cohort (n = 25) and tested each participant in a heat chamber (73°C, 10% RH) for 30 minutes and during a control condition (~26°C) for 30 minutes 7-10 days apart. Some participants were heat treated while some underwent control conditions on the first test period and the conditions were reversed on the second test period. Each participant completed three forearm MVC (via elbow flexion, each lasting ~5s) one minute apart and then completed a seven minute fatigue task comprising

of 35 MVCs (seven seconds per contraction) at each session. Following the RE protocol a post fatigue task of single seven second MVC at one and ten minute time points was conducted. They observed no significant difference in MVC torque pre fatigue in either iteration. Overall, they did not observe an acute effect on MVC immediately post heat treatment at either time point compared to control. However, the authors reported an intriguing outcome in that the subset who underwent the heat treatment first showed a faster recovery in comparison to the subset who underwent the control test first ($p < 0.05$) (Iguchi and Shields, 2011). This observation points to more long term attenuation of muscle damage by HS. However, the acute results contradict the reports by Nosaka and colleagues (Nosaka et al., 2007). No muscle temperatures were reported in this study. The authors further report a >30 minute gap between heating and the exercise. Additionally, the heat application is reported as 73°C at face level, the exact methodology, the procedure of application and the implications are unclear, making the results ambiguous.

In a more comprehensive study, Goto and colleagues combined HS with low intensity RE. They heated the non-dominant arm of nine healthy men with steam/heat sheets for 60 minutes (achieving a mean muscle temperature rise from ~36 to 38°C at depth of 1.5 cm at 30 minutes). After 30 minutes, both arms were subjected to flexion-extension exercise (3 sets of 30) of the elbow, four days a week for 10 weeks (Goto et al., 2007). Following the termination of the intervention, they reported that CSA of the non-dominant heated arm increased significantly from baseline, 7.5% ± 5.5% compared to the non-heated dominant arm which showed no significant change (Goto et al., 2007). Yoon and others investigated the muscle response to HS and RE in an elderly cohort of women between 65-75 years. They applied heat via the same heat/steam sheet of the aforementioned study to the *quadriceps femoris* of the non-dominant legs for eight hours a day (three times a week) for 12 weeks with one group receiving HS with low intensity exercise (n = 8), one group receiving just moderate intensity (n = 6) leg extensions along with one group just receiving HS (n = 7). The observed that mean muscle temperature in the heated legs (38.8°C ± 0.4°C) was on average 4°C higher compared to the contralateral non-heated leg (Yoon et al., 2017). At the conclusion of 12 weeks, they reported significant changes in the CSA in the cohort that received HS in tandem with RE (pre 36.85 ± 4.91cm^2 to post 38.57 ± 4.52cm^2) compared to HS or RE alone (Yoon et al., 2017). In this investigation, it has to be noted that given the participants mean age of 73.63 ± 2.88, the response to the stressors would be less pronounced compared to a younger cohort given the dose response nature between age and RE, however, no differential response to HS was seen between the young and the elderly

(Kumar et al., 2009) Moreover, the results of this investigation were further weakened by the fact two different intensities of RE were prescribed.

Importantly however, Frier and Locke contested the positive effects of HS on RE, reporting that it inhibited muscle hypertrophy. A batch of 25 rats, maintained in the same 12h light/dark cycle, in accordance with other rodent studies, was subjected to full body HS at 42°C for 15 minutes prior to muscle overloading against a control batch of 25 rats receiving no HS prior to overloading. In both groups, the contralateral plantaris of each rat served as the intra-subject control for the treatments applied. Consequently, the surgically removed *gastrocnemius plantaris* muscles were weighed dry and wet. They reported no significant difference between muscle mass or total body weights over seven days between the groups (Frier and Locke, 2007). Moreover, they reported no difference between the total protein content at any of the days between the RE and RE and HS groups. MCH I content decreased following HS (Frier and Locke, 2007). In a similar vein, Stadnyk and colleagues reported that there is no clear benefit in muscle heating on hypertrophy. A mixed cohort of ten participants were trained for 12 weeks, 2-3 days a week for a total of 48 sessions. The RE element consisted of four sets of eight concentric/eccentric knee extensions at 70% 1RM. Heat was applied to one leg via an electric heat pad (the surface or the output temperature was not reported). When averaged across depths 1, 2 and 3cm, the temperature rose from 35.2 ± 1.1°C to 38.2 ± 0.9°C immediately post intervention. However, specific depth data have not been reported (Stadnyk et al., 2017). The tissue/muscle mass was measured via DXA scans. They reported total muscle gains of 752 ± 304g in the control group against 761 ± 280g in the HS group. The percentage increases were HS 15 ± 7% the control 15 ± 6% ($p = 0.94$) indicating that there is no additive effect on hypertrophy (Stadnyk et al., 2017). However, there are a number of limitations in this study including the aforementioned heat application method and the uncertainty over the exact muscle temperatures achieved during the interventions.

It is evident through the above reviewed evidence that the effect of HS on phenotypic muscle responses to RE is far from distinguished. The mode of heat application, duration, mode of combination (pre, concurrent or post) with RE have produced improvements and no improvements at a similar ratio in the limited body of literature. Therefore, further investigation is required in this area.

1.4.1 Adaptive changes in muscle performance to heat and resistance exercise

Another aspect of adaptive changes parallel to the phenotypic adaptations are the changes in muscle performance in response to HS (acute or chronic), RE or combination of both. The number of investigations which have examined this aspect is similarly sparse, particularly investigating HS.

Exercise and HS has been combined in a number of ways to investigate potential additional benefits in improving or maintain skeletal muscle performance (Hyldahl and Peake, 2020). In a series of early studies, Asmussen and colleagues reported of a significant positive relationship between increasing muscle temperature and maximal dynamic strength. They tested the effect of temperature on vertical jumps, with and without countermovement. The heating was achieved via active exercise (mode not reported) and muscle temperature was measured at a depth of 3cm of in the *vastus lateralis* (Asmussen et al., 1976). Bergh and Ekblom, in their seminal and wide ranging study, measured the effects of temperature on both isometric and dynamic strength, power output, force production as well as speed, using a different experimental modality to that of Asmussen's. In order to raise body/muscle temperature they utilised intermittent exertions on a bicycle ergometer achieving peak muscle temperatures between 38ºC-39°C measured at depths 3-5cm (similar to Asmussen's approach). In addition, the researchers measured maximal strength via measuring the torque of maximum voluntary knee extensions at three different velocities. The force was measured via vertical jump height (facilitated by an initial countermovement) and power via maximal effort ergometer sprinting (Bergh and Ekblom, 1979). They reported clear improvements in peak torque, jump height, maximum cycling speed and power with increasing muscle temperature (Bergh and Ekblom, 1979). Critically, both of the above cited studies used a mode of exercise to heat the muscles, instead of using a heat source. Therefore, the possibility exists that the improvements observed were due to prior neuro-muscular activation in the exercised muscles. To this matter, they stated that the improvements in jumping and sprinting were driven by the improvements in torque, rather than temperature, given the actions were performed in sequence. However, while investigating the relationship between temperature and muscle performance (force- velocity in this study), Binkhorst et al, reported that with the increase of muscle temperature (at a depth of 1.5cm in either the *medial palmaris longus* or the *medial digitorum superior*) maximal power and velocity increased significantly. Maximal force however, did not change accordingly. Interestingly, they further concluded that while maximal dynamic strength is positively correlated to temperature in agreement with Asmussen's reports, that maximal isometric strength is independent of muscle temperature (Binkhorst et al., 1977).

Binkhorst's method of heating was localised hot water immersion (elbow to wrist). HS was applied for 30 minutes before the first contraction at 18ºC, 25ºC and 39°C with the highest muscle temperature achieved was 38ºC. All parameters were measured via handgrip dynamometer (Binkhorst et al., 1977). These claims are further established by the reports of Sargeant (Sargeant, 1987). In his investigation, he reported an ~11% increase in peak power and peak force during a 20 second maximal ergometer sprint, following 45 minute of leg immersion in 44ºC water bath (Sargeant, 1987). Notably, all of the above tested populations were recreationally active non-athletes. Casadio and colleagues recruited a mixed cohort (n =16, male = 8, female = 8) of highly trained power athletes competing at national or international level in weightlifting, powerlifting, athletics and netball (>3 years of consistent RE) for a randomised crossover study conducted in acute hot (~30°C, 40-60% relative humidity) and cold (~20°C, 40-60% relative humidity) (Casadio et al., 2017). They tested for the difference in strength and power in back squats, power cleans, vertical jumps, and medicine ball throws in two separate sessions conducted five to seven days apart. They reported possible increments in upper body power (medicine ball throw) in females (3.4%, 90% CL −1.5, 8.6) and males (3.3%, −0.1, 6.9) and an improvement in lower body power (vertical jump) only in males (3.2%, −0.4, 6.9). They reported trivial core temperature increments in response to HS (0.03°C) and a mean increase skin temperatures of 3.05°C in females and 1.97°C in males (Casadio et al., 2017).Contrasting these reports, Nosaka and colleagues reported that maximal isometric force in elbow flexion did not improve in a cohort of untrained (n=10) females after receiving ten minutes of localised heating via microwave diathermy to the *biceps brachii* immediately pre exercise intervention compared to the non-heat treated control arm (dominant non-dominant randomised) (heat = 112 ± 4.9 N, control = 110.6 ± 4.0 N. They reported an average deep muscle temperature increase from 33.9 ± 0.8 °C to 37.5 ± 1.7°C at a depth of 1.5-2 cm (Nosaka et al., 2004).

In a contemporary study by Goto and colleagues, they investigated the changes in strength in response to chronic HS. As discussed in a prior segment, in this investigation, eight healthy men (mean age 45.1 ± 2 years, height 170 cm ± 2 cm and body weight pre-treatment 67.4 ± 1.9 kg post 67.3 ± 3.5 kg showing no significant change over the test period) were tested . During the investigation, the selection of leg for HS was randomised with five participants receiving HS in their non-dominant leg and three on their dominant. The *vastus lateralis* was targeted and the area of heating was identical for each participant. The method of heating is discussed in part 2 of this review. The chronic treatment was applied eight hours a day, four days a week for ten weeks (Goto et al., 2011). Isometric torque of each test leg was recorded one day prior to the

commencement of the intervention and repeated identically one day post termination of intervention. The maximal isometric torque of voluntary extension/flexion at 90º, with total contraction time of five seconds, was measured on both treatment and control legs, two repetitions five seconds apart. They reported a statistically significant mean increase in isometric torque of 5.8 ± 2.5% in the heat-treated legs compared to non-significant 3.7 ± 6.9% in the control legs, during extensions implying a HS driven performance improvement. Intriguingly however, they did not observe a significant difference during flexion (Goto et al., 2011). This observation could be due to the fact that the torque generated during flexion is multi-fold lower compared to extension and therefore, the increments were negligible. More likely however, was the fact that the heating of *vastus lateralis* did not impact the muscle involved in flexsion However, the improvements in isometric torque seen by Goto were further verified by Racinais and colleagues who reported that fully body, passive HS, applied via heat controlled climate chamber (44-50°C, 50% relative humidity) for one hour day for 11 consecutive days, where rectal temperature maintained ~39°C in the hot conditions compared to ~36°C in thermoneutral, also increased isometric torque of plantarflexion in anthropometrically matched cohort (age 38 ± 7 years, weight 74 ± 7 kg, height 177 ± 7 cm). They were also able to discern that the improvement in torque was due to the improvements in muscle contractility (Racinais et al., 2017). Kim and colleagues conducted a longitudinal study in a mixed cohort (n=12, 10 males, 2 females, mean age 23.6 ± 4.8y), in which they applied localised HS to the thigh via a hot water circulating perfusion garment (~52°C) for 90 minutes reaching skin temperatures between 39.5-40°C, five days a week for eight weeks with intra-subject contralateral control (Kim et al., 2020). Muscle temperature was not measured, but was estimated from similar studies that used a similar method (Heinonen et al., 2011, Kuhlenhoelter et al., 2016) reaching ~38°C. At the termination of the intervention, strength was assessed via maximal torque of knee extensors (pre-familiarised) and single bout of 40 consecutive maximal contractions. The total work out put during contractions was quantified in order to measure fatigue after five minutes of ergometer warm-up. They reported significant improvements ($p = 0.007$) of 6% and 5% of isokinetic peak torque at weeks 4 and 8 respectively in the HS leg compared to 2% and 1% in the non-heated leg. They further reported that fatigability did not change from baseline in either leg (Kim et al., 2020). However, in contradiction, Labidi and colleagues found that localised HS applied directly to the lateral and medial *gastrocnemius* via adhesive heat pads for eight hours a day for five consecutive days for six weeks did not improve isometric, concentric or eccentric torque. The mixed cohort (n = 15, male = 8, female = 7, age 35 ± 6 years, weight 70 ± 14kg, height 173 ± 7cm) did not show any improvements in muscle contractility either. Muscle temperature was measured at a depth of

~2cm and was measured to have increased 4.6 ± 1.2 °C after six hours of heat treatment (Labidi et al., 2020). The lack of improvements seen in Labidi's study compared to the others could be due to the fact the peak muscle temperature at a depth of ~2cm was 37.6°C, was lower in comparison to the other studies which saw ~38°C at depth of 3.5-4cm, was not enough to induce changes.

Only two studies have combined RE and HS concurrently. Goto and colleagues reported that after localised HS to the *biceps brachii* four days a week for ten weeks in combination with of low intensity flexion-extension RE improved maximum isometric torque over the unheated contralateral arm (Goto et al., 2007). It is important to note however, that HS and RE combination was always to the non-dominant arm. Therefore, the improvements were due to the expedited adaptations of the non-dominant arm and not HS is a possibility. Conversely, Stadnyk and colleagues saw no improvements in peak and mean concentric torque after 12 weeks of concurrent localised HS combined with RE to the *vastus lateralis* (Stadnyk et al., 2017). Both studies indicated a muscle temperatures of ~37-38°C based on previous and pilot studies. The contradiction between the reports further bring in to focus whether Goto's results were due to the training of the non-dominant arm.

Collectively, the impact of muscle temperature is of note, as improvements appear to occur above temperatures of ~38°C. However, the limited evidence on concurrent application is decidedly equivocal.

The above evidence indicates that HS or HS combined with RE has the ability to improve upon the performance aspect in the skeletal muscle. However, some studies, especially chronically, did not see performance improvements when HS was utilised. The lack of improvements appear to align with lack of improvements in deep muscle temperature. Therefore, the combination of HS concurrently with heavy RE, where muscle temperature increased over a threshold, may improve performance aspects chronically.

1.5 Anabolic synthesis responses to resistance exercise and heat stress

The above reviewed morphological adaptations are driven by chronic and acute molecular responses to the stress inputs as RE and HS are received as stressors by the skeletal muscle (Wackerhage, 2014). Hypertrophy via anabolic protein synthesis and to a much lesser extent hyperplasia as well as the underlying adaptive responses such as satellite cell response, mitochondrial biogenesis, angiogenesis (capillarisation), heat shock protein (HSP) response as well as Ca^{2+} movement all contribute to mount the compensatory countermeasures to potential

damages that would be caused by the continuation of the stressors manifesting as muscle adaptations (Chesley et al., 1992, Ross et al., 2014, Groennebaek and Vissing, 2017, McCarthy, 2013, Bloor, 2005). As mentioned above the hypertrophic response is the most well documented and most observable adaptive response RE. It is characterised by the positive difference between muscle protein synthesis (MPS) and protein degradation resulting in net anabolism within the target muscle group (Wackerhage, 2014, Phillips et al., 1997). It has been established that muscle protein accretion occurs in the recovery phase rather than during the active loading phase (Chesley et al., 1992). Biolo and colleagues reported that a recreationally active male cohort (n = 5, age 24 ± 2 years), subjected to an acute bout of intense lower body RE (with tracer amino acids injected in to the right femoral artery concurrently), showed significantly elevated intracellular levels leucine, lysine and alanine at three hours post exercise, increasing significantly by 60%-120% ($p < 0.05$) compared to the same time point at rest (Biolo et al., 1995). It is key to note however, that this simulates a fed state which has a direct effect on the rate of MPS. The molecular response is also load and intensity dependent. In the presence of excess amino acids, against a heavy load the body is able to maintain a positive difference of proteins resulting in hypertrophy. Miller and others reported that, after an acute bout of one legged kicking at 67% percent of the maximum work load (defined as Wmax) (in a fed state where protein synthesis is maximally facilitated), protein synthesis was elevated up to 72 hours (Miller et al., 2005, Møller et al., 2015). Supporting the notion of intensity driven adaptations, it has further been reported that below 40% of an individual's 1 RM, there is no detectable peaks in acute MPS whereas it is increased by two to three-fold above intensities of 60% (Kumar et al., 2009). However, muscle fatigue requires consideration as well where volume of exercise is prioritised over intensity. Burd and colleagues tested a group of males (n = 15, age 21 ± 1 years) with four sets of unilateral leg extensions at different load/volume programmes, 90% 1RM until volitional failure, 30% 1RM work-matched to 90% till failure or 30% 1RM until volitional failure, reported that MPS at 30% 1RM to failure is comparable or better to 90% 1RM to failure or low volume (30% 1RM) work matched to resemble high volume to failure (Burd et al., 2010). Furthermore, when discussing muscle molecular adaptations, it is key to distinguish between acute and chronic RE as the two molecular responses differ between the acute, predominantly single session interventions and the long term coordinated training protocols, where varying RE elements are arranged in order to achieve the desired objectives such as hypertrophy (Francaux and Deldicque, 2019).

A number of rodent studies have shown that HS is capable of inducing anabolic synthesis response and thereby improving the muscle protein content in the skeletal muscle as well has facilitate the recovery of injured muscle as well as attenuate or recover atrophied muscle (Kobayashi, 2003, Uehara et al., 2004, Goto et al., 2004, Kojima et al., 2007). Yoshihara and colleagues indicated that HS activates anabolic synthesis pathways in the rodent skeletal muscle and the activation is potentially temperature dependent (Yoshihara et al., 2013). Conversely however, Frier and Locke indicated that HS may attenuate hypertrophy in the rodent skeletal muscle (Frier and Locke, 2007). In humans, the evidence is limited for HS driven induction of anabolic synthesis limited. However, in an extensive recent investigation Ishan and colleagues demonstrated that acute HS (discussed in detail below), especially full body HS is capable of instigating anabolic synthesis in the skeletal muscle (Ihsan et al., 2020).

The flow of information from load as well as heat sensing to subsequent protein synthesis can be broken down to three separate steps. The molecular response occurs following the activation of the signal transduction cascade and during the effector process. The first expression response is marked by the elevation of the signal transduction pathway molecules. In the signal transduction phase, several molecular mechanisms are at play (Wackerhage, 2014, Song et al., 2017). Deciphering the often overlapping mechanisms for each key complex of molecules as well as how precisely is the mechanical signal converted to a chemical signal is at the forefront of the expanding research scope in molecular exercise physiology and by extension the broader discipline of exercise sciences.

Of the so far recognised regulatory pathways that drive hypertrophy, the mammalian/mechanistic target of rapamycin (mTOR) pathway is the most crucial (Wang and Proud, 2006, Goodman et al., 2011, Hornberger, 2011, Song et al., 2017). It plays a role in both protein synthesis as well as protein degradation. It has been observed that a decrease in mTOR signalling is associated with muscle atrophy (Goodman et al., 2011). Based on the existing body of research, the mTOR pathway is both necessary and sufficient to trigger adaptive responses (Goodman et al., 2010). Central to the mTOR pathway is the mTOR protein complex which manifests in two major variations, mTORC1 and mTORC2 (Goodman et al., 2011). Each complex is a unique set of proteins with mTOR in common and each complex is activated by and responds to unique combinations of stimuli and is localised in different cellular compartments (Laplante and Sabatini, 2012, Song et al., 2017). The stimuli could be standalone or a combination of inputs such as amino acids, growth factors, insulin as well as by the overloading of the muscles by intense exercise. mTORC1, commonly localised at the lysosome, is more central to the muscle

molecular response to RE (Wackerhage, 2014, Song et al., 2017). It has been well established that the mTOR activation occurs via GDP bound Ras homologous enriched in brain (Rheb) (Betz and Hall, 2013).

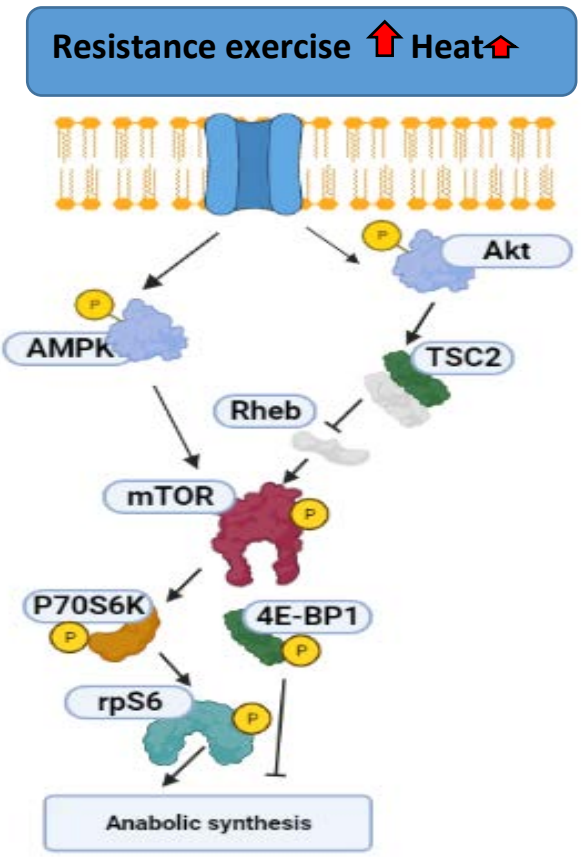


Figure 1.1 A simplified diagram of mTOR driven anabolic synthesis pathway. Scale of the red arrows indicates the strength of each stressor to induce synthesis (not to scale).

1.6 Anabolic synthesis

1.6.1 mTOR

As was established prior, RE and HS both drive phenotypic adaptations such as hypertrophy which is the endpoint outcome of increased MPS driven at large through mTOR pathway. In an early *in vitro* study Goto and colleagues conditioned four batches of rat myoblast cells (L6- 90% cultured cells had induced myotubes at the point of conditioning) with either 97 hours of incubation at 37°C (unstressed control), one hour incubation at 41° C followed by 96 hours at 37 °C, one hour incubation at 37°C with a subsequent 96 hours of cyclic stretching or one hour incubation at 41°C followed by 96 hours of cyclic stretching. They reported significant elevations of total muscle protein content at all three test conditions by 20%, 13% and 28 % ($p < 0.05$) respectively. This a clear cumulative effect of HS pre mechanical stretching was indicated over

the standalone effect of each stressor (Goto et al., 2003). While the total protein content elevation under HS was higher than that of mechanical stretch, the difference was not significant. However, the results illustrate the significant proteins synthesis response by muscle to load and HS.

It has been reported that localised acute HS has the potential to enhance mTOR signalling after a bout of low intensity RE in humans (Kakigi et al., 2011). It is evident, though limited in the case of HS, that this key pathway is triggered by both the stress modalities utilised in this study. The investigation of the levels of mTOR by prevalence, to high intensity RE (performed to muscular failure) applied concurrently with targeted exogenous HS of varying intensities to drive muscle adaptations contribute to and expands on the current knowledge body significantly. In their seminal report, Kakigi and colleagues reported that, HS enhances mTOR signalling in human muscle following RE. A recreationally active male cohort (n = 8, 22.3 ± 0.7 years) was tested three weeks apart in a randomised crossover manner for the effects of HS on an acute bout of one-legged knee extensions (four sets of six repetitions). One leg received the RE bout and the contralateral leg received the exact RE intervention pre- treated with targeted HS to the *vastus lateralis* (time not cited, muscle temperature 41.1°C). They received nutrient identical meals prior to test day. The exercise intervention was conducted in a fasted state (Kakigi et al., 2011). Control biopsies were withdrawn ten minutes prior to the RE intervention from the non-treated leg. Post treatment biopsies were withdrawn from the treated leg immediately and at hour post-RE. The authors reported significant changes from baseline in phosphorylation levels of mTOR at site Ser2448 ($p < 0.05$) at one hour post-RE in the heat-treated leg compared to RE alone. Although phosphorylation levels increase during the recovery phase following RE, the phosphorylation levels at one hour post-HS were significantly higher compared to one-hour post-RE alone. Since, the positive relationship between increased mTOR phosphorylation and MPS is already established, this is a critical result that indicates a clear HS driven auxiliary effect on mTOR (Kakigi et al., 2011). Fuchs and collgues tested if hot water immersion following a single bout (45 minutes) of RE (leg press and extensions to failure) increases post exercise MPS in a cohort oft young males (n = 12, mean age 23 ± 1 years) (Fuchs et al., 2020).Immediately, post exercise, one leg was immersed in 46°C water while the contralateral control leg was put in 30°C for 20 minutes. Biopsies were extracted at two and five hours from the *vastus lateralis*. They cited a CMT of ~37.5°C at a depth of ~4.5cm in the heated leg immediately after heating (Fuchs et al., 2020). They reported no difference between heated and control in myofibrillar protein fractional synthesis rate (measured via L-phenylalanine and L-leucine tracers) at two or five hours. Moreover, total protein phospho-mTOR (Ser2448) levels were not different between the

groups at two or five hours (Fuchs et al., 2020). Using HS as the lone stimulus, Yoshihara and colleagues reported that a phosphorylation response on Ser2448 of mTOR occurred in the hind legs of male rats were immersed in hot water (41°C) for 30 minutes. However, this response was not significantly greater from baseline in overnight fasted conditions (Yoshihara et al., 2013). More presently, Ihsan and colleagues tested the muscle molecular response to localised (single leg, *vastus lateralis)* and full body acute HS applied for one hour on an active male cohort w (n = 9, mean age 35 ± 4 years). All participants underwent both heat treatments, in a counterbalanced design, a week apart in a fasted state (Ihsan et al., 2020). Biopsies obtained at pre, at 30 minutes and 180 minutes, with control biopsies extracted from the contralateral leg during single leg treatment. Full body HS was applied at 50°C (50% RH) via environmental chamber, maintaining core temperature at approximately 39°C. In addition, localised HS was provided via a full leg water perfusion sleeve with mean final contact temperature at 45.3 ± 2.2°C, achieving mean temperatures 38.1 ± 0.6°C (Ihsan et al., 2020). The researchers reported a significant 64% increase of total phosphorylation levels of mTOR in the full body treatment group when compared to the control ($p = 0.038$) and no significant increments in localised HS. While the acute full body treatment observations further supports the previous reports on HS driven mTOR activation, the non-significant response to acute localised HS indicates the existence of a threshold muscle temperature, given Kakigi and colleagues reported significant changes in mTOR Ser2448 phosphorylation levels at >41°C compared to <39°C in this investigation. Notably however, the intervention does not include an RE element. However, the number of studies that investigate acute or chronic mTOR response in terms of total protein content, phosphorylated content to HS or a combination of RE and HS are extremely limited and warrants more investigation. Especially in context of chronic, concurrent HS applied with RE.

mTORC1 and mTORC2 are regulated by different sub-pathways and for mTORC1 to form, it requires activation by Rhas homologous enriched in brain (Rheb), a small guanosine triphosphate binding protein, and the best characterised positive regulator of mTOR (Wackerhage, 2014, Sancak et al., 2008, Song et al., 2017). Therefore, it is a critical component, and a potential limiting factor in the mTOR signal transduction pathway. Rheb is centrally involved in fully activating mTOR (via driving phosphorylation of Ser2448) complex post translocation to the lysosomes (Song et al., 2017, Inoki et al., 2003a). Rheb directly binds the catalytic domains of mTOR (Sarbassov et al., 2005, Song et al., 2017). However, in basal, non-stimulated conditions, Rheb is negatively regulated (via GDP bound state) by the tuberous sclerosis complex (TSC1-TSC2). In the absence of either protein, mTOR is hyper activated by Rheb (Sancak et al., 2008). Furthermore, It has been reported that the over-expression of Rheb GTPase can overcome the

inhibitory effects of the absence of leucine and that it plays a key role in the activation of key regulators of protein synthesis and cell growth, downstream of mTOR such as P70S6K and 4E binding protein 1 (4E-BP1) (Long et al., 2005, Inoki et al., 2003a). Song and colleagues assigned a recreationally active male cohort (n = 14, age 25 ± 2 years) in to two RE groups (n = 7 in each), a non-feed group and one receiving a protein-carbohydrate beverage post exercise (10 minutes). A control biopsy was extracted prior to the intervention from the *vastus lateralis*, followed by immediate and 10 min -post biopsies (from the same leg) following the RE intervention of five set each of leg press and knee extensions of eight to ten repetitions until failure, and two further biopsies (from the contralateral leg) at one and three hour marks after the feeding. Interestingly, they reported a ~30% increase in Rheb-mTOR co-localisation level in both fed and non-fed groups at the 10 min time point. These results indicate similar activation levels of mTOR by RE compared to RE combined with an amino acid rich nutrient intake at immediately acute muscle molecular response. They further reported a ~20% decrease in Rheb-TSC2 association over the three hour recovery period, alluding to the negative regulatory influence of the TSC complex (Song et al., 2017). However how HS or HS in combination with RE effects the expression of Rheb has not been focused on in previous studies.

1.6.2 TSC2

It is evident that Tuberous sclerosis complex 2, a GTPase-activating protein (TSC2) is a central regulator in the load sensing mTORC1 sub- pathway, given its role in regulating Rheb. TSC2 is upstream of mTOR and Rheb and requires activation via phosphorylation by AMP activated protein kinase (AMPK) and is directly and negatively regulated by Akt (Inoki et al., 2003b, Inoki et al., 2003a). So far in the body of research, it has been well established that TSC2 negatively regulates the key protein synthesis and cell growth mediators, ribosomal protein S6 kinase (P70S6K) and 4E-BP1 via the inhibition of Rheb (Inoki et al., 2003b, Wackerhage, 2014). Phosphorylated AMPK has been found to transfer the phosphate group to TSC2 deactivating it, thereby, allowing the phosphorylation of Rheb (Dreyer et al., 2006b). As stated above, Song and colleagues in their seminal study, reported that following an acute bout of RE lead to reduced levels of TSC2 near the cell membrane, concurrently dissociating from Rheb (Song et al., 2017). According to Jacobs and colleagues, mice subjected to eccentric contractions of the lower legs via stimulation of the sciatic nerve (left stimulated, right control) showed 20% ($p \leq 0.05$) reduction of localisation near the membrane (Jacobs et al., 2013) which is supported by the work of Song et al (Song et al., 2017). However, the total content of TSC2 was not altered significantly, but the levels phosphorylation increased (> 6 fold from basal) indicating deactivation and

dissociation (Jacobs et al., 2013). Interestingly, it has been noted that during RE through a yet unidentified mechanosensor, TSC2 is inhibited to promote mTORC1 activation (Wackerhage, 2014, Philp et al., 2011). However, the effect of HS or HS in combination with RE on TSC2 responses has not been investigated before in the human skeletal muscle.

1.6.3 Akt

Akt (also known as protein kinase B PKB) is expressed directly upstream of TSC2 and is a main negative regulator of mTOR via its phosphorylation. It is also a key downstream target of PI3K and is central to the activation of mTOR, via its phosphorylation and subsequently forming the Akt/mTOR complex. This complex is key in transducing the mechanical load signal generated by RE. It has also been reported that the Akt/mTOR pathway plays a key regulatory role in attenuating muscle atrophy (Bodine et al., 2001). The influence of localised HS on Akt phosphorylation (Ser473) was further established by Yoshihara in their rodent study, where they reported significant increments from basal (< 37ºC) at 37ºC (~0.7 fold change), 39ºC (~0.7 fold change) at 41°C (~1 fold change). At 41°C a significant increase was further observed from 37 ºC (~0.5 fold change) ($p < 0.05$) (Yoshihara et al., 2013). Camera and colleagues reported a ~100-200% increase in Akt phosphorylation levels at sites Thr308/Ser473 ($P < 0.05$, mean age 28.4 ± 1.6 years) at 30 minutes and one hour post-exercise respectively, in a group of recreationally active males (n = 8), after an acute RE intervention eight sets of five repetitions of seated leg extensions (Camera et al., 2010). Interestingly however, Kakigi and colleagues did not observe a significant increase in Akt phosphorylation levels one-hour post an acute lower body RE intervention (method outlined above). However, the authors reported a significant increase ($P < 0.05$) in Akt phosphorylation at one-hour post-exercise when the RE bout was preceded by 20 minutes of localised acute HS (Kakigi et al., 2011). The difference in phosphorylation levels to RE is intriguing given the interventions and the anthropometrics of the groups are similar. It is possible that the lower total volume in the latter is the cause. Camera's eight sets of five repetitions compared Kakigi's four sets of six repetitions. Phosphorylated Akt levels in skeletal muscles have also shown to increase after 60 minutes of full body acute HS, 58% from basal ($P < 0.02$) and the total Akt content by 93% from pre-treatment ($p < 0.01$) (Kakigi et al., 2011). Interestingly however, the authors did not report any significant changes for acute localised HS (Ihsan et al., 2020). While these observations establish that the expression and phosphorylation levels of Akt increases with temperature and the temperature dependant activation can possibly augment the effect acute RE, there is significant degree of uncertainty.

Furthermore, the effects of repeated bouts of concurrent HS and RE on the Akt response remains to be investigated.

1.6.4 AMPK

AMPK is a key marker in the MPS pathway as it has been referred to as a molecular gauge in determining cellular energy status (Hardie and Carling, 1997, Dreyer et al., 2006b). AMPK is heterotrimeric and contains a catalytic (α- in two isoforms 1 and 2) and two non-catalytic subunits (β- in two isoforms 1 and 2,γ) (Hardie et al., 1998). It has been reported to be upregulated significantly in rodents during and acutely post aerobic exercise, nerve stimulated muscle contractions as well as *in vitro* mechanical contractions (Fujii et al., 2000). In a seminal report, Fuji and colleagues observed a two and a 2.5 fold significant increase (from baseline) in AMPKα2 in humans but saw no difference in α1, in a mixed cohort of (n = 7) individuals exercising (ergometer) at 70% VO_2 max for 20 minutes and 1 hour, respectively. They did not however, observe an elevation in α1 levels at the same time points nor did they observe a similar pattern at 50% VO_2 max, indicating an intensity driven and isoform specific nature of AMPK activation (Fujii et al., 2000). The intensity or a high stress-activation correlation has been further confirmed by Chen and colleagues where they reported fivefold and eight fold elevations during an hour of medium and high intensity ergometer cycling (60% and 80% of VO_2 max respectively) (Chen et al., 2003). In an acute bout of RE (ten sets of ten repetitions of leg extensions (70% 1RM), Dreyer and colleagues observed that AMPKα2 activity increased significantly immediately post (75% from baseline, $p < 0.05$) and one hour following (increase not reported) ten sets of ten repetitions of leg extensions (70% 1RM) in a mixed cohort of participants (n = 11). They did not observe changes in α1 levels (Dreyer et al., 2006b). Based on these observations, it is evident that AMPKα2 is activated and increased acutely following both endurance and RE while AMPKα1 showed no change. Interestingly, Kakigi and colleagues noted that after an acute bout of RE (four sets of six repetitions of knee extensions), activated AMPK (phosphorylated at Thr172) did not improve at one hour post from baseline in a cohort of male (n = 8). More intriguingly, they observed a similar lack of elevation when the same cohort underwent an identical bout of RE post a 20 minute, localised application of HS to the same leg (*vastus lateralis*) (Kakigi et al., 2011). Collectively, these results indicate a lack of activation via RE and a lack of additive effect from HS when combined with RE which contradicts the earlier observations. The contravening nature of the available information on AMPK and the limited body of work available on the response to HS combined with RE makes it a key target for further investigation

1.6.5 P70S6K

P70S6K is a direct downstream target of mTOR that is central in the Akt/mTOR complex signal transduction. It has been a key correlative marker associated with increasing muscle mass following RE (Baar and Esser, 1999, Kumar et al., 2009). In the process of signal transduction subsequent to load sensing, the above mentioned mTORC1 complex directly phosphorylates P70S6K (Thr389). This activates P70S6K as a translation initiator (Camera et al., 2010) however, the exact mechanism of how this process occurs is yet to be fully elucidated (Wackerhage, 2014). Furthermore, mTORC1 plays an inhibitory role blocking elongation regulatory factor eEF2, to further promote translation elongation (Naito et al., 2012). Terziz and colleagues tested a cohort of (n = 10) males split into long term RE intervention (n = 6) and sedentary control (n = 4) group and observed a several fold (as reported) increase in phosphorylated P70S6K (Thr389) 30 minutes post the initial RE bout session comprising nine upper and lower body RE. Furthermore, the authors reported high correlations between fat free whole body and leg muscle mass ($r = 0.89$ and $r = 0.81$ respectively, $p < 0.01$) as well as the type IIa fibre CSA ($r = 0.82$, $p < 0.05$) and the initial elevation of P70S6K (Thr389) after 14 weeks RE (three sessions a week). Thus, these results strongly indicate a central role in RE driven protein synthesis (Terzis et al., 2008). Mitchell and colleagues however, did not observe an increase immediately, at 30 minutes or at one hour post exercise in the phosphorylation levels of P70S6K (Thr389) after an acute bout of lower body RE (Mitchell et al., 2013) which contradicts the findings of Terzis (Terzis et al., 2008). However, the authors did report a significant increase in the phosphorylation levels of phospho-P70S6K (Thr389) at five hours post training from baseline (~ 1.5 fold change). Interestingly however, it has been reported that phosphorylated levels of P70S6K increased significantly, at both Thr389 and Ser424/Thr421 one hour post an acute bout of RE preceded by a 20 minute application of HS to the *vastus lateralis* (~fold change 1.5, $p < 0.05$) (Kakigi et al., 2011). Critically, the levels of phosphorylation levels in the combinatory stress group was significantly higher compared to the RE only group ($p < 0.05$) (Kakigi et al., 2011). This fold change is comparable to Mitchell's findings at five hours post, possibly indicating a cumulative effect of RE and HS. Further clouding the available body of work on P70S6K, in a more recent investigation, Fuchs and collgues saw P70S6K (Thr389) levels decrease significantly immediately after bout of hot water immersion following RE compared to a control leg, but increase significantly over control at two and five hours ($p < 0.05$).However, they saw no differences in Ser424/Thr421 levels between heated and control groups. Heated leg showed a peak CMT of ~37.5°C immediately after treatment (Fuchs et al., 2020). Ihsan and colleagues reported the effects of localised and whole-body HS on the

activation of P70S6K. In a crossover design, a group of (n=9) males underwent full body HS (50°C, 50% RH maintain a core temperature of ~39°C) and localised HS to the *vastus lateralis* one week apart for one hour in each instance. They found a 174% increase from baseline in phospho P70S6K levels at 30 minutes post intervention for the full body HS intervention and found no such elevations in the localised HS iteration at the same time-point. They did not report whether the phospho-P70S6K (Thr389) levels also increased during the full body heat intervention (Ihsan et al., 2020). After reviewing the current findings, the response of P70S6K, especially in the case of HS combined with RE remain ambiguous.

1.6.6 4E-BP1

Another key downstream target of mTOR (mTORC1) is 4E-BP1. It is a parallel initiation factor to P70S6K that has been found to play a key role in RE driven protein synthesis (Fingar et al., 2002). It has been reported to supress mRNA suppression when in its non-phosphorylated form and is phosphorylated (multiple site) in to deactivation via the mTOR/Akt pathway (Gingras et al., 1998). Kumar and colleagues reported that after a bout of RE, in young men, the levels of phosphorylated 4E-BP1 (site not reported) increased significantly (~25%) one hour post intervention ($p < 0.05$). In contradiction, Camera and colleagues observed that phosphorylated levels of 4E-BP1 (Thr37/46) did not elevate at one hour post RE intervention, they further reported no significant elevations of phosphorylation at immediate post, 15 minutes, 30 minutes and one hour post at Thr70 after the same RE intervention (Kumar et al., 2009, Naito et al., 2012, Camera et al., 2010). Furthermore, it has also been observed that after a bout of one-legged knee extensions (isokinetic concentric contractions), where HS was applied before and continuously during the exercise (microwave), the levels of 4E-BP1 decreased during the post exercise period. The decrease was significantly lower compared to RE only instance (Kakigi et al., 2011). Interestingly, it has been also suggested that 4E-BP1 might be playing an inhibitory role against protein synthesis during RE (Kakigi et al., 2011). This discrepancy, along with the above outlined key positioning of 4E-BP1 in the mTOR pathway, as well as the fact that 4E-BP1 has been linked, along with P70S6K in a role increasing mammalian cell size (Fingar et al., 2002), makes it a critical factor to investigate in the protein synthesis pathway. Importantly, the role of 4E-BP1 merits investigation as a factor in its response to acute and chronic RE combined with full body concurrent HS.

1.6.7 Ribosomal biogenesis

Ribosomes are a key component in the protein synthesis pathway and act as the translational apparatus. The synthesis of ribosomes is a highly regulated and specific process within cells

(Figueiredo et al., 2015). While some rodent studies have established the involvement of ribosome biogenesis in muscle hypertrophy (Wen et al., 2016) only a very limited amount is known about how its modulated by active mechanical loading in humans. It has been proposed to be mTOR dependent for activation (von Walden et al., 2012). Key among molecular markers that have been investigated to quantify the effect on ribosomal biogenesis are ribosomal protein S6 (rpS6 or S6rp), phospho-rpS6 (Ser235/236) as well as cell cycle regulating cyclins (Cyclin D1) (Voit et al., 1999, Wilson and Cate, 2012). Figueiredo and colleagues investigated the effect on ribosomal biogenesis by a single bout of RE as well as the chronic effects of RE. In a cohort ($n = 14$) of males, they observed a fold change of ~4.5 from baseline in phsospho-rpS6 (Ser235/236) ($p < 0.05$) in both 70% and 90% 1RM groups one hour post an acute of RE consisting of leg press, knee extensions/flexions (four sets of each to failure). Furthermore, they reported a fold change of 4.2 from baseline in phospho-rpS6 (Ser235/236) ($p < 0.05$) after eight weeks of lower body RE (two sessions a week). Moreover, they reported a fold change of 1.9 from baseline in Cyclin D1 both post-acute and chronic RE interventions (Figueiredo et al., 2015). In a second report, they observed that in a cohort ($n = 9$) young males, who underwent an acute lower body RE bout comprising of leg press, knee extensions and walking lunges (three to six sets until failure), Cyclin D1 increased significantly at time points two hours, 24 hours and 48 hours (Figueiredo et al., 2016). The absolute fold change numbers have not been reported. Interestingly, they reported that ten minutes of cold stress (cold water immersion) prevented significant elevations at the same points (Figueiredo et al., 2016). It is evident however, that RE does increase ribosomal biogenesis signalling. However, the body of work investigating the effects of HS or the combined effect of HS and RE is very limited. Ihsan and colleagues reported that the levels of phospho-rpS6 (Ser235/236) increased by 302% ($p=0.0038$) after a bout of whole body HS 50°C 50% RH (method outlined above) for one hour. In contrast, the same level of elevations were not observed after an acute bout of localised HS for the same period of time (Ihsan et al., 2020). Interestingly, in an earlier study, Kakigi and colleagues reported (study outlined above) that phospho-rpS6 (Ser235/236) levels increased significantly following an acute bout of RE preceded by an acute localised bout of HS (20 minutes). This response was significantly higher compared to the response to RE bout alone at the same time point ($p < 0.05$), implying an additive effect on the phosphorylation of rpS6, thereby ribosomal biogenesis by HS (Kakigi et al., 2011). However, the effect of chronic RE combined with HS on ribosomal biogenesis has not been investigated prior.

1.6.8 Ion Channels (Ca^{2+} response)

Transient receptor potential (TRP) channels (ion channels) will be another key focus of this study. This family of channels are responsible for sensing physiological changes in the tissues such as pH and temperature and triggering intracellular pathways such as mTOR, in response by allowing ions, predominantly calcium (Ca^{2+}) into the cell (Hudson et al., 2016). It has been found that eleven TRP channels are highly sensitive to temperature. Among these thermos-sensitive channels, eight are activated by heat. Importantly, these receptors are polymodal, and are therefore activated by other stimuli besides heat, such as physical exercise (Hudson et al., 2016). Transient receptor potential vanilloid type (TRPV1) is one such polymodal receptor that carries significance in the context of this study. They are activated by high temperatures (~40^{o}C) (Hudson et al., 2016), and have been found to be present in skeletal muscles (Lotteau et al., 2013). Therefore, the level of prevalence of this receptor is a key indicator of the molecular level muscle adaptions driven by HS and RE. Regardless of this tether, there have not been any studies that have investigated the potential key relationship between TRPV1 and mechanical stress combined with HS. However, Ca^{2+} influx following RE is associated with triggering muscle protein synthesis (MPS). Ito and colleagues have shown clearly that activation of Ca^{2+} via TRPV1 has the ability to trigger hypertrophy in rat skeletal muscle (Ito et al., 2013b, Ito et al., 2013a) NFATc1, Calcineurin is centrally involved in this pathway. It has been observed that Ca^{2+} activated calcineurin then activates NFATc1 to drive hypertrophy in cardiac muscles. The calcium influx (spike) depending on the frequency, duration of the influx and spatial distribution can carry a multitude of information (Crabtree, 1999). It has been suggested that during RE Ca^{2+}/calmodulin activated calcineurin subsequently activates NFATc1 translocating it in to the nucleus which then promotes the transcription of genes that drive muscle adaptations. It has been observed that HS also has the ability to trigger Ca^{2+} response that drives NFATc1 translocation (Naito et al., 2012). Therefore, these markers, especially TRPV1, present themselves as key targets to be investigated for the impact of chronic, concurrent HS during RE.

1.7 Heat shock protein response

In addition to the above outlined key junctures in the signalling process, the direct cellular response to HS is mounted via heat shock proteins (HSP) also known as stress proteins. HSPs are induced by temperature predominantly, and also by other stresses such as oxygen free radicals, transition heavy metals, metabolic inhibitors and amino acid analogues. These proteins are expressed in all cells as a response to proteotoxic stressors and by extension cytotoxic stressors (Morimoto, 1994, Becker and Craig, 1994, Skidmore et al., 1995). They have been

identified as a vital component in the process of protein synthesis. They are involved in a chaperone capacity (cyto-protective) in protein formation, maturation degradation as well as folding and translocation and assembly, effectively shielding other proteins from potential denaturing from heat (Kim et al., 2006, Morimoto, 1998). The precise mechanism by which the cell senses the stress to consequently trigger the HSP response remains unclear.

HSPs are generally identified in two different groups, small and large, based on their structural (molecular weight) and biochemical properties. The most cyto-abundant HSPs have been identified as HSP60, HSP70 and HSP90 (Becker and Craig, 1994). Among these the HSPA superfamily (HSP 72) containing of 13 members (Kampinga et al., 2009, Krüger et al., 2019) is the most widely investigated. HSP72 is the most abundant inducible form of HSP70 in mammalian cells (Brown et al., 1993).

It has been reported that HSP72 binds other proteins, especially unfolded proteins, regardless of the cellular loci. HSP72 expressed in endoplasmic reticulum of the mitochondria have been posited to bind other precursor proteins with a high degree of exclusivity, enabling translocation of the primitive proteins it binds (Haas and Wabl, 1983). Furthermore, it has been found that this binding also pertains to stabilising denatured or misfolded proteins (Bole et al., 1986). The ability of HSP72 to bind and subsequently sequester from its target at different location of the cell determines its significance in critical cellular processes such as protein synthesis and cyto-protective responses to stress insults (Becker and Craig, 1994, Brown et al., 1993). A supposition for the increased levels of HSP72 during stressors such as heat and RE is that free residing HSP70 binds the denatured proteins from the stress, and the depletion of available HSPs within the cell (Abravaya et al., 1992). It has been reported that HSP70 might be well suited as a biomarker to examine the thermal stress history of the cell, given its sensitivity as a stress response (Ryan et al., 1991). Moreover, it has been shown that the loss of inducible HSP72 attenuates muscle regeneration in the in the rat skeletal muscle and overexpression prevents muscle atrophy (Senf et al., 2013, Senf et al., 2008). Indicating a key role in skeletal muscle growth adaptations. The acute HSP72 response to HS is well established. Ogura and colleagues reported that four males treated with 20 minutes of microwave hyperthermia (2.5 GHz, 150W) at the *vastus lateralis* improved HSP 70 (72) levels significantly ($p < 0.05$) (Ogura et al., 2007). Skidmore and colleagues attempted to delineate the response to HS from the response to exercise in rats. They allowed a subset of rats to exercise (treadmill running) at ~14ºC (cool) to eliminate the effect of the increasing core temperature. They reported two-threefold increase in HSP72 levels compared to the sedentary control group indicating that exercise (endurance) is an instigating stressor for

HSP72 confirmed by the unchanged colonic temperature during the intervention (Skidmore et al., 1995). Morton and colleagues found that in a cohort (n = 8) of young males who completed 45 minutes of non-damaging treadmill running, HSP72 content increased significantly at 48 hours ($p < 0.05$, 179%) and at seven days (178%) from pre intervention (Morton et al., 2006b). They further reported marked individual variation among the cohort (Morton et al., 2006b). This response had previously been identified to be in a protective capacity (Kilgore et al., 1998). It's prevalence in cells during and after a combination insult of HS and exercise has been noted to be higher as well as longer lasting (Goto et al., 2004). Interestingly however, Morton and colleagues, in a subsequent study reported that muscle temperatures raised to levels comparable to exercise (3.6 ± 0.5ºC) by lower body hot water immersion (one hour at 45ºC) in a male cohort (n = 7), did not improve HSP70 levels at time points at 48 hours and 7 days (Morton et al., 2007). Moreover, Hafen and colleagues saw HSP72 levels increase significantly after ten and six days of repeated HS to the *vastus lateralis*, with peak CMT reaching ~40°C (Hafen et al., 2018, Hafen et al., 2019). It has also been reported that the deletion of certain HSP72 group proteins affect the calcium (Ca^{2+}) handling and contractile ability of the cardiac muscle (Kim et al., 2006). While a clear role in growth response has not been elucidated for HSP72, other than its role as a chaperone, it has been indicated that HSP70 may have a role in fibre type shift during hypertrophy. (Locke et al., 1994, Tsika et al., 1987, Folkesson et al., 2013). Folkesson and colleagues tested a mixed cohort (n = 26) split into resistance trained (n = 6; 11 ± 6 years of training experience) endurance trained (n = 8; 8.3 ± 2.1 years of experience) and healthy active (n = 12; recreationally active) groups for basal levels of HSP72. They reported that while no difference were observed between the groups, significant differences were observed between type I (197 ± 7 pixel intensity (PI)) and type II (202 ± 8 PI; $p = 0.01$) (Folkesson et al., 2013). Therefore, HSP72 bears a key importance in being investigated in response to chronic concurrent HS and RE.

HSP60 is another subset of HSPs that are critical in the stress response process (Bukau and Horwich, 1998), HSP60 is found predominantly in the mitochondrial reticulum (approximately a third have been reported to be found in extra-mitochondrial locales). It has been defined as an intra-mitochondrial protein and a critical component in cell viability, especially under stress (Marino Gammazza et al., 2018). In eukaryotes, it has been reported that HSP60 plays a similar role to HSP70 in mitochondria, stabilising nascent proteins and it appears to be playing a similar role within the cytosol (Becker and Craig, 1994). The number of studies that have investigated the HSP60 activity in human skeletal muscle is very limited. Morton and colleagues reported

that a male cohort (n = 7) locally stressed via one hour of lower body single leg hot water immersion (45ºC) did not show an improvement in levels of HSP60 at 48h or 7 days post heating (Morton et al., 2006b). In a subsequent study, they reported that while active (endurance) athletes have higher basal HSP60 levels compared to a sedentary cohort, they did not improve HSP60 levels post a non-damaging treadmill run at 48 hours or seven days (Morton et al., 2008). Hafen and colleagues did not see improvements in the HSP60 levels after six repeated consecutive bouts of HS to the *vastus lateralis* (Hafen et al., 2018). Folkesson and colleagues tested a mixed cohort (n=26) split into resistance trained, and healthy active groups for basal levels of HSP60. They reported (measured via fluorescent densitometry), no differences between any of the test groups or fibre type specific expression levels (Folkesson et al., 2013). However, given the ubiquitous nature of expression of HSP60 in the skeletal muscle as well as its role as chaperone (in mitochondria), it is a key HSP target to be investigated against chronic concurrent HS combined with RE.

HSP90 is the third group highly conserved large HSPs found in all cells and remains a much less investigated class compared to HSP60 and HSP72. It has been reported that HSP90 plays chaperone role in binding and folding nascent proteins in to their native conformation (actin and tubulin) (Becker and Craig, 1994). It has been reported that HSP90 maintains HSF-1 (discussed later in the section) in an inactive state (Anckar and Sistonen, 2011, Zou et al., 1998). HSP90 also has been especially identified to be a designated chaperone, when the nascent protein is a product of a stress induced DNA alteration (Krüger et al., 2019). Ogura and colleagues reported that after 20 minutes of microwave hyperthermia, HSP 90 levels increased significantly ($p < 0.05$) at the *vastus lateralis* (Ogura et al., 2007). Hafen and colleagues saw HSP90 levels increase in the *vastus lateralis* after ten as well as six days of localised HS at peak CMT of ~ 40°C (Hafen et al., 2018, Hafen et al., 2019). However, the body of work on HSP90, especially with regards to exercise is extremely limited and has not been investigated in the context of chronic HS and RE.

Another two major HSPs that are important in the adaptive stress response in the skeletal muscle are HSP27 and αB-crystalline, recognised in the subclass of small heat shock proteins. HSP27 and αB-crystalline are involved in cyto-protection and is ubiquitously expressed in a majority of bodily tissues (Koh, 2002). It has been supposed that similar to large HSPs, HSP27 and αB-crystalline can bind partially denatured proteins and aid in proteolysis. Ogura and colleagues reported that after 20 minutes of microwave hyperthermia HSP27 and αB-crystalline levels increased significantly ($p < 0.05$) at the *vastus lateralis* (Ogura et al., 2007). An important role

has been assigned to HSP27 and αB-crystalline in short term cyto-protection from exercise induced muscle damage. They (αB-crystalline predominantly) have been reported to localize around the z-disks after stress and interacts with actin and desmin protecting the cytoskeleton and remodelling the contractile components during high-stress eccentric overloading (Koh, 2002). Thompson and colleagues tested a mixed cohort (n = 8), who performed 25 repetitions (x 2 sets; dynamometer) maximal eccentric only bicep curls on the non-dominant arm and reported that at 48 hours post-exercise HSP27 levels increased by 234% ($p < 0.01$) from pre intervention in the *biceps brachii* (Thompson et al., 2001). Folkesson and colleagues had two recreationally active male cohorts undergo an acute endurance (n = 6; one-legged ergometer cycling, 30 minutes, at two intensities 6-9 days apart) and RE protocol (n = 9; leg extension at 70% 1RM, ten sets of eight repetitions) respectively and extracted biopsies from the *vastus lateralis* immediately pre and post interventions (whether the protocols were randomized was not reported). They reported granular accumulations of HSP27 in RE but not in the endurance exercise cohorts. They further reported HSP27 relocation mainly appeared in type II fibres (Folkesson et al., 2008). Interestingly, the same authors further reported that in three cohorts, highly resistance trained, highly endurance trained and recreationally active individuals, HSP27 levels (measured via densitometry) showed a significantly higher levels of intensity in type II fibres (207 ± 10 PI) compared to type I (212 ± 6 PI, $p = 0.02$) in both active and resistance trained (type I; 223 ± 4 PI, type II; 218 ± 7 PI, $p = 0.04$) groups but not in the endurance group. The opposite pattern was observed in the same cohort for αB-crystalline where type I fibres showed significantly high level over type II in both active and resistance trained groups but not in the endurance group (active type I; 173 ± 5 PI verus type II; 189 ± 7 PI $p = 0.003$, resistance type I; 215 ± 6 PI versus type II; 224 ± 5 PI, $p = 0.04$). The levels of both proteins were similar in each fibre type for endurance athletes (Folkesson et al., 2013). While the results here contravene the findings of Morton and colleagues (Morton et al., 2008), who found a higher levels of αB-crystalline in endurance athletes, it could be due to the higher level of oxidative type I fibres in their cohort. Regardless, these intriguing observations indicates the fibre type and activity specific expression of HSP27 and αB-crystalline and indicates training specific expression patterns. (Vicart et al., 1998). It has further reported that post translational modifications such as phosphorylation occurs during mechanical loading via MAP kinase activation. These modifications appear key to the oligomerisation, which appears to be the natural state of the majority of small HSPs in unstressed cells (Koh, 2002). The evidence is conflicting as to whether HSP 27/αB-crystalline phosphorylation is contributory towards cyto-protection or weather

phosphorylation plays a different role. Furthermore, it has been reported that phosphorylation HSP27 may play an important role in regulation of microfilament dynamics following oxidative stress and maybe an adaptive response in cyto-protection (Huot et al., 1996). It has also been reported that in the human lens (eye), phosphorylation of αB-crystalline may influence its chaperone like activity negatively (Kamei et al., 2001). Therefore, could be a potential marker for strength of cyto-protective response. The exact role of the modifications during exercise remains to be elucidated (Koh, 2002). Furthermore, the effects of post translational modifications (phosphorylation) of small HSPs to HS and RE has not been previously investigated.

It has been established that the heat shock genes are under the auto-regulatory control of a family of DNA binding proteins identified as heat shock transcription factors (HSF). Therefore, stress induced gene transcription requires the activation of HSFs. It has been found that larger mammals express multiple HSFs. HSFs 1, 2 and 4 appear to ubiquitous among vertebrates. The function of these HSFs seems to share a redundancy, differentially controlling and regulating the heat shock protein transcription. It has been reported that, *in vivo,* fibroblasts derived from HSF-1 deficient mice were not able to initiate stress induced HSP transcription (Lis and Wu, 1993, Morimoto, 1993, Sorger and Pelham, 1988, McMillan et al., 1998, Becker and Craig, 1994, Anckar and Sistonen, 2011). Interestingly, HSF- 1 has been found to be expressed at 37ºC at a size of 70kD (native and denatured) and at 42°C it has been found to be expressed at 178kD (Cotto and Morimoto, 1999). Additional to its role as a regulator of HSP expression, HSF-1 also acts as a regulator of non-native proteins in the stressed cell, capturing and maintaining them at their intermediate fold states, preventing them from becoming proteotoxic subsequently aiding in their refolding or degradation upon the cells recovery (Tanabe et al., 1997). In their *in vitro* study Tanabe and colleagues reported that chicken embryos (cultured at 37°C) subjected to temperatures ranging from 39-46°C for a time period up to two hours, there reported differential activation of HSF-1, identifying it as the first to be activated with increasing temperature, acutely. However, they reported similar expression levels total HSF-1 from 37- 44°C, indicating that total expression levels do not alter with temperature in the reported range (Tanabe et al., 1997). Furthermore, they reported extremely low levels of DNA binding activity at both 39°C becoming more prominent at 41°C increasing up to 45°C. The activation of HSF-1 is understood to be reached via hyperphosphorylation via a two-step process. While many phosphorylation sites have been investigated as potential activation sites, it appears Ser326 phosphorylation is strongly correlated with HS driven transcriptional capabilities of HSF-1 (Guettouche et al., 2005). Furthermore, it has been identified that HSF-1 is negatively regulated via the

phosphorylation of sites Ser303/307, whereby the transcriptional competency is attenuated (Knauf et al., 1996, Chu et al., 1998).

1.8 Myonuclear proliferation and accretion

Muscle fibres are one of the very limited multinuclear cell types in vertebrates (Bruusgaard et al., 2003). Myonuclear proliferation and accretion is an established response to short and long term RE driven muscle hypertrophy and are posited to be added to the existing fibres via mitosis (fusion) of SC (Kadi et al., 2005, Bruusgaard et al., 2010). However, it has also been suggested that muscle hypertrophy can occur in the absence of SC, but the degree of hypertrophy in that instance has not been compared to SC^+ (Bodine et al., 2001). In a rodent study, Bruusgaard and colleagues reported that 14 days after overloading the extensor digitorum longus via partial ablation, myonuclear density increased by 37% (49 ± 1.8 to 67 ± 2.4 per mm). They suggested based on their findings, that increasing number of myonuclei is a major cause of hypertrophy given the high translational levels in proteins synthesis driven by the increasing number of myonuclei (Bruusgaard et al., 2010). As mentioned in section 1.9, there exists a debate whether SC and thereby addition of myonuclei is required for hypertrophy (Rehfeldt, 2007). Petrella and colleagues recruited 66 adults in two age groups (20-35 and 60-75 years) with no lower body RE five years preceding. The 16 week progressive RE intervention consisted of three sets of 8-12 repetitions of knee extensions, squats and leg presses (Petrella et al., 2008). Load based on 80% baseline 1RM maximum. Biopsies were extracted pre intervention and 24 hours post last session of the intervention from the left *vastus lateralis*. Cohort was K means cluster (3) divided based on fibre area response (nXtr (extreme) =17, nMod (moderate) = 32, nNon (none) = 17). They reported a 9% increase in the myonuclei per fibre in Mod cluster ($p < 0.05$) and a 26% increase in the Xtr cluster ($p < 0.001$) and no increase in the Non cluster ($p = 0.877$) (Petrella et al., 2008). These results agree partially with their previous observations where they observed a myonuclei per fibre increase by 19% ($p < 0.02$) from baseline in a 16 week lower body RE intervention identical to Petrella et al., 2008 (Petrella et al., 2006). These observations do support the suggestion that there exists a correlation between muscle fibre CSA (hypertrophy) and myonuclei density. The evidence on myonuclear proliferation by HS extremely limited. In a rodent (rat) study, Oishi and colleagues applied a muscle degradation agent in to the soleus muscle and heat treated via lower body hot water immersion (42 ± 1°C) for 30 minutes for either three bouts (one week) or seven bouts (two weeks). They reported no difference in myonuclear number between untreated control and the HS only control at one or two weeks (Oishi et al., 2009). However, they reported increased myonuclear numbers (per cross section) in HS muscle damage group

compared muscle damage control at week one and two by 49%-77% respectively ($p < 0.05$) (Oishi et al., 2009). Indicating a potential induction of myonuclear proliferation in damaged muscle but not healthy muscle. These results support observations by Uehera and colleagues who reported increased levels of proliferating cell nuclear antigen positive myonuclei (PCNA) and 5-bromo-2′-deoxyuridin (BrdU) positive myonuclei in HS rats (41°C for 60 minutes, single bout). PCNA levels increased 38.9 ± 3.9% compared to control 17.9 ± 3.9 % at 24 hours ($p < 0.05$) however, decreased significantly at 14 days. $PCNA^+$ positive cells in HS group 13.2 ± 1.4% compared to control 2.6 ± 1.2 % ($p < 0.05$) at 24 hours, also decreased at seven and 14 days (Uehara et al., 2004), indicating an acute response to HS. Goto and colleagues indicated that after ten weeks of localised HS to the *vastus lateralis* for eight hours a day, four days a week, myonuclear density visually increased (pre and post count not reported). Moreover, they reported ~8.3% ($p < 0.05$) increase in the mean CSA in the HS group (Goto et al., 2011). While there are no studies investigating the combinatory effect on RE and HS on myonuclear proliferation and accretion, the above evidence from each isolated stress suggests a potential cumulative effect.

1.9 Satellite cell response

Satellite cells (SC) are categorised as myogenic precursor cells that are yet to be differentiated. From a non-activated (quiescent) state, they have the ability to transform in to either new muscle cells or fuse themselves with existing muscle fibres adding new volume to muscle (hyperplasia) or more commonly, provide new myonuclei. SC play the most central role in muscle regeneration and are situated beneath the basal lamina that encapsulates each muscle fibre and they also have the ability to self-renew (Collins et al., 2005, Kadi et al., 2005). The major molecular markers of myogenesis (the conversion to muscle cells) include myogenic transcription factors (MRFs) - key among them MyoD and paired box transcription factor (Pax7). It has been observed that, Pax7 knock-out mice have virtually no SC. The marking feature of an activated satellite cell is the novel expression of MyoD. Quiescent cells express only Pax 7 (Wackerhage, 2014). While the role of SC in maintaining muscle quality via the regenerative process is well understood (Zammit et al., 2006), the SC response in RE driven muscle hypertrophy is not well understood. Two schools of thought exist as to whether SC addition is required or not required for muscle hypertrophy (McCarthy and Esser, 2007, Rehfeldt, 2007, Petrella et al., 2008, Smith et al., 2001). However, a number of rodent studies have posited the role of SC as the provider of precursor cells involved in muscle repair and hypertrophy (Phelan and Gonyea, 1997). In the instances of muscle damage, SC have been shown to undergo division providing novel myonuclei to existing fibres (Zammit et al., 2006). It has been demonstrated, in rats, that when SC proliferation is

inhibited, so is the growth and recovery of the damaged and lost muscle mass (Rosenblatt et al., 1994). A limited number of human studies have investigated the effect of RE on SC response. Crameri and colleagues reported that in a cohort of (n=8) young males tested after an acute bout of single leg RE (50 one leg drop down jumps, 30° and 180° knee extensions, eight sets of ten repetitions each) with the contralateral limb serving as a control, there was a significant elevation in the percentage of NCAM positive cells from baseline as well as from control at day two (6.38 ± 3.36) and at day four (6.94 ± 8.27) (Crameri et al., 2004). Dreyer and colleagues observed that a bout of acute eccentric RE improved SC content in young (0.18 ± 0.05, $p<0.001$) as well as older (0.10 ± 0.02, $p < 0.001$) groups compared to post exercise. Moreover, they reported that the younger cohort improved significantly over the older cohort (Dreyer et al., 2006a). It is evident then that RE triggers an acute SC response. However, it is key to note that in both the studies, the test populations were untrained, prompting the notion whether the observed response is a general stress response. In a 16 week RE intervention (five lower body and six upper body exercise) four times a week in a group of (n = 14) of young men, Nederveen and colleagues observed that total SC count, measured via Pax7 and MyoD quantification (Pax7/MyoD per 100 myofibres), increased significantly from pre intervention at 72 hours post the last exercise session of the intervention. Pax7 (17.7 ± 1.3, $p < 0.05$), Pax7/MyoD (activated SC) also improved significantly at 24 hours post (3.1 ± 0.2, $p < 0.05$) and 72 hours (3.1 ± 0.4, $p < 0.05$). They further reported that compared to the ~35% increase in active SC 24 hours post a single bout of RE, there was significant increase of~55%, in the number of SC 24 hours post the last session of the 16 week intervention, indicating a positive effect of chronic RT in SC activation. This supported the earlier observations of Bellamy and colleagues who conducted a very similar 16 week RT intervention in a cohort (n = 23) of young males (Nederveen et al., 2017, Bellamy et al., 2014). The number of studies which have investigated the effect of HS on the SC response is limited. In a rodent study, Kojima and colleagues observed that in a group of male rats, subjected to 60 minutes of HS at 41°C, the Pax7 positive cells improved significantly ($p < 0.05$) compared to the non-heated control group at three days post HS (Kojima et al., 2007). These findings were supported by Takeuchi and colleagues who reported that, in crush injured rats, subjected to 20 minutes of HS at 42°C, Pax7 marked cells improved significantly ($p < 0.05$) at days four and six compared to the non-treated group (Takeuchi et al., 2014). These results indicate that HS has the ability to promote SC response. However, there are no studies that have investigated the effect of HS on SC response driven by chronic RE.

1.10 Mitochondrial adaptations

Mitochondria are the most critical cellular apparatus in cellular energy turnover. It has been well established that intense physical activity increases the mitochondrial content (generation of new reticular components) within muscle (Jornayvaz and Shulman, 2010). Mitochondrial biogenesis could be defined as division and growth of pre-existing mitochondria. It has been found to be driven by exercise (predominantly endurance), oxidative stress as well as temperature (Jornayvaz and Shulman, 2010, Holloszy and Coyle, 1984, Hood, 2009). It has been reported that in humans, type I fibres house the greatest mitochondrial content, followed by type IIa and IIx and endurance exercise is a major instigator of mitochondrial biogenesis in all three types of skeletal muscle fibres (Irrcher et al., 2003). However, the body of work on the effects of RE on mitochondrial biogenesis is not as extensive. In investigating the effects of RE, the majority of studies have used the citrate synthase (CS) activity for mitochondrial content, mitochondrial complexes I-V (OXPHOS) for respiratory capacity, mitochondrial fractional synthesis rate (FSR), as well as peroxisome proliferator activated receptor γ co-activator 1-alpha (PGC-1α) levels as a quantifying markers of mitochondrial biogenesis (Hafen et al., 2018, Groennebaek and Vissing, 2017, Wilkinson et al., 2008). Conducting a single bout chronic RE study, Wilkinson and colleagues reported that, in a cohort of (n = 10) men who performed three sets of 10-12 repetitions of single leg knee extensions for ten weeks where sessions number alternated between two and three a week. While mitochondrial biogenesis is widely reported to be a chronic process, Wilkinson and colleagues reported a significant increase in muscle mitochondrial FSR four hours post the first RE session. Interestingly, the authors did not observe a significant change from baseline post the ten-week intervention (Wilkinson et al., 2008). Similarly, the limited number of studies who have investigated the effect on mitochondrial biogenesis by chronic high load RE, ranging from 6-24 weeks (two to four sessions a week) did not improve mitochondrial biogenesis (Groennebaek and Vissing, 2017). With the exception of the reports of Robinson and colleagues, who reported an increase in the FSR after 12 weeks of RE in a mixed cohort (n = 9) of older (> 65 years) adults, interestingly however did not find the same effect in the younger cohort (18-30 years) who underwent a similar training protocol (Robinson et al., 2017). On the current body of evidence, it can be argued that chronic RE does not improve mitochondrial biogenesis. However, whether mitochondrial biogenesis improves with the application of HS or the combined application of a secondary stressor, such as HS along with RE, has only been investigated within a very limited scope. Hafen and colleagues conducted a single leg intervention study, where they randomly assigned a leg to either a control group or a test group,

where HS was applied locally to the *vastus lateralis* using short wave diathermy for two hours a day for six days. They reported no improvements in elevations in concentrations of mitochondrial complexes (I-V) or PGC-1α 24 hours post intervention. Moreover, they did not observe change in CS activity. However, they noted significant increases in complex I and V (37 ± 19%, p = 0.028 and 39 ± 22.4%, p = 0.046) and PGC-1α (10 ± 3.3%) from baseline after six days of consecutive heat application (Hafen et al., 2018). They further supported their findings in a follow up study where they reported the ability of localised HS to maintain the mitochondrial quality against single leg immobilisation in a mixed cohort (n = 23). They observed a significant decrease in PGC-1α levels by 7.4% in the immobilisation group (n = 11) compared to the heat treated immobilised group where a 10.4% increase in PGC-1α levels occurred (Hafen et al., 2019). In contrast, Kim and colleagues did not observe an increase in OXPHOS or CS activity in a mixed cohort of (n=12) young adults who underwent an eight-week, single leg localised HS intervention (via water perfusion garment). Supporting these claims, Mang and colleagues observed that a mixed cohort (n = 13) who underwent ten days of heat acclimation (over 14 days) in a climate chamber set to 42-44ºC (30-50% RH) while performing 3% incline walks at 30-40% of their maximal velocity showed no improvements from pre intervention in levels PGC-1α at 48-72 hours post intervention (p = 0.119, ES = 0.84), Interestingly, however they reported a significant positive correlation between PGC-1α and HSP72 levels (r = 0.585, p = 0.046), which could be an indication of HSP response to mitochondrial biogenesis albeit not a significant level (Mang et al., 2020). It is evident that the effect HS has on mitochondrial biogenesis has not been fully resolved. Furthermore, the effects of heat applied in combination with chronic RE on mitochondrial biogenesis have previously not been investigated.

1.11 Angiogenesis

Angiogenesis refers to the process where the capillary density and the adaptive remodelling of the blood vessels within the muscles is driven through exercise, endurance more than resistance, or others forms of stress (Bloor, 2005, Hudlicka et al., 1992). Furthermore, it has been suggested that angiogenesis is positively correlated to muscle blood flow (Laughlin and Armstrong, 1982). This process increases the efficiency of the muscle to perform via increased circulation. It has been observed that the capillary density may vary within the muscle depending on the predominant muscle fibre type. Factors other than mechanical stress also have the potential to trigger angiogenesis and include oxidative stress, Ca^{2+} and cell produced nitric oxide (NO) (Wackerhage, 2014, Gavin, 2009). All these factors trigger multiple signalling pathways

including CaMK/AMPK which involves the key molecular marker PGC-1α. However, the particular pathway via which the response occurs is yet to be determined (Gavin, 2009). Other key markers such as vascular endothelial growth factor (VEGF), Angiopoietin1 (Ang1), which are two factors involved centrally in capillary proliferation and remodelling respectively (Bloor, 2005b). Another key marker that promotes angiogenesis is endothelial nitric oxide synthase (eNOS) which is involved in regulating blood flow via the production of NO (Gavin, 2009). It has been reported that inhibition of NOS may attenuate the VEGF response to acute treadmill exercise in rats by 50% (Gavin et al., 2000). The effect of aerobic exercise in promoting angiogenesis in humans is well established (Brodal et al., 1977, Bloor, 2005). However, the acute and chronic effects of RE is less well defined. Gavin and colleagues reported that in a mixed cohort (n = 7, one female) who underwent an acute bout of knee extensions (three sets of ten repetitions or until failure during the third set at 60-80% 1RM), VEGF levels increased significantly from baseline at two and four hours post intervention. They saw no such elevations in the levels of Ang1 at the same time points (Gavin et al., 2007). Following a chronic eight week RE intervention, Yeo and colleagues reported that VEGF and Ang1 levels increased significantly ($p < 0.05$) (Yeo et al., 2012). While the effect of RE on angiogenesis is better investigated the evidence on the effect of HS is further limited in availability. Kuhlenhoelter and colleagues tested a cohort (n = 55) of adults randomly allocated to three test groups (two heating interventions and no heat control) for the acute effects of localised HS on angiogenesis (via hot water perfusion garment). Test conditions were total lower body heating and thigh-heating. The lower body heating intervention was 90 minutes in length and blood and muscle samples were withdrawn at 30 minutes and two hours post intervention. They observed a significant increase in the VEGF mRNA fold change from baseline ($p < 0.05$) at 30 minutes following the lower body HS compared to the untreated control group. However, a similar change was not observed with Ang1. They saw no such elevations at the two-hour time point indicating an immediate acute response (protein content was not measured) (Kuhlenhoelter et al., 2016). Ihsan and colleagues supported these observations in their reports of 107% ($p = 0.011$) increase in VEGF mRNA levels after an acute bout of whole-body HS. Interestingly however, they did not report similar changes post a bout of localised HS (Ihsan et al., 2020). Furthermore, in a chronic, localised HS (eight weeks, 40 sessions) intervention, Kim and colleagues reported a significant change in the eNOS levels ($p = 0.003$) post intervention, however saw no changes in the VEGF levels (Kim et al., 2020). However, these results indicate that HS does drive an acute and chronic antigen

response. However, the effect of chronic HS combined with RE has not been previously investigated.

A number of chronic RE interventions have shown to improve capillarisation in the human skeletal muscle. Following eight weeks of RE (two session a week, bench press and bilateral leg press only), capillary to fibre ratio increased by 52% ($p < 0.01$) in the *vastus lateralis* in a cohort of untrained males, who underwent a total 40 minutes of hypoxic (14.4 % O_2) exposure before and after each training session (10 before, 30 after). Moreover, a similar cohort who performed the same RE program in normoxic conditions showed a strong trend ($p = 0.06$) towards improvements (Kon et al., 2014). After 16 weeks of full body RE saw capillary contacts, capillary to fibre ratio, capillary density as well as capillary to fibre perimeter index (CFPE) improved significantly ($p <0.05$) in recreationally young ($n = 23$, 24 ± 3 years) as well as old ($n = 22$, 67 ± 4 years) cohorts (Nederveen et al., 2016). They further saw that capillary contacts, capillary density and CFPE was significantly higher in the younger cohort ($p < 0.05$) (Nederveen et al., 2016). These results indicate that full body RE, even at low volumes (Kon et al., 2014) is capable of improving capillarisation in the human skeletal muscle. The evidence on the ability of HS to improve capillarisation chronically is limited. However, the available evidence indicates that HS has the ability to improve or maintain capillarisation in the skeletal muscle. Hyldhal and colleagues saw that in an active mixed cohort ($n = 11$) ten days (two hours day) of localised HS to the *vastus lateralis* was able to attenuate the decline of vascular function caused by limb immobilisation (Hyldahl et al., 2021) Kim and colleagues reported that after eight weeks of (five days a week, 90 minutes a day) localised HS to the *vastus lateralis*, in which muscle temperature was raised to a peak of ~37°C , HS arrested the decline of capillarisation indices compared to the control, contralateral leg. Significant heat treatment effects were seen for capillary contacts ($p = 0.016$), capillary to fibre ratio ($p = 0.007$) and CFPE ($p <0.001$) compared to the control leg. Group of sedentary young males ($n = 10$) undergoing full body HS at 40°C (40% RH) three times a week, Hesketh and colleagues saw, CFPE improve by 15% ($p = 0.012$), capillary contacts by 9% ($p = 0.049$) and capillary to fibre ratio by 12% ($p = 0.025$) post intervention. Interestingly, these improvements were highly comparable to an aged matched group ($n= 10$) undergoing six week of moderate intensity continuous training (nil group effect) (Hesketh et al., 2019). However, the effect of HS combined concurrently with heavy RE for potential additive effects on capillarisation has not been investigated previously.

1.12 Section summary

Performance gains as well as aesthetic muscle adaptive improvements to RE is highly sought after by athletes as well as recreational fitness enthusiasts. Moreover, the use of RE as a mode of muscle rehabilitation against injury, muscle atrophy and general physical wellbeing is common. As reviewed above, RE has been shown to improve performance aspects such as strength, power, force and speed. Moreover, mTOR driven anabolic synthesis pathway, ribosomal biogenesis, Ca^{2+} response, heat shock protein response, myonuclear proliferation, satellite cell response, mitochondrial biogenesis and angiogenesis have been shown to be adaptively responsive to various modalities of acute and chronic RE in varying degrees depending on the type of load-stimulus. A variety of HS applications, chronic and acute, localised as well as full body, have also been identified as a stressor that can influence performance as well as skeletal muscle adaptations. However, the evidence is limited and somewhat contradictory. A potential method whereby performance and muscle adaptive gains from RE could be enhanced or expedited upon has significant performance and clinical benefits. To this point, a number of studies have combined RE with HS in order to investigate potential additive effects of HS on performance and muscle adaptive gains by RE. Here as well, the evidence is ambiguous, but suggest that HS could be impactful predicated upon reaching a certain muscle temperature threshold. This might be dependent upon the mode of heat application.

1 Literature review part 2: Heat application

In the variety of studies reviewed above in section 1, an assortment of localised and full body heating methods were utilized. Each method carries its own set of limitations and advantages. While some methods are high in efficacy for increasing CMT, they lack the ability heat a large body surface area. In contrast, some methods are able to heat a large area, but do not allow for the concurrent performance of exercise and impede range of motion (ROM). Other methods allow for adequate ranges of motion, but do not generate a heating gradient steep enough to raise CMT at a high enough rate for the stressor to be concurrent. Another subset of methods are efficient in heating however, low in comfort for the participants during the heat exposure. Therefore, for a heating method to be effective as a chronic, concurrent heat application method, it needs to fulfil a number of key criteria. To be able to heat a large body surface area while not impeding the ROM whilst simultaneously raising core temperature and CMT at a high rate.

In order to therapeutically or recreationally heat the body or body segments to raise core and CMT, the body's heat dissipating mechanisms must be overcome. The thermoregulation of the body to maintain the homeostatic balance is critical for optimal function as well as survival. Therefore, the body temperature regulation is a highly refined process. All the tissues in the body act as thermal reservoirs and possess the capability to retain heat. When under HS, in the instances in where core or peripheral tissue temperatures are rising above the homeostatic threshold, different mechanisms are activated, such as the dilation of cutaneous blood vessels increasing skin blood flow as well as the activation of the endocrine sweat glands in order to facilitate heat loss (Taylor and Cotter, 2006, Taylor, 2014). Therefore, in order to increase body temperature, the heat input must be greater than the rate of dissipation. This would create the disturbance in the homeostatic state which is necessary to generate a stress response.

With regards to skeletal muscle, heating is affected by a multitude of factors. Technical, environmental, physiological, anatomical. Technical factors predominantly involve the method of heating, the length of the intervention, mode of heat transfer, rate of heat decay (dissipation). The modes of heating involved can be first be broadly classified in to wet and dry heating. Steam baths, saunas, hot water immersion represent wet heating methods, while ultrasound, microwave, shortwave diathermy, dry sauna, heat packs, electric heat wraps and water profusion garments represent dry heating methods. Each of these methods will be extensively reviewed in the following section. The heat generated by these methods are conveyed through to tissues via four modes of heat transfer. Conduction (in contact with skin), convection (using heated air or fluid), advection (combination of convection and conduction), radiation (wave based radiation). Where

heating of the tissue is concerned, these four modes are of great relevance (Cameron, 2017). The rate of heating depends on the mode of heating, method of heat transfer, factors such as subcutaneous fat layer thickness of the target muscle as well as the specific heat capacity of the target tissue, depending on the water content, fat to muscle ratio, temperature gradient between the source and the target muscle, the nature of contact (direct, air gap or barrier), area of contact (Cameron, 2017) as well as the distance from the heat source. In choosing an effective heating method, all of these factors require consideration.

Given all other factors are constants, the rate of heat transfer can be condensed in to the below equation (Cameron, 2017):

$$Rate\ of\ heat\ transfer = \frac{(area\ of\ contact\ x\ thermal\ conductivityx\ temperature\ gradient)}{tissue\ thickness}$$

The environmental factors include and humidity and room temperature. Humidity affects the rate of cooling. As the muscle gets heated, a number of cooling mechanisms intervene to maintain homeostasis. Sweating and vasodilation are among the key phenomenon for this purpose (Lele, 1954, Taylor, 2014). The higher the humidity of the room the more it will impede the evaporation of sweat and will critically depend upon the humidity of layer of air closet to the skin. A higher humidity will slow the rate of evaporation (Gleeson, 1998, Wilson et al., 2002), which in turn aids to retain high body temperatures for extended periods of time. The muscle temperature further depends on the temperature gradient between the skin and the environment. If it is a steeply negative gradient, the heat loss is expedited. In the case of acute localised heat application, the influence of this factor is enhanced even further, as the heat source is in direct contact with the target, is within close proximity (Anderson, 1999, Cameron, 2017).

The physiological factors include core temperature, tissue temperature (at rest and active), rate of metabolism, venous and arterial blood temperature, the specific thermal conductivity of the muscle, the flow volume and rate of blood through the target tissue, skin blood flow, as well as hormonal and cytokine input (Taylor, 2014). For instance, interleukin-1 has been found to be acting to raise the control set point mediated by the hypothalamus (Gleeson, 1998). Additionally, it has been reported that the inhibition of dopamine at the hypothalamus attenuates the dose response rise in core temperature that is driven by exercise (Gleeson, 1998). Furthermore, osmo receptor signalling and pressure receptors affect heating by sensing the changes in plasma volume and osmolality (Petrofsky and Laymon, 2009, Gleeson, 1998). The heat generated in the muscle during activity is a key factor. During exercise, heat production of the body spikes due to the inefficiency of the metabolic process that supplies energy for force production. It has been

reported that for every 1L of oxygen, approximately 20kJ is produced via the oxidative metabolism (Gleeson, 1998). However, only 4kJ is being used for mechanical driving and the remaining balance is expelled as heat. It has been reported that during high intensity ergometer exercise, CMT will increase by 1°C per minute during the initial stages, however is then transferred to body core during prolonged activity (Gleeson, 1998).

In regulating temperature, the contributing anatomical factors include the blood vessel density of the muscle (which varies in response to the chronic load and activity the muscle is under). Which can be divided in to two major manifestations, growth of existing capillaries as well as development of new capillaries (Bloor, 2005). This affects the rate of heating given that more capillaries allow a higher volume of blood moving through the muscle carrying heat away (Bloor, 2005, Petrofsky and Laymon, 2009). Elevated local temperature increases skin blood flow (Gleeson, 1998, Taylor et al., 1984) and the rate of heat dissipation away from the site of heating is directly correlated to skin blood flow. It has been reported that, in response to extreme HS, cutaneous vasodilation can increase up to 6 to 8 L/min (Taylor et al., 1984). Taylor further reported that when the local skin temperature is increased to 42ºC, the local vasodilation is maximised (stress time of 20-40 minutes). Once the local skin temperature is above 42 ºC, it significantly attenuates the sub cutaneous blood flow of the target area (forearm) and its overall contribution to the body's thermoregulation. The authors further verified this observation by increasing the local temperature to 43.5ºC and reported that forearm blood flow remained stagnant at the same rate as 42ºC (Taylor et al., 1984). This directly indicates an upper limit to the rate of cutaneous and sub cutaneous vasodilation and the rate of blood flow which directly affects the local and full body thermoregulation beyond skin temperatures of 42 ºC. Therefore, it is critical to identify the role of the skin (as the largest organ), in thermoregulation of the body both systematically and locally. The thermal stress regulation within the body, rising internal temperature as well as rising skin temperature, directly and negatively received by the preoptic/anterior hypothalamus and cutaneous vasodilation is triggered as primary response along with sweating. (Boulant and Gonzalez, 1977, Boulant, 2010). The flow rate and volume and the rate of flow of blood through the deep muscle is another key factor in deep muscle heating. The rate of flow and the consequently increased flow volume is exponentially proportional to the rising temperature, as part of the body's thermoregulatory interventions. During local heating, the blood entering the target area is at or below the homeostatic temperature of ~37 ºC, and muscular vasodilation allows a higher volume through the muscle (Greenberg, 1972). The temperature gradient between the site of heating and the flowing blood allows the

blood to receive heat and carry it away. The thickness of the sub cutaneous fat layer is another anatomical factor that has been shown to play a role in the deep muscle heating. Petrofsky and Laymon reported the thickness of the sub cutaneous fat layer is directly and inversely proportional to the rate of deep muscle heating (Petrofsky and Laymon, 2009). Interestingly, they further reported methods such as hydrocollator heat packs and whirlpools were significantly ineffective against a thicker subcutaneous fat layer. In addition, the authors indicated that raise in muscle temperature was slower by 66% in overweight participants compared to lean participants. They concluded that moist heat (steam, sauna) is more effective in penetrating the fat layer compared to dry heat (heat wraps) (Petrofsky et al., 2009). However, these findings contradict the findings published by Lehmann and others in 1978 in which a 915MHz microwave applicator as a deep muscle heating intervention in two cohorts with subcutaneous fat layer thicknesses $\leq$ 1 cm and $\leq$ 2 cm. Lehmann and colleagues reported higher deep muscle temperatures in the individuals with fat layers thicker than $\leq$ 2 cm. This observation could be due to the fact wave based systems heat deep muscle more effectively, the thicker ($\leq$ 2cm) fat layer shields the heat loss more effectively compared to the leaner ($\leq$ 1 cm) fat layer. However, it is key to note that Petrofsky colleagues did not use wave based (short and microwave) heating methods in their investigations when inquiring the effects of dry heat methods. Therefore, this discrepancy further reinforces the fact that the heating modality plays a key role in the heating patterns observed in the muscle. This invites for further investigations on the sub cutaneous fat layer and the causal effect on deep muscle heating. These ambiguities however, make it difficult to deduce the role of the subcutaneous fat layer in deep muscle heating. The effects seems to be predicated on the heating modality, the time of application and locale of application. Furthermore, as subcutaneous fat layer thickness appears to be contributing factor to the rate of heating, the method of measurement of the sub cutaneous fat layer thickness becomes key in investigating deep muscle heating.

It had been previously reported that skinfold calliper measurements are higher in precision compared to ultrasound (Nordander et al., 2003). However, more recent studies have validated the use of ultrasound to measure muscle as well as subcutaneous fat layer thickness as the most accurate technique and now has been standardised. St. Störchle and colleagues took duplicate measures from eight standardised sites in the lateral thigh from a cohort (n = 8, BMI < 28.5 kgm^{-2}) males, as well as tested 216 random sites (thickness ranging from 3-10 mm) distributed all over the body. They reported the accuracy over all sites with a standard deviation of 0.05 mm (8%) (Störchle et al., 2018). Ultrasound further minimises operator error as well as the

discrepancies introduced by the compression fat layer (which is common error with the skinfold calliper) given the real time visual indications of the target areas (Störchle et al., 2018).

The neural mediation of heat response regulation is not comprehensively understood. It has been found that with local heat application, the consequent local vasodilation is initiated by the neurogenic reflexes (Pergola et al., 1993, Minson et al., 2001). As emphasised above, local and systemic heat regulation responses vary depending on a number of factors such as the temperature utilised, time of application as well as rate of heating, and it has been posited that varied combinations of these factors could instigate different regulation mechanism (Minson et al., 2001). In the neural mediation of local heat, two distinct branches of sympathetic nervous system appear to be involved. The adrenergic vasoconstrictor system and a cholinergic vasodilator system, where acetylcholine is released in tandem with a yet unrecognized neurotransmitter. However, depending on the rate of heating or nature of heating the neural response could vary between the lessened effectiveness of the noradrenergic vasoconstriction (which only seems to be effective in short term and only contributing minimally (Kellogg Jr et al., 1999, Minson et al., 2001). This observation is further supported by Kellogg and others who demonstrated that even when the neurogenic response is abolished (via botulism toxin), the vasodilation in response to heating does not diminish (Kellogg Jr et al., 1995). Interestingly, it has also been posited that neural regulation might not play a role at all in local thermoregulation, based on the observation that skin denervated by burns and skin grafts still preserved the ability to activate vasodilation (Aulick et al., 1977). Therefore, when considering an effective mode of local heating, neural regulation can be omitted as a contributing factor.

1.1 Dry and wet heat

Dry heat is defined as a heat source that does not involve a moisture element while wet or moist heat involves water-based heating transfer element. It has been previously reported that moist heat is more effective at raising the subcutaneous temperature compared to dry heat (Brandt, 1998). When used in a therapeutic capacity, wet heating modalities are more commonly utilised compared to dry heating methods (Brandt, 1998). However, it has been proposed that as long as skin temperature is raised to the same temperature by either modality, it should not impact the temperature achieved 1 cm below the skin surface (Stillwell, 1965). While this notion is upheld during wet and dry heating, the efficacy of wet and dry heating is not predicated upon the skin temperature, but on the medium of heat transfer to the skin. For instance, stationary air is a strong insulator (Anderson, 1999), therefore, when using conductive heating methods, it is key to ensure

there are no air pockets between the two surfaces. With the exception of heating via diathermy, all of the studies discussed in part one of the literature review reported highly comparable CMTs. Therefore, it can be deduced that the heat transfer method is not a contributing factor in improving the efficacy of skeletal muscle heating.

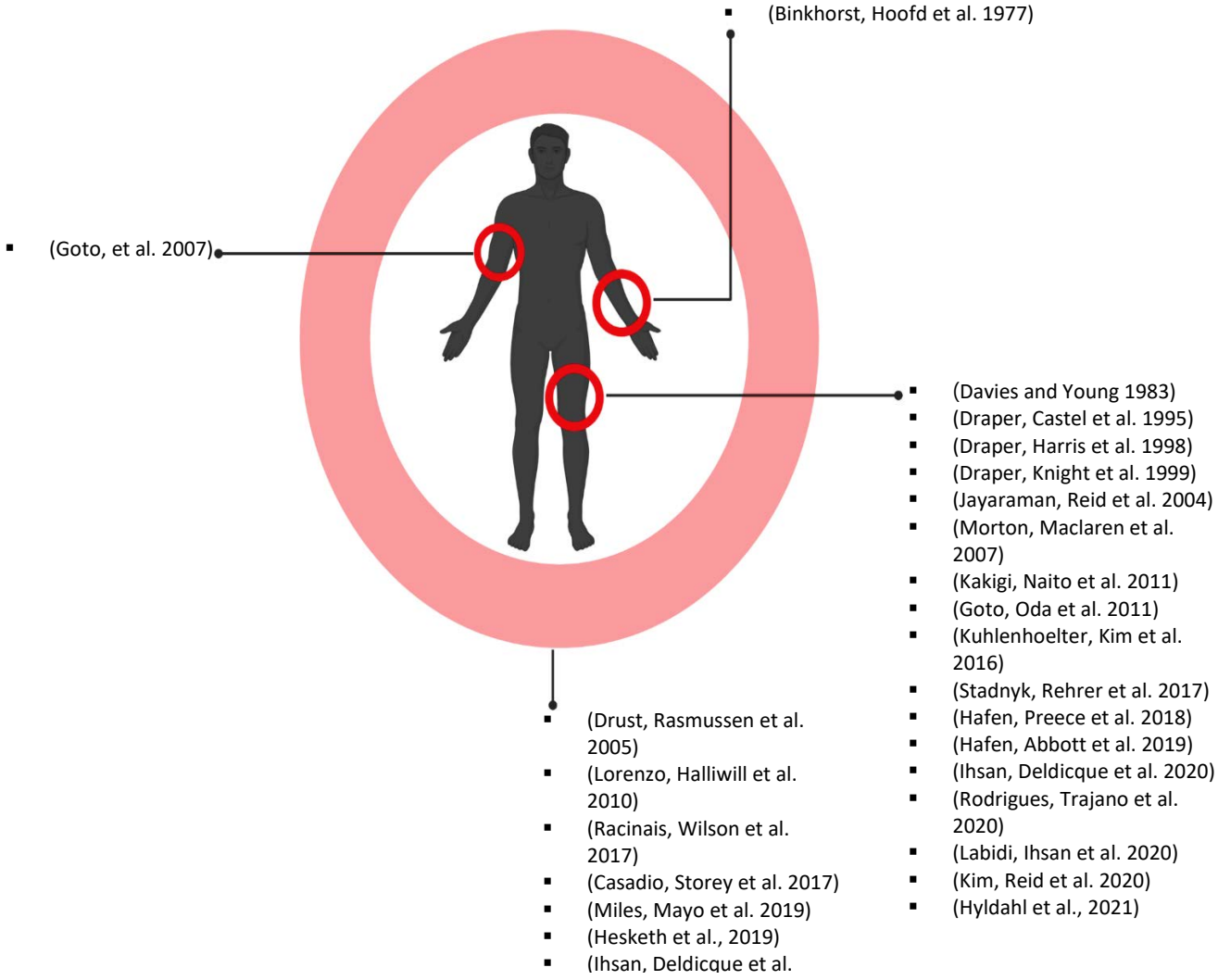


Figure 1.2. Schematic categorisation of heating methods by cite of the most relevant studies. Circles indicate sites of heating with the large circle indicating full body heat.

1.2 Heating modalities

A number of heating modalities have been used for medical purposes for centuries. Heating was utilised as a method to promote healing in the deep musculature (Draper and Ricard, 1995). In the current body of literature there are number of heating methods that have been utilised for skeletal muscle heating in varying contexts. These include therapeutic, rehabilitative, potential performance enhancement in the form of heat acclimation (Petrofsky et al., 2009, McGorm et al., 2018, Hyldahl and Peake, 2020). The modalities can be divided in to two major categories,

localised and whole body (figure 1.2). In the following section each heating modality will be discussed in depth for their effectiveness in improving muscle temperature.

1.2.1 Steam sheets

There are a number of commercially available variants of steam sheets. They are a commonly used non-pharmacological method in relieving muscle and joint pain as well as increasing joint range of motion (Seto et al., 2008). Steam sheets are an evolution of dry heat sheets. Dry heat sheets generate heat via a chemical reaction. Most commonly an iron compound reacting with oxygen exothermically (Seto et al., 2008). In the steam sheet, a dry heat pack is modified with a compartment containing water that is converted to steam when the chemical reaction occurs. The heating is considered to be more efficient given the fact that condensation of steam directly offers water convection which is more efficient compared to air convection. Furthermore, Seto and others established that the application of the sheet has the ability to conform to the shape of the muscle and had the ability to deliver a homogenous heat distribution across the target area for prolonged periods (six hours per day at the longest). However, this investigation did not report the length of time one sheet will effectively maintain peak output. Furthermore, the peak skin or muscle temperature achieved has not been reported precisely (Seto et al., 2008). Goto and others applied a similar heat-steam sheet to *biceps bacchii* for 30 minutes and measured temperature at a depth of 1.5cm and reported temperature increments ~2 °C (Goto et al., 2007). In the *vastus lateralis,* Goto and colleagues saw muscle temperature increase to peak of 38.3 ± 0.1°C at a depth of 1.5cm after three hours of heating (Goto et al., 2011). Similar temperatures were seen (37.6 ± 1.0°C) at depth of ~2cm by Labidi and colleagues after six hours of heat application (Labidi et al., 2020). Importantly, the sheet also allows for unimpaired movement of the target area (Seto et al., 2008). Based on these findings, the steam sheet appears to be a viable option for localised muscle heating. However, they do not offer control over the heat output which is a hindrance to achieving specific temperatures.

1.2.2 Heat (hot) packs

Heat or hot packs are a commonly used method of therapy in treating muscle soreness and strains. Furthermore, heat packs have been heavily utilised in muscle related research. There are a number of heat pack modalities. They can be mainly categorised in to chemical and heat storage (Petrofsky et al., 2009). The chemical heat pack commonly contains sodium acetate, calcium chloride or magnesium sulphate compartmentalised from water. When combined, it drives an exothermic reaction producing temperatures up to 54°C for a period of 15-20 minutes (Petrofsky

et al., 2009). Heat storage packs are either microwaved or hydrocollator energised. Microwave heat packs contain wheat or flaxseed. The entrapped water in these seeds store heat via high frequency vibration. Both chemical and microwave heat packs produce dry heat although their final temperatures are variable due to the high heat loss gradient (Petrofsky et al., 2009). Therefore, not highly efficient in raising deep muscle temperature. Hydrocollator heat packs are activated by placing in heated water at 70 °C- 75°C until the gel reaches thermal equilibrium with the water. This method provides moist or wet heat and is more efficient in raising deep muscle temperature compared to the dry heat packs. Petrofsky and others reported that the hydrocollator heat pack drove skin temperature increments up to 9°C from baseline, while deep tissue temperature (measured at a depth of 2.5 cm in the quadriceps) only rose by ~ 1 °C with an application time of 15 minutes (Petrofsky et al., 2009).

1.2.3 Sauna

Sauna bathing is a commonly used full body heat application method. Also known as the Finnish bath, this modality has been used for therapeutic and recreational purposes. This produces heat by dry air at high temperatures (Hannuksela and Ellahham, 2001). The temperatures range from 80°C - 100°C with a relative humidity of between (5%-25%). Accepted treatment times vary between 5 - 20 minutes depending on an individual's level of heat tolerance (Hannuksela and Ellahham, 2001, Pilch et al., 2014). The acute physiological effects of sauna bathing mainly affect the circulatory system (cardiac output, blood flow to muscles, skin blood flow), endocrine system as well as the homeostasis of body temperature. However, skin temperature rises significantly faster compared to core temperature with some investigations reporting skin temperatures above 40°C within minutes (Kauppinen, 1989). Pilch and colleagues measured core temperature rectally and showed an increment of 1.16°C at a chamber temperature of 91°C and 5%-18% of relative humidity (Pilch et al., 2014). Critically, it must be noted that in achieving this temperature, they employed 5 minute cooling periods at 22°C in between the 15 minute heat treatments in a total intervention time of 60 minutes (Pilch et al., 2014). The evidence is non-direct and sparse on deep muscle temperatures caused by dry sauna in the current body of literature. However, based on the observed core temperature elevation it is discernible that it would be highly difficult to raise deep muscle temperature to levels above ~38.5-39°C within practical parameters of time and participant wellbeing.

1.2.4 Steam baths (Wet sauna)

Steam baths or wet sauna is another modality of heat treatment that is used therapeutically, though the applications are more recreational than medical. The temperatures used in steam bath can vary between 40°C-70 °C degrees when the chamber is fully saturated with steam (Pilch et al., 2014). The increased humidity in the wet sauna, prevents evaporation of sweat, inhibiting the dissipation of heat. Thereby, aiding the increment of core and muscle temperature at a higher rate compared to dry sauna. In an investigation conducted by Pilch and others they reported a maintained temperature of 59°C at the humidity of 60.5%. While they did not measure muscle temperatures, a mean core temperature increment of 1.8°C in 45 minutes of whole body exposure with three five minute cooling intervals following every 15 minutes of heating (Pilch et al., 2014). Moreover, this method poses major logistical and practical limitations when applied with exercise concurrently. Furthermore, similar to dry sauna, wet sauna is more a superficial heating method (Hannuksela and Ellahham, 2001).

1.2.5 Hot water immersion

Also known as thermotherapy, this method has been tested for its effectiveness in muscle heating for well over a century. Lefèvre, in 1911, reported that immersing the forearm in water temperatures up to 35ºC, had no discernible effect on the muscle temperature 1cm below the fascia (Lefevre, 1911). However, contrary to his findings, Barcroft and Eldholm found that muscle temperature of a limb fully immersed in water of varying temperatures did have a significant effect on the muscle temperature. Among a range of other temperatures, they tested 38 ºC, 40 ºC,42.5 ºC and 45 ºC water baths with the target forearm immersed for a duration of two hours. In some cases, they prolonged the sessions by changing the water temperature after two hours (Barcroft and Edholm, 1943). Critically, they observed that with the higher water temperature, the deep muscle rate of heating decreases. They further observed that at bath temperatures 42.5ºC and 45ºC, the deep muscle temperature was identical at 39ºC (Barcroft and Edholm, 1943). In the trials where the bath temperature was higher than the core body temperature, the deep muscle temperature was significantly lower than the bath temperature (Barcroft and Edholm, 1943). Alluding to a possible threshold in thermoregulation. Presently, hot water immersion is employed for recovery and rehabilitation and as a potential acute treatment for delayed onset muscle soreness and extreme fatigue, predominantly in sports as a means of expedited postgame recovery method (Pournot et al., 2011). The set temperature of the treatment varies between protocols and most available evidence is anecdotal. Water temperatures from 20°C- 35°C are considered thermoneutral due to the fact that they do not increase core-

temperature following prolonged immersion (Versey et al., 2013, Barcroft and Edholm, 1943). In the instances of body immersion, the times of treatment vary from 10-24 minutes in the published investigations. The two main immersion methods are neck high and waist high immersion. It has been reported that, while effective in raising skin temperature significantly within 10-24mins, it may not be effective in raising muscle temperature (Barcroft and Edholm, 1943). Furthermore, there are a number of adverse effects in prolonged hot water immersion. The comfort levels have been observed to plateau with temperatures reaching 42.5ºC with 45ºC being the highest threshold of tolerability for the average, untrained individual (Barcroft and Edholm, 1943). The primary derogatory effects are potential burns and heat illness at high water temperatures. Furthermore, at temperatures between 45°C-50°C protein denaturation occurs (Michlovitz, 1990), as well as causing hypotension, ectopic beats among other potential dangers. Which limits the highest applicable temperature to drive potential high deep muscle and core temperature (Wilcock et al., 2006, Nagasawa et al., 2001, Morton et al., 2007 (Fuchs et al., 2020, Rodrigues et al., 2020). Furthermore, hot water immersion in any form is not a pragmatic solution as method of concurrent heat application during an exercise bout. It does not allow for the performance of RE with concurrent heat application as the limb requires to be immersed in water for constant heat application to be maintained at the desired temperature.

1.2.6 Ultrasound heating

Ultrasound (ultrasound diathermy) is another commonly used deep muscle heating modality. Therapeutic ultrasound has been in use for a few decades now as a mode of treatment for soft tissue injuries, osteoarthritis, joint dysfunction, increased joint range of motion and musculoskeletal pain (Draper et al., 1995a). It has also been employed as a method for expedited tissue repair in muscle and bone as well as treatment of scar tissue (Sellani et al., 2016, Draper et al., 1999). Ultrasound achieves tissue heating by absorption. The mechanical energy of the travelling wave (energy stored as high frequency vibrations) is converted in to heat when disrupted during collisions with the tissue. Tissues with a low water content such as fat do not absorb heat at the same level compared to protein and water rich tissues such as muscle (Dyson, 1987). Ultrasound can be applied in multiple frequencies between 0.75MHz-3MHz, with low frequency waves offering more in depth penetration but lack focus. 1 MHz ultrasound can increase the muscle temperature by 3°C-4.5ºC at depths of 3-5cm below the epidermis, which makes it the better solution for deeper lesions or for individuals with a thicker sub cutaneous fat layer. 3 MHz is utilized for comparatively superficial lesions at depths of 1-2 cm (Draper et al., 1999, Rashidi, 2017). The rate of heating also depends on the area covered by the applicator

head, given that the energy output is dispersed through a larger area therefore, heating is inversely proportional to the size of the area heated (Draper et al., 1999). While ultrasound diathermy can deliver muscle temperature elevations from baseline to above 41ºC at depths of 3-5 cm, it poses a number of difficulties that eliminates it as a potential method of heating with concurrent RE. Chief among them is the lack of control and precision over the temperatures achieved in the muscle. Additionally, it is difficult to maintain the muscle at the desired temperature and perform a bout of exercise given the positioning of the applicator encumbers on the range of motion. It also further decreases the rate of heating due to altering focus caused by the movement of the muscle during the exercise while the applicator is stationary.

1.2.7 Shortwave diathermy

Shortwave radio frequency diathermy or shortwave diathermy (SWD) is another deep muscle heating method. This method has been employed as a means of enhanced recovery supplement from ligament injuries as well as to increase joint range of motion by relieving stiffness. It has been shown that SWD is more efficient in heating a larger area compared to ultrasound at a similar rate (Garrett et al., 2000). It has been reported that SWD, at a frequency of 27.13MHz, can increase the muscle temperature by 4°C at a depth of 3cm with 15 minutes of heat application (Draper et al., 1999). Touchberry and colleagues investigated the effects of SWD heating in the *vastus lateralis* and reported significant changes in the molecular response pre and post the treatment. However, they have not reported the depth of heating or the peak temperature achieved (Touchberry et al., 2008). In another investigation, Robertson and colleagues reported that deep heating via SWD improved the range of motion in the ankle (Robertson et al., 2005). However, they did not report temperature data possibly due to the difficulties posed by the target site. Hafen and colleagues showed that with a two hour bout of heat application to the *vastus lateralis*, muscle temperature at a depth of 3.5cm could be increased by ~4°C from baseline reaching peak temperatures of ~40°C (Hafen et al., 2019, Hafen et al., 2018). However, in applying with concurrent RE application, it poses the same issues as the other two diathermy methods. The applicator is large. The positioning of the applicator impedes upon the range of motion required by a concurrent bout of exercise. Additionally, the area of heating is dependent on the size of the applicator head which by extension limits the effective target area.

1.2.8 Microwave heating

Microwave diathermy is another deep muscle heating method. The mechanism of heating is similar to short wave and ultrasound diathermy where tissues absorb the energy via molecular

vibration where the kinetic energy of the wave is converted to heat (Goats, 1990). It has been suggested that higher the protein (and water) content of the targeted muscle, the faster it will gain heat and at a faster rate compared to ultrasound and shortwave diathermy (Ichinoseki-Sekine et al., 2008). It allows for the raising of the core muscle temperature without raising the skin temperature by the same degree. Some microwave hyperthermia systems utilize a thin silicon bolus filled with thermostatic water to prevent the contact overheating (Ichinoseki-Sekine et al., 2007). This method is highly directional, which permits the heating of a small, targeted area. Additionally, it operates at a higher range of frequencies compared to shortwave diathermy which makes it comparatively more efficient (Goats, 1990). The effective depth of heating varies with tissue type and the applied frequency (Ichinoseki-Sekine et al., 2008). The most commonly used frequencies are 434 MHz and 915 MHz which are both effective at heating the muscle to a depth of 4cm (Giombini et al., 2007). Guy and colleagues reported that microwave diathermy had the ability to heat the muscle to physiologically significant temperatures (above 37 °C), while maintaining the skin temperature under 36 ºC (Guy et al., 1974). In a more recent investigation, a direct contact 434MHz (with an integrated deionized water circulation system to cool the skin simultaneously) at a total power output of 60W, reported a mean peak muscle temperature of 43.7 ± 0.8°C with mean a temperature rise of 8.9 ± 1.4 °C from baseline. With aid of the active cooling system, the skin temperature was held below 40 °C, with the highest observed being 39.2 °C (Ichinoseki-Sekine et al., 2007). The same investigators also reported that 2450 MHz application achieved a mean peak muscle temperature of 41.1 ± 1.3°C with skin reaching a mean peak of 39.2 ± 0.8 °C (Ichinoseki-Sekine et al., 2008) although, 715 MHz has been reported as the optimum frequency for therapeutic heating (Goats, 1990). However, similar to the other diathermy methods, the application requires the target area to be stationary. And if the applicator is in contact with the target area, a concurrent about of exercise cannot be performed.

1.2.9 Electric heating wraps

Electric heating wraps are a commonly utilised dry heat method for therapeutic and recreational heat application. This the most widely commercially available heat application method and there are number of platforms available that offers range of power outputs. The underlying principle is high resistance electrical conduction, encapsulated in a variety of fabric sleeves. In order to make the heat flow uni-directional, the sleeves commonly comprise of an insulating material such as polycholoroprene (Neoprene) on one side. However, the number of investigations that have utilized electric heat wraps as a method of heat application is limited. Draper and colleagues tested a commercial heat wrap (knee) and reported that during 120 minutes of heat application,

temperature of the *vastus medialis oblique* increased by a mean of 3.19 ± 1.27°C at a depth of 1.5 cm (Draper and Hopkins, 2008). In a more recent investigation, Stadnyk and others employed a rheostatically controlled heat pad on the *vastus lateralis* and measured temperatures at depths of 1, 2 and 3 cm and reported temperature increments from 35.2 ± 1.1 °C to 38.2 ± 0.9 °C when averaged across all depths (Stadnyk et al., 2017). However, this report does not provide accurate deep muscle temperatures. Average temperature is misleading as higher shallow temperature compensates for lower deep muscle temperature. This investigation did not report the corresponding skin temperatures. This is the more viable option for application with concurrent exercise as the impedance of ROM is minimal. However, deep muscle temperature elevations appear insufficient (≤ 38°C).

1.2.10 Paraffin treatment

In paraffin treatment, a precise mix of paraffin wax and mineral oil (at 6:1 or 7:1 ratio) is utilized. The mix when melted will hold at a temperature range of 45 - 50°C. This range allows for safe, direct application of heat on to the skin. However, insulating mitts are utilised to minimise heat loss. There are three methods of application, dip- wrap, dip- immersion and paint (Cameron, 2017). The dip-wrap and immersion methods can only be used on the extremities (hands and feet). However, the paint method can be used anywhere in the body (Cameron, 2017). This approach involves painting on multiple layers of wax (six to ten) on the target site. The wax layers store heat and transfer it to the target area. However, the efficacy of this method for deep muscle heating is low and available literature is minimal. Abraamson and others reported that even when the wax temperature is at 52.2 °C the corresponding skin temperature only reached 38.89 °C (dip- immersion). This discrepancy occurs due to the fact that paraffin itself is an insulator (Borrell et al., 1980, Abramson et al., 1964). This along with the fragility of the wax layer which is susceptible to defragmentation are major limitations of this method as a means of deep muscle heating. Along with the obvious limitation of not being able to use concurrently with exercise.

1.2.11 Infrared heating

Infrared heating is another wave-based method in which the heating is achieved via energy absorption and is predominantly a superficial heating method (Gale et al., 2006). The sources of infrared used for muscle rehabilitation are sunlight, infrared light emitting diodes, low intensity lasers, supraluminous diodes and infrared lamps. It has been shown that only 50% the infrared radiation (wavelength 1200 nm) will penetrate beyond 0.8 mm, which indicates that the majority

of the energy fails to reach the muscle (Cameron, 2017). Furthermore, the level of heating is predicated upon the distance between the target and source as well as the angle of incidence. The effectiveness of this heating method is further varied depending on the skin colour, with darker tissue being more receptive (Cameron, 2017). The use of this method has been extremely limited past 1950. However, more recent investigations have shown that infrared does have the capability to alleviate lower back pains as well as increase range of motion in joints (Gale et al., 2006). However, the lack efficacy in deep muscle heating is a major limitation.

1.2.12 Fluidiotherapy

Fluidiotherapy is another superficial dry heating method where heating is achieved via the circulation of heated air through a suspension of particles (Borrell et al., 1980). In the contained conditions of the specially designed cabinet, the specialised particles simulate a fluid delivering uniform heat. It has been reported that a chamber set to 47.8 ± 0.8°C was able to raise muscle temperature by 3.8 °C at a depth of 0.43 cm. Given the depth, it is questionable whether it qualifies as muscle (Cameron, 2017, Borrell et al., 1980). Vardiman and colleagues reported a muscle temperature increase of 5.66 ± 0.78°C to a peak of 39.08 ± 0.39°C at a depth of 3 cm, in the calf (Vardiman et al., 2013) However, this method has not been shown to be effective below 1.2 cm (Borrell et al., 1980), which poses a major limitation. Additionally, the treatment cabinet effects the range of motion significantly.

1.2.13 Misted hot water spray

This is a custom developed heating methodology where the target limb is suspended within a frame to which a number of fine mist sprayers are attached. The sprayers are fed with water heated to the desired temperature. When activated it covers the target limb area with an even coating of heated fine mist (Taylor et al., 1984). Taylor and others reported achieving localised skin temperatures up to 43ºC. However, they did not measure muscle temperature (Taylor et al., 1984). In terms of practical applicability, this method poses a number of difficulties. It requires the target to be stationary which hampers a concurrent exercise element. Pre or post RE heating is also affected by the facts the heat supply is non-constant and the energy-carrying fine droplets are susceptible to a steep and negative environmental heat gradient.

1.2.14 Water perfusion garments

This utilises the circulation of heated water through a garment worn over the target muscle area. In one investigation, a closely laid concentric tubing (medical grade polyvinyl chloride tubes) network is worked into the wearable, elastic, garment, and water, heated to the prescribed

temperature is pumped continuously until the target muscle temperature is reached. The water is fed through a heated bath circulator (Kuhlenhoelter et al., 2016). In two separate protocols, the perfusion garment was worn on the buttocks and thigh, thigh and lower leg. In both occasions, the temperature of the circulating water was held between 48°C-52°C and heat was applied for 90 minutes. While the authors did not measure muscle temperatures during this time, they reported mean skin temperature increments of 6.5 °C (from 33.1 °C-39.6 °C), and core temperatures increments of ~0.6 °C in one protocol and no significant changes in the other (heat application to the quadriceps) (Kuhlenhoelter et al., 2016). Kim and colleagues used a highly comparable water perfusion system and reported similar skin temperatures to Kuhlenhoelter (Kim et al., 2020). Ihsan and colleagues saw customised water circulating sleeve (reservoir temperature 49.5 ± 1.4°C) improve muscle temperature to 38.1 ± 0.6°C at a depth of ~3 cm after an hour of heating (Ihsan et al., 2020). While this method provides continuous and homogenous heating, it has limitations. Based on this evidence, it does not provide the level of deep muscle heating required. However, it is possible that these difficulties can be overcome by utilizing a higher water temperature. Given that that circulating water is heated to a specific temperature, the target muscle receives hot spot free homogenous heat. MedElite4 (Gameready®,Comcord, California), a water perfusion system that uses expandable bladders built in to wraps instead of tubes, is perhaps more efficient at providing homogenous heating given the total area coverage. Furthermore, as the bladder has the ability to conform to the shape of the limb, coupled with the fact that the built-in compression mechanism allows the heat application to be further uniform. Once site specific wrap is secured over the target area, it does not hinder the range of movement.

1.2.15 Environmental chamber

Environmental or climate chambers are a full body HS method, which is similar to sauna in the level of coverage it offers, albeit in a dry environment. Environmental chambers offer the ability to control the desired temperature (and humidity) via a computer controlled thermo-sensor system, heating is achieved via consistently pumping and recirculating hot air (heated to the specified temperature) through the fully insulated chamber. In their full body heating investigation, Racinais and colleagues reported achieving peak mean skin and core temperatures of 38.9 ± 0.7°C, and 39.2 ± 0.3°C (rectal) respectively after 1 hour of intervention with no activity at 50°C (50% RH) (Racinais et al., 2017). The identical heating protocol was followed by Ihsan and colleagues and reported core muscle temperatures of 38.8 ± 0.5°C, significantly increasing from pre intervention ($p < 0.0001$) and almost identical core temperatures 39.1 ± 0.3°C (Ihsan et al., 2020). A clear advantage of the environmental chamber as full body heating method, and as

a heating method in general, over other methods, is the built-in validation of the consistency of the intervention temperature as evidenced by the above cited studies. Moreover, given the relatively large and dry area available during the intervention, environmental chambers are ideal for a concurrent training intervention with no impedance on the ROM or the consistency of the heat application during complexed movements.

1.3 Summary of the review

Methods by which performance as well as muscle adaptive gains by RE could be improved or expedited upon is highly sought after by athletes and fitness enthusiasts. Moreover, in clinical settings, methods by which muscle rehabilitation capabilities of RE can be enhanced upon are hotly researched. RE is a highly capable stressor of the skeletal muscle. On both acute and chronic time frames, RE has shown the capability to promote muscle as well as performance adaptations in humans. Similarly, HS has demonstrated the ability to improve anabolic synthesis as well as other key muscle adaptations. A limited number of studies have investigated HS in combination with RE for potential additive benefits upon the skeletal muscle adaptations to RE with ambiguous outcomes. In reviewing the limited body of literature, HS driven muscle adaptations appear to be predicated upon a threshold temperature. However, this notion has not been previously investigated in humans. Moreover, additive benefits of HS on RE maybe be further influenced by the heating modality. Previously, a number of heating methods have been used to examine the effects of HS on skeletal muscle. However, the muscle temperatures achieved and overall efficacy in each method has shown to be highly variable. A reliable skeletal muscle heating protocol is yet to be established. Moreover, concurrent application of HS and RE is further dependent upon the area of heating, preservation of ROM as well as the overall user safety and well-being.

In addressing the existing gaps in the knowledge base, this thesis will seek to develop a reliable muscle heating protocol that can be used concurrently with RE. Subsequently, the impact of HS on chronic performance adaptations such as strength, force, speed and agility will be investigated. Moreover, how the cellular signal transduction pathways regulating skeletal muscle adaptations to RE are impacted with long term concurrent HS will also be explored.

Chapter 2: Development of a reliable heating protocol and validating heating device

2.1 Introduction

Localised heating of the human skeletal muscle has previously shown to induce anabolic synthesis, angiogenesis as well as mitochondrial adaptations (Goto et al., 2011, Hafen et al., 2018, Kuhlenhoelter et al., 2016). Some studies have shown that localised heat stress (HS) may improve skeletal muscle performance while some studies have seen no impact (Binkhorst et al., 1977, Labidi et al., 2020). Importantly, combining localised heating with resistance exercise (RE) as shown improve upon the anabolic synthesis response to RE (Kakigi et al., 2011). The variability seen on the impact of localised HS appears to mainly be predicated upon muscle temperatures achieved. It has been suggested that an effective clinical response is only observable when core muscle temperature (CMT) is over 38.5-39°C (Lehmann et al., 1974, Draper et al., 1995b). In the current body of literature, the muscle temperatures achieved and the rate of heating is highly variable. This variance appears to be predicated upon the method of heating, duration and intensity of application.

Acute, localised exogenous heat application methods have previously involved microwave heating, ultrasound, shortwave diathermy, infrared heating, paraffin treatment, hot water immersion, heat packs, electric heat pads, steam sheets, fluidotherapy and water perfusion garments (Ichinoseki-Sekine et al., 2007, Draper et al., 1995a, Draper et al., 1999, Goto et al., 2011, Hafen et al., 2019, Kim et al., 2020, Chiesa et al., 2016). Their efficacy for achieving deep muscle heating is highly variable. While a number of these methods are effective at raising CMT by 4°C-5°C, they each carry limitations. The size of the device, the inability to heat the muscle from all directions, and the viable time of application without an increased risk of overheating need to be considered when using methods such as microwave or ultrasound. In the case of steam, heat pads, or hot water immersion, they do not offer the ability to control the heat with any degree of precision as there is no precise control of the output. Moreover, the concurrent combination of RE and HS further requires the device to preserve range of motion (ROM) during exercise. Methods such as immersion pools, fluidotherapy and paraffin treatment methods impede the ROM required to complete an effective bout of exercise within the context of concurrent heat application and RE given the target area is hindered during heat treatment. Furthermore, rate of heating (time taken to increase CMT by 1°C) and the ability to control temperature incrementally in order to achieve specific muscle temperature is key in reliable heating protocol. The rate of

heating through the muscle ultimately depends on the heat gradient between the heat source and the muscle temperature (Petrofsky and Laymon, 2009). This requires a heat source that allows a high starting temperature in order to set a high gradient. To this point, the difficulties associated with raising deep muscle temperature are many. The main among which is user safety compromised by high contact skin temperatures of the heating method.

The level of heating achieved depends on factors such as the duration of application, energy per cm^2, rate of heat dissipation, the amount of adipose tissue, ambient conditions and the amount of muscle generated heat (Draper et al., 1995a, Draper and Ricard, 1995, Draper et al., 1999, Petrofsky and Laymon, 2009). Thus, the six key factors that were considered when determining the appropriate method to apply heat stress were; *(1)* CMT achieved *(2)* time period of application against user comfort, *(3)* the ability to precisely control the total temperature output, *(4)* area/coverage of heating, *(5)* utilizing a device that does not impede the natural range of motion (ROM) needed for exercise bout and, *(6)* homogeneity of heat application (Table 2.1).

Table 2.1. A summary of the most commonly reported localised heating methods and their efficacy to be used concurrently with resistance exercise ✔ =Yes x = No, NR= not reported,? = has not been tested before,*=investigated in this study.

Device	Highest core muscle temperature difference reported (average °C)	Large area of heating	Control over output	Range of motion preserved	Homogeneity of heat application	Participant comfort rating
Microwave diathermy	**~5-6°C**	**x**	**✔**	**✔**	**x**	**NR**
Shortwave diathermy	**~3.5°C**	**x**	**✔**	**✔**	**x**	**NR**
Steam Sheets	**~1-2°C**	**✔**	**x**	**✔**	**x**	**NR**
Hot water immersion	**~3°C**	**✔**	**x**	**x**	**✔**	**NR**
Ultrasound heating	**~1.8°C**	**x**	**✔**	**✔**	**x**	**NR**
Heat pad	**~2°C***	**✔**	**✔**	**✔**	**?**	**NR**
Water perfusion system	**? ***	**✔**	**✔**	**✔**	**?**	**NR**

At the inception of this investigation, only two studies had been conducted on humans with concurrent localised HS during RE (Kakigi et al., 2011, Stadnyk et al., 2017). Kakigi's investigation used microwave heating, while Stadnyk's used a heat pad. Kakigi predicted a peak CMT > 41°C based on a previous report (Ichinoseki-Sekine et al., 2008). Stadnyk predicted a

peak CMT of ~38°C based on their pre experimental pilot study. Both studies investigated the *vastus lateralis*. Neither study directly reported depth specific CMT. Kakigi saw HS acutely improve upon anabolic synthesis to RE but no chronic improvements in strength (Kakigi et al., 2011). Whereas Stadnyk saw no chronic effect of HS on lean muscle mass response to RE, nor did it impact performance (Stadnyk et al., 2017). These variabilities in outcome highlight the impact of heating modality. In order to investigate the impact of muscle temperature as well as to further focus on the possibility of a threshold muscle temperature for improved adaptations to RE, a heating method that is more versatile is required. Therefore, the development of the novel method of localised heating for concurrent application with RE is warranted.

2.2 Methods

2.2.1 Overview

Three localised heating solutions were tested in this preliminary study. The primary objectives were to *1)* test the skin and muscle temperatures generated by each solution (validity) and, *2)* test the repeatability and the perceived comfort levels of participants under each solution. All solutions were initially tested for skin thermal sensation and comfort and were eliminated as a viable option if the comfort levels reported were deemed unsuitable for prolonged (>30 minutes) exposure. The first two solutions were powered by a purpose designed power platform, while the third was a commercially available heating device.

2.2.2 Thermal sensation and comfort scale and feedback

The subjective thermal sensation and comfort scale developed by Gaoua and Granthem was utilised to determine the heat-comfort feedback (Gaoua et al., 2012). Participants were familiarised with the scale before the first testing session. The thermal sensation and comfort scales were presented to them abreast (Figure 2.1). The thermal sensation scale was presented in colour for anchoring purposes and the comfort scale in monochrome. Participants were asked to indicate a position (and colour) on each scale. The corresponding rating scores were obscured and was only accessible to the investigator. Thermal sensation and comfort feedback were recorded simultaneously, immediately pre- heat application and at 15 minute intervals thereafter. Two consecutive reports of ≥ 17 on the heat scale, that were taken five minutes apart was deemed as the cut off threshold for heat application.

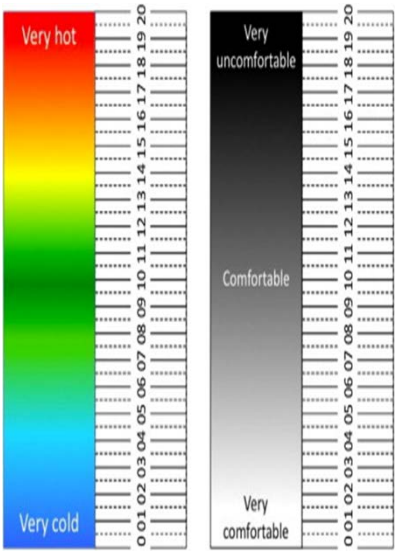


Figure 2.1. Gaoua – Grantham thermal sensation and comfort scale

2.2.3 Heat application solutions

2.2.3.1 Purpose developed power regulation platform

In this phase of the investigation, a regulator platform was developed with the capability of powering and regulating wrap solutions one and two, which will be expanded upon in forthcoming sections. The platform was developed in collaboration with the engineering department, within the College of Sports and Exercise Science at Victoria University, Melbourne.

This regulator had the ability to simultenously regulate two separate power outputs (Figure 2.2). Furthermore, it was equippied with built in temperature sensors, which provided a negative feedback control to activate an electronic thermostat allowing the desired temperature to be preset. These presets included two different temperatures up to a maximum of 45°C. Once the target temperature was exceeded by 0.25°C (the margin of error ± 0.25°C), the power supply was automatically terminated. The temperature feedback however was maintained continuously and was programmed to reinitiate the power supply once the temperture reached 0.25°C below the target temperature. If at any instance the skin temperatue was to reach 45°C, the automatic safety cut-off intervened to terminate the power supply. This pre-programmed auto regulatory mechanism allowed for a constant temperature output to be maintained over the chosen site for a deemed period of time. Using this platform, a commercially available heat wrap and an industrial grade heat mesh fabric were powered and regulated as two of the three heat application methods tested in this phase of the study.

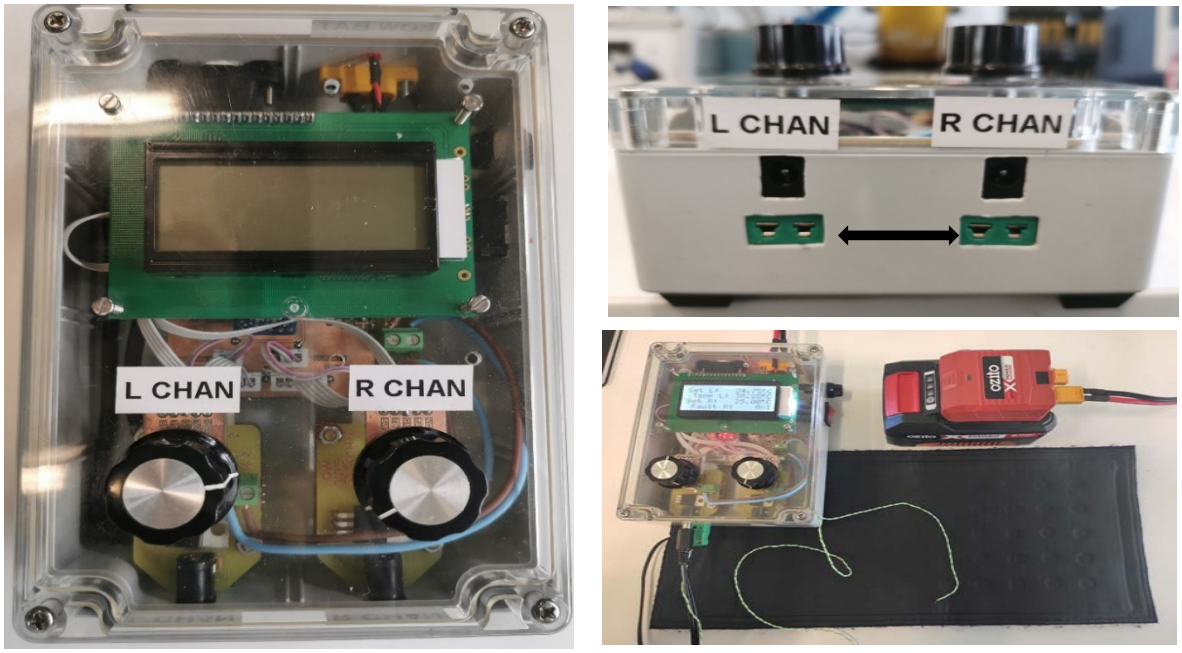


Figure 2.2. The power regulator platform comprised of two separate outputs for simultaneous use on two targets. The regulator platform controls the power output provided by 12V volt battery pack.

2.2.3.2 Preliminary testing protocol for heating solution one and two

The preliminary testing protocol investigated two variables; the peak skin temperatures achieved and the time elapsed to achieve the target temperatures. The tested wraps were 270.96 cm^2 in area (solution one: 15.24 cm x 17.38 cm) and 1500 cm^2 (solution two: 30 cm x 50 cm) respectively. The chosen target area was the thigh (quadriceps). Room temperature was recorded (22.4 ± 1.6°C) at each pilot session. The two solutions were tested on four participants and two participants, respectively (Figure 2.3 & 2.4).

Solution one: Recreational heat wrap (powered by a purpose designed, battery powered platform)

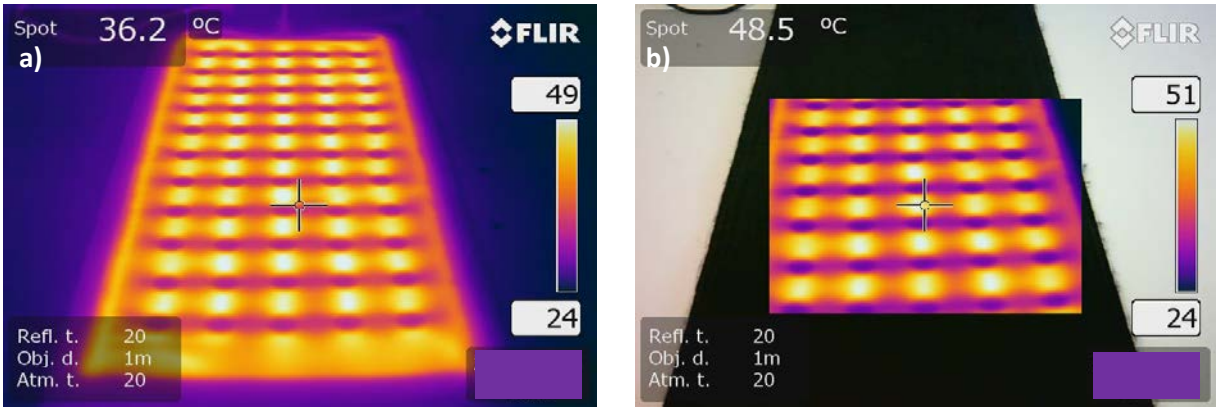


Figure 2.3. FLIR® infrared images of the Exo2® heat wrap; a) full scale infrared image b) sectional image. Cross hairs aimed at heating element (or chain) in image (b).

The first method tested was an electric heat pad/wrap, known as the FabRoc^{TM,} which was produced by EXO2® (McDonough, Georgia, USA). The fabric substrate is a blended polymer and carbon formulation that contains carbon particles evenly dispersed through-out the substrate. In tandem, it has a conductive chain built into the material which carries electrical current through (Figure 2.3b). As the temperature increases, the material expands, allowing the fabric to be homogenously resistive to the provided current with minimal hotspots (Figure 2.3a), which is a critical factor when delivering localised acute HS. The design of the fabric allows it to convert ~99% of the energy to heat. Furthermore, a refined balance between increasing temperature and resistance allowed it to self-regulate the incoming current, which by extension aids in maintaining a stable surface temperature and therefore a positive temperature coefficient (Exo2® product specifications sheet). The material is conducive to a broad voltage range from 12 V- 21 V and has a top end temperature point of 300°C. Furthermore, it is light and mildly elastic and does not apply excess compression. In this solution FabRoc® is insulated within a proprietary bonding film on one side and heat shielding 3 mm neoprene on the other side. This made the heat flow unidirectional as well as moisture proof.

Heat wrap one was applied to the thigh for 30 minutes with two skin temperature probes retrieving temperature at 0.2 Hz, from *vastus lateralis* and *biceps femoris*. The temperature applied to the skin at was 43 ± 0.5°C. Participant comfort was recorded every 5 minutes until termination of the test using the Gaouo-Grantham 1-20 (10 = comfortable, 20 = very uncomfortable, very hot) thermal- comfort scale (Figure 2.1).

Solution two: Flexible heating mesh fabric (SEFAR® Powerheat NT)

The second solution tested was a flexible heating mesh fabric developed by SEFAR® (Thal, Switzerland) (Figure 2.4). The highly conductive alloy mesh material is a combined weave of breathable polyethylene terephthalate (PET) microfilaments, micro metal wires and conductively coated fibers. The entire fabric is conductive and has free emitting connection bars from one locus and can be powered directly from a power source between voltages of 5 V-25 V. Depending on the voltage, the top end surface temperature varies from 60°C - 90°C. It is conducive to morphing and can be shaped and molded into a multitude of shapes as well as cut into desired shapes due to the continuous nature of the conductive fabric. The material is lightweight (140 g/m^2) and has a thickness of ~0.3 mm. Due to the high conductivity of the alloy mesh it presented a low thermal resistance, resulting in immediate and efficient heating. Given the uniform heat transfer across the fabric it, when immobile, delivered heat at a steady rate to the target muscle.

This allowed it to be laminated or sleeved without effecting the overall conductivity, which improved participant safety and repeatability.

Heat wrap two was applied to the thigh for ~10 minutes, at which time participants reported high levels of discomfort so testing was ceased (Table 2.2).

Given the high discomfort ratings by the participants, these were discontinued without testing for repeatability.

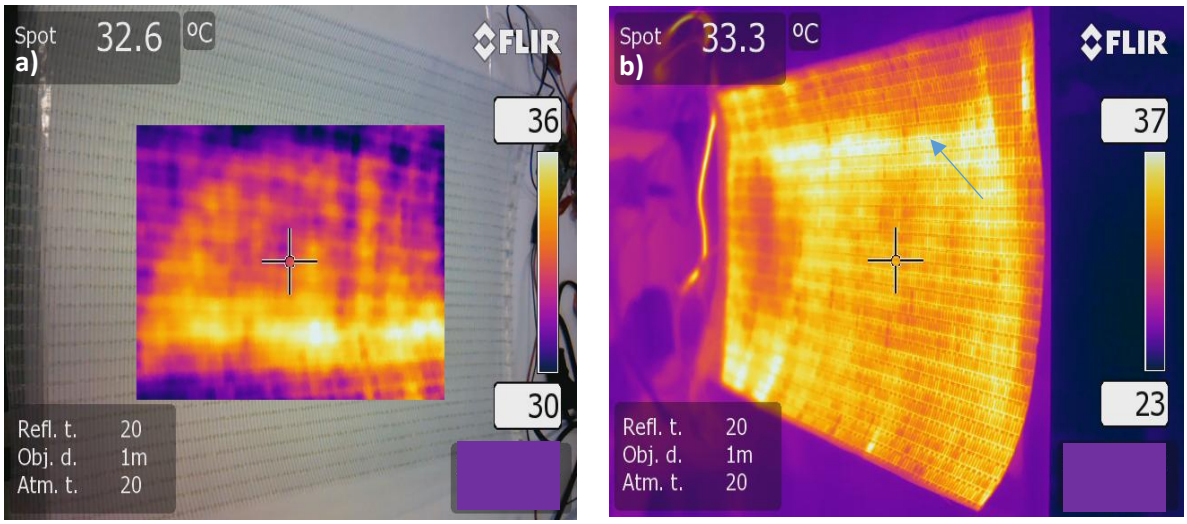


Figure 2.4. a) The FLIR® infrared images of SEFAR® PowerNT™ heat mesh. The hotter area depicted in b) was caused by the mesh being less in contact with its resting platform.

Solution three: Med4Elite™ Hot/Cold/Contrast therapy device developed by Game Ready®

Med4Elite™ is a heat, cold, contrast and compression therapy platform developed by Game Ready® (Concord, CA, USA) (Figure 2.5a). It allowed targeted heat application via heated water perfusion through shape conforming wraps. It offers a top end water temperature of 45°C and can be controlled real time via computerised temperature management. It allowed two limbs to be treated simultaneously at the same temperature via two separate outlets. The wraps were two part with an outer sleeve housing a thin bladder with guided perfusion. Highly homogenous heat can be delivered to the target areas due to the constant unbroken circulation of heated water, the conformation of the wrap to the target area as well as the compression generated by the expanding bladder (Figure 2.5b).

The MedElite® system provided two variants of water perfusing wraps (straight knee wrap, full leg boot). Each allowing for different area sizes to be heated (Figure 2.5b).

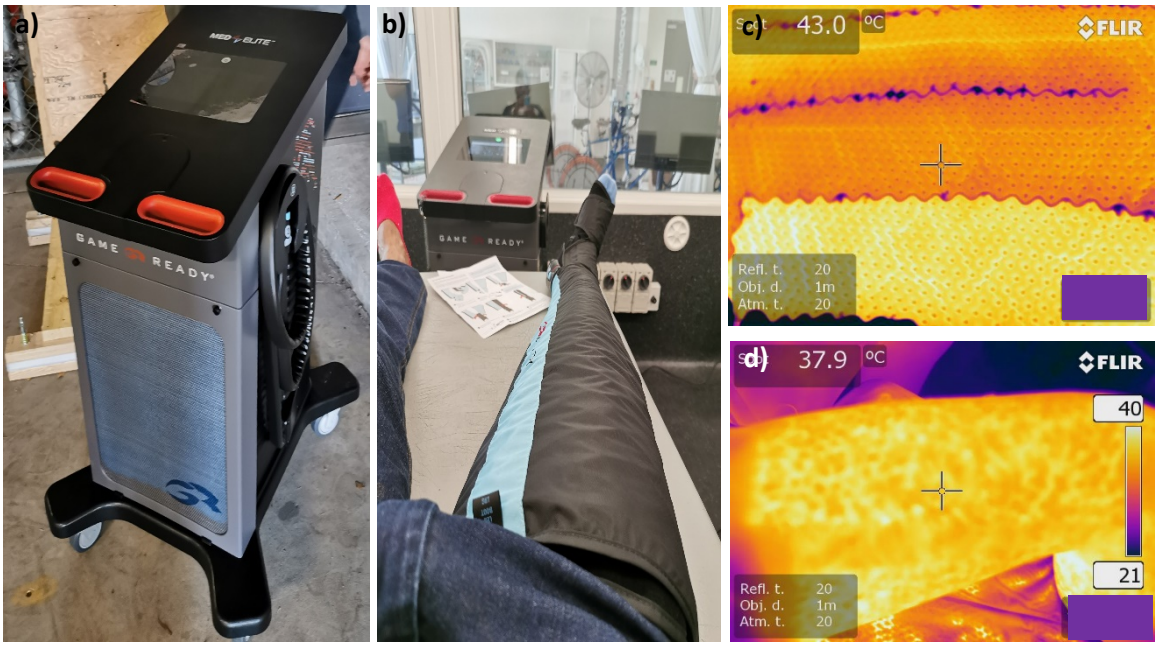


Figure 2.5. a) The Med4elite™ heat, cold, contrast and compression therapy platform by Game Ready®. b) The full leg boot applied in a testing session, c) the infrared profile of the wrap closer to maximum temperature. The water is guided through by sewn in seals which are visibly much colder, d) the thermal profile of the quadriceps 60 seconds after the removal of the wrap. The homogenous heating is evident.

2.2.4 Testing of the apparatus and protocol of heat application

2.2.4.1 Participants and sample size calculation

The heating apparatus was tested on a mixed cohort of ten participants. The sample size was based on a similar study conducted by Kuhlenhoelter and others (Kuhlenhoelter et al., 2016), who tested a water perfusion device for thigh as well as lower body heating.

The Victoria University Human Research ethics committee approved (HRE18-229) this research project. All participants were informed of the voluntary nature of the project, and all participants provided written informed consent.

2.2.4.2 Measurement of thigh dimensions

Length and circumference measurements of the thigh were obtained prior to heat application. Length was recorded as the distance between the participant's anterior superior iliac spine and the head of the patella. Circumference was obtained from 5 cm below the *gluteus maximus*, the mid-point between anterior iliac spine and the head of the patella, and 1cm above the patella. These were measured in order to endure the use of the correct wrap for each participant as two different sizes (medium and large) were provided by the supplier

2.2.4.3 Measurement of sub-cutaneous fat layer thickness

Prior to heat application, an approximate area of 8 cm x 5 cm on the *vastus lateralis* was shaved with a sterile razor. The positioning of the site was 5 cm lateral to the mid-point between anterior superior iliac spine and the head of the patella as the centre. A general amount of ultrasound transmission gel was applied and three measurements of the subcutaneous fat layer were obtained via ultrasound (CX50, Philips Healthcare, Netherlands) scans from three locations within the site. Two readings vertical to the site and one horizontal comprised the three measurements.

2.2.4.4 Intramuscular and skin thermistors

An intramuscular temperature probe was inserted at 5 cm lateral to the mid-point between anterior superior iliac spine and the head of the patella. The site was cleaned and sterilised, and 18 gauge catheter (Optiva IV catheter 18G x 4 cm, Smith's Medical, USA) was inserted into the *vastus lateralis*. The needle was removed leaving the catheter embedded in the muscle. A sterilised, dual sensing, thermistor (Model T-204A, Physitemp Instruments, Clifton, USA) was then inserted to a depth of 3.5 cm - 4 cm and the catheter was withdrawn slightly, leaving the tip of the probe exposed to the muscle. The thermistor was then secured to the leg and was anchored to ensure stability. An identical thermistor was placed on the skin adjacent to the intramuscular probe. The site was then covered with a sweat proof membrane (Tegaderm; 3M, North Ryde, NSW, Australia). Temperature was logged real time at a rate of 0.2 Hz at a depth of 3.5 cm-4 cm and 1.5-2 cm.

2.2.4.5 Heat application

Before the window of heat application, each participant rested for 30 minutes in a temperature-controlled chamber (HEUCH, INNOTECH®, VIC, Australia), allowing for the CMT to stabilize (all of the above listed procedures took place in the interim). The room conditions were maintained at a thermo-neutral 23°C with 30% relative humidity standard for each volunteer. Circulating water temperature was set at 45°C. The safety threshold for highest skin temperature tolerated was set at 44°C. Previous human studies have investigated the effects of heat on muscle at 44 ± 1 °C where the *vastus lateralis* was in direct contact with hot water (Skurvydas et al., 2008, Morton et al., 2007) and another instance where the forearm was immersed in 46°C water (Clarke, 1963). Furthermore, in another similar study construct, (hot water perfusion suit) water temperature was maintained at 48°C for 90 minutes and skin heaters were used at 43°C for 20 minutes in direct contact with the skin (Kuhlenhoelter et al., 2016). Therefore, the 44°C was justified in terms of participant wellbeing.

Given the probe is a flexible wire, it allowed for the heat wrap to go over the probe without impedance. Skin temperature was monitored via a skin temperature sensor. Both skin and muscle temperature were recorded in real time (at a rate of 0.2Hz). Heating was applied until core muscle temperature plateaued and remained at a given temperature for ten minutes. Feedback was obtained from the participants on the overall level of comfort via the thermal sensation-comfort scale as described above. The skin temperature pattern was also monitored via a thermal camera (FLIR T1010, FILR, Oregon, USA) at the start and the end of each test, as a method of visualizing pre and post intervention heating patterns (Figure 2.5d), enabling for direct comparisons among the cohort on the area of coverage and the heating pattern and the homogeneity of heating (Ring and Ammer, 2012). In between imaging, the participants' legs were covered with a thermal foil blanket to prevent heat loss via radiation.

Gameready's short, straight knee wrap was compatible to use concurrently with RE (resistance exercise) and was available to us in different sizes to accommodate for the variability in thigh girth between participants. Furthermore, the wraps themselves are configurable to preserve range of motion during exercise.

The two sessions were conducted in an identical fashion 48 hours apart. In the second session, heat was applied to the contralateral leg. This was done to duplicate the peak CMT outcomes for each participant, in order to minimise the variability introduced by potential differences in the temperature probe depth.

Given across ten participants (20 test sessions in total) core muscle temperature failed to reach ~38°C, further test –retest repeatability was not conducted as it was evident that the heating was inadequate to continue with further testing.

2.2.4.6 Statistical analysis

All data was analysed using GraphPad Prism 9.2 (San Diego, CA). Non parametric paired T-tests were performed on pre and post intervention muscle temperature data with the significance accepted at $P < 0.05$. Additionally, a simple linear regression analysis was performed to generate correlations between time to peak core muscle temperature and subcutaneous fat layer thickness and peak core muscle temperature. All data reported as mean ± SEM unless otherwise stipulated.

2.3 Results

2.3.1 Thermal sensation and comfort testing

For solutions one and two, thermal sensation-comfort scale feedback was above the deemed ≥ 17 threshold for thermal sensation and comfort (Table 2.2).

Table 2.2 Initial thermal sensation and comfort results against mean peak skin temperatures achieved. Data presented as mean ± SD.

Number of participants	**Peak skin temperature (°C)**	**Time to peak (min)**	**Thermal sensation rating**	**Comfort reading**
	Exoglo®, Exo2 heat wrap			
4	42.8 ± 0.37	15.4 ± 0.21	17 ± 1	18 ± 1
	SEFAR® PowerNT heat mesh			
2	45.8 ± 0.0	10.1 ± 0.9	19 ± 0	19 ± 0
	Gameready® MedElite water perfusion device			
10	41.1 ± 0.76	17.1 ± 3.4	13 ± 1	12 ± 1

2.3.2 Sub-cutaneous fat layer thickness

Sub cutaneous fat layer thickness was obtained from both legs (Table 2.3 and Figure 2.6).

Table 2.3. Absolute and average sub-cutaneous fat layer thickness of all participants tested for solution three

Participant	Sub cutaneous fat layer thickness (left) (cm)	Sub cutaneous fat layer thickness (right) (cm)	Average (cm)
1	0.8033	0.756	**0.779**
2	0.397	0.470	**0.433**
3	0.675	0.697	**0.686**
4	0.271	0.264	**0.267**
5	1.440	1.740	**1.590**
6	0.378	0.558	**0.468**
7	0.509	0.400	**0.454**
8	0.485	0.509	**0.497**
9	0.511	0.392	**0.451**
10	0.525	0.539	**0.532**

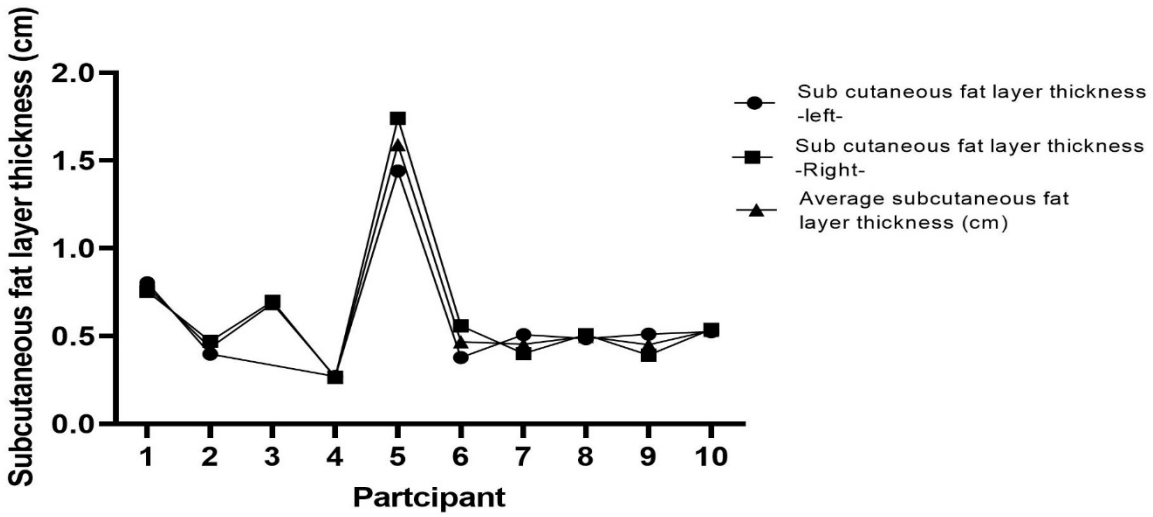


Figure 2.6. Sub-cutaneous fat layer thickness

2.3.3 Core and shallow muscle temperature

The Game Ready, MedElite® produced a mean peak CMT (at 3.5cm) of 37.56 ± 0.52°C which increased significantly from mean pre intervention CMT levels (35. 81 ± 1.06°C) ($p < 0.0001$) (Figure 2.7). In addition, the device produced a shallow mean muscle temperature (at 1.5 cm) of 38.65 ± 1.87°C (Figure 2.8) with an average difference between mean peak CMT and mean pre intervention CMT of 2.18 ± 0.82°C.

The average time elapsed to reach peak CMT was 78.3 ± 13.7 minutes (Figure 2.9a). A negative correlation was observed between mean CMT and sub cutaneous fat layer thickness ($r = -0.2502$, $p < 0.0001$) (Figure 2.9b)

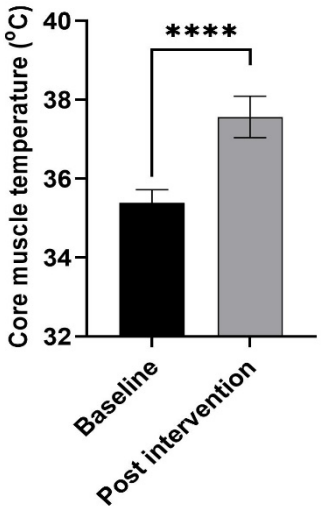


Figure 2.7. Mean core muscle temperature at 3.5cm. Reported as mean ± SEM; **** p < 0.0001

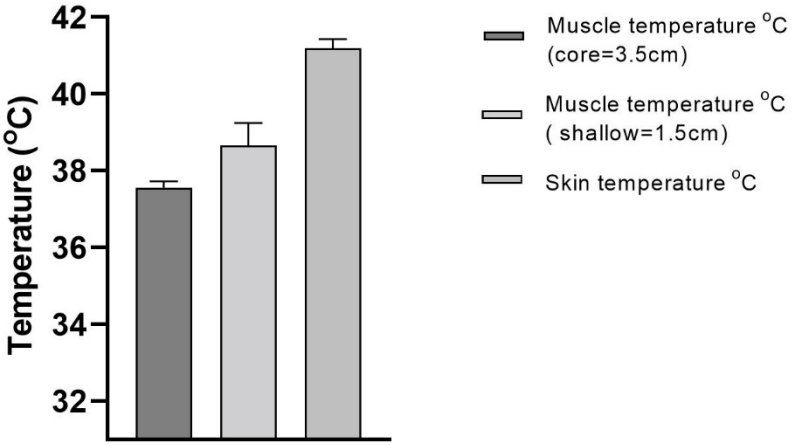


Figure 2.8. Core, shallow muscle temperatures and skin temperature. Reported as mean ± SEM

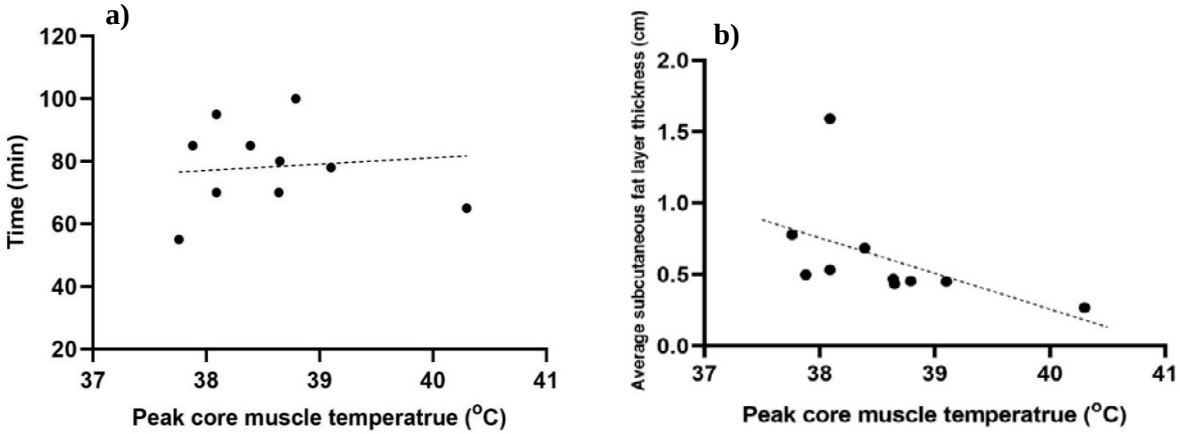


Figure 2.9. a) Scatter plot of individual times taken to reach peak core muscle temperature and b) the relationship between mean peak CMT and average sub cutaneous fat layer thickness

2.4 Discussion

Localised, exogenous heat application is commonly used as a recreational recovery method and a multitude of heat application methods are commercially available. A range of reports exist on the effectiveness of these methods in increasing CMT in humans (refer to part two of the literature review). However, only a limited number of studies have investigated the effect of localised HS on muscle adaptations as well as performance adaptations to acute and chronic RE.

At present, there is a lack of evidence on which method is most effective when used as a stressor in tandem with RE to potentially enhance the training adaptations. Crucially, while a limited number of prior investigations have used heat concurrently in combination with acute bouts of RE, they have reported equivocal results in terms HS impact on muscle adaptations.

As outlined in the introduction, we defined six criteria to assess the currently available heating methods for concurrent heat application during RE; *1)* CMT achieved *2)* participant comfort, *3)* area of effective heat application, *4)* control over output, *5)* homogeneity of heating and *6)* preservation of ROM during performing of exercise. Given that none of the previously tested methods fulfilled the criteria, the main aim of this investigation was to develop and test three novel methods of localized heating that have not been previously used. Secondarily, to develop a novel heating protocol that could be used concurrently with RE.

While solutions one and two (Exoglo®, Exo2 and SEFAR®, PowerNT respectively) both preserved ROM, they exceeded the participant comfort and wellbeing threshold, eliminating them as viable localized heating methods. In addition, the development of hot spots meant that they failed to deliver the homogeneity required in the target area. Solution one was robust, however, failed to conform to the muscle during movement thereby creating inconsistent contact patterns which lead to the development of the hot spots. Solution two performed better in conforming to the shape of the thigh with movement. However, the high conductivity of the mesh substrate lead to rapid heating of the contact interface with the skin. The rate at which the skin temperature was rising was not compensated by the rate at which heat was being dissipated, leading to high levels of participant discomfort within a short time frame (< 15 minutes).

The Game Ready® MedElite water perfusion device produced an acceptable thermal sensation and comfort feedback over prolonged periods of time (< 30 minutes) on all iterations of testing (straight knee and full leg boot wrap). Furthermore, it produced excellent homogenous surface heating levels (based on post intervention infrared imaging) and was deemed suitable for CMT testing.

The rate of heating (Figure 2.9a), defined as: $\frac{termperature\ (℃)}{time\ elapsed\ (\mathrm{min})}$, was relatively constant until equilibrium was reached. Equilibrium was defined as a fluctuation of temperature by only ± 0.2 °C within a time period of 15 minutes. CMT increased significantly (37.56 ± 0.52°C, $P < 0.0001$) from pre intervention (35.38 ± 1.06°C), fulfilling five of the six criteria delineated. The wrap maintained the homogeneity of heat supply during light movement of the leg, however, significantly encumbered the ROM (flexion) when performing a knee extension. Moreover, while the CMT increased significantly, it did not reach the expected 38.5-39°C, therefore deemed not suitable as well.

Localised or full body heat stress?

The human circulatory system is optimised to actively dissipate heat in order to maintain homeostasis (Benzinger, 1969). Furthermore, heat is lost to the environment via sweat evaporation, radiation, convection and conduction (Benzinger, 1969). Additionally, it is evident from the results of this investigation that factors such as the sub-cutaneous fat layer thickness negatively correlate with the rate of heating and peak CMT. These factors heavily influence the balance between comfort and intensity (applied temperature) during heat application, influence the muscle temperatures achieved during heating interventions.

At the inception of this project, the available data (Table 2.1) justified the testing of a novel localised heating protocol given the number of difficulties each method presented in using as a method of concurrent heat application. However, evidence generated in the present investigation implied that developing a localised heating method where all the criteria required for ensuring high efficacy in raising CMT > 38.5-39°C during concurrent application with RE, whilst maintaining unhindered ROM, homogenous heating and acceptable levels of participant comfort may not be feasible.

In order to maintain unhindered ROM while simultaneously ensuring the homogeneity of heat application, as well as providing full coverage of the target area during concurrent RE, full body, HS appeared the most viable option. At the stage of planning of this investigation, there were a number of human studies which have utilised full body concurrent HS during endurance type exercise as well as to test for heat acclimation driven performance enhancements in humans (Racinais et al., 2017, Lorenzo et al., 2010). Lorenzo and colleagues used a climate chamber conditioned at 40°C (30% RH) as a full body heat acclimation devise, where the participants performed two bouts (each 45 minutes) of ergometer rides separated by a ten minute rest, followed by exercise testing at 38°C (30% RH). They found improvements in performance (Lorenzo et al., 2010). Racinais and colleagues reported that full body passive heat acclimation at 44-50°C (50% RH), improved muscle contractility in humans (Racinais et al., 2017). Webb and colleagues tested six subjects for skin and muscle temperature (two cohorts of n = 3), in an iterative test where each cohort went through temperature conditions of 27°C, 45°C and 15°C or 27°C, 15°C and 45°C in an environmental chamber with exposure times of 2-3 hours. Participants were only dressed in an athletic supporter to endure full exposure to conditions (Webb, 1992). They reported mean peak CMT in the thigh ~38 °C at 3.5-4.0 cm (including the subcutaneous fat layer) at 45°C, which is almost identical to the CMT reached in this study (37.56 ± 0.52 °C) in the same target muscle at the same depth. Mean shallow muscle temperatures at

1.5-2.0 cm were also comparable ~39°C. These temperatures were obtained within an average time of 88 minutes (they report high degree of variability within the three participants tested), which is also highly comparable to the time to mean peak CMT observed in this investigation at 78.3 ± 13.7 minutes. This data collectively served as the justification for full body HS to be utilised during concurrent heat application and RE as opposed to the use of a localised HS method. It is key to note that RE alone is capable of raising the muscle temperature ~1 °C (in the thigh) (González-Alonso et al., 2000, Racinais and Oksa, 2010).

In combining full body HS and RE, we took in to consideration that participants will be performing high intensity RE to failure in a heated environment. In piloting the heat conditions, we found that when performing RE, 40° degrees was the highest tolerable environmental temperature. Therefore, we did not use temperatures similar (45°) to that of Racinais and Webb, as we expected the culmination of HS and the RE to improve CMT above 38.5-39°C, while maintaining the wellbeing of the participants during the performance of concurrent exercise in heat. However, as stated above, heavy RE itself is capable of raising core muscle temperature by ~1°C. Number of studies had shown that resistance exercise alone can improve muscle temperature by 0.8°C -1.0°C. Morton et al., (2006) showed that after 45 minutes of non-damaging treadmill running muscle temperature increased > 39°C with a peak core temperature of ~39°C. Moreover, Morton et al., (2007) showed that core temperature increased by ~1.7°C when a single limb (leg) was immersed in hot (45°) water which increased core muscle temperature by ~4°C to a peak of 39.7°C . Lorenzo et al., (2010) showed that performing moderate intensity ergometer exercise in 40°C elevated core temperature to ~39°C. This evidence allowed us to strongly deduce that the maximum tolerable temperature of 40°C would elevate core temperature above ~39°C during exercise while raising muscle temperatures over 38.5-39°C. Based on this evidence we predicted that RE would actively compensate and create comparable conditions to 45°C of HS alone while ensuring the well-being of the participant.

Therefore, we hypothesized that full body HS applied at 40°C (30% RH), combined with high intensity RE should increase the CMT to >38.5 39°C, which has been posited to drive long term performance and phenotypic adaptations in the skeletal muscle.

Chapter 3: Effects of long-term concurrent heat stress on performance adaptations to resistance exercise

3.1 Introduction

Strength, speed, power and mobility are key physical traits required for different modalities of athletic or physical performance. Resistance exercise (RE) is widely established as an effective exercise training approach to improve hypertrophy, strength, speed, agility, power, force and coordination across various levels of athletic development and age (Schoenfeld and Aragon, 2018, Harries et al., 2012).

It has been established that in order to maximize the gains of RE (hypertrophy, strength, power, force) three basic principles are to be adhered to. Progressive overload, variation and specificity (Kraemer et al., 2002). Progressive overload is the gradual increase of the load, and/or volume in order to maintain the muscles in the stressed state. Variation refers to the systematic adjusting of one or more program variables such as frequency, intensity, exercise duration and modality during the course of a training period to ensure that the nature of the stimulus is challenging to prevent the plateauing of the adaptive response. The principle of specificity dictates that training adaptations are distinctive to the stimulus applied, therefore tailoring of the program to accentuate selected or desired adaptations. Therefore, characteristics of force, velocity, directionality of loading, and mechanics of the movement during training will produce distinct adaptations. It also implies that to train to improve a physical quality, there may be certain methods of training that will lead to more rapid and/or larger adaptations in particular qualities, for instance, power training for power production (Ratamess et al., 2009, Dudley et al., 1991, Häkkinen et al., 2003, ACSM, 2009). Additionally, Dudley and others submitted that for long term RE programs to be effective in improving dynamic strength significantly, eccentric muscle contractions are key and in order to further maximize performance gains, the inclusion of a variety of functional isometric contractions is paramount (Dudley et al., 1991, Keogh et al., 1999). Many individual factors such as motivation, general and musculoskeletal health, genotype and environmental conditions affect the performance gains via RE (Thomis et al., 1998). These factors will impact the extent of an individual's response to a given training program, compared to another. Therefore, a well-balanced program containing concentric, eccentric and isometric contractions, designed based on the above mentioned principles is the most effective method in order to maximize the results across the population (Ratamess et al., 2009).

Interest has been increasing to discover supplementary interventions that can be applied in combination with RE to further improve the potential performance gains, possibly on a shorter time frame. Heat stress (HS) is one such among those (Hyldahl and Peake, 2020). There exists a very limited body of work on HS and its ability to improve performance aspects such as strength, power, speed and agility following periods of training. In addition, the influence of HS during chronic training time frames is further limited. In early reports, Asmussen and colleagues observed a strong relationship between acute HS (muscle temperature) and maximal dynamic force and power, where jump height improved from 20-30 cm at 30°C to 40-45 cm at 40°C. Importantly however, they stated that the temperatures reported were not mean core muscle temperatures, but arbitrary values for comparison between hot and cold muscles (Asmussen et al., 1976). Therefore, the fidelity of the reports are questionable. Bergh and Ekblom reported clear improvements in peak torque, jump height, maximal cycling speed and power with increasing muscle temperature (reported temperature range 30°C-39°C) (Bergh and Ekblom, 1979). However, given they used active exercise to heat the muscles, the improvements could also be explained by acute neuro-muscular adaptations, where the prime movers are already recruited and synergist and antagonistic muscles are pre-activated (Sale, 1988). Binkhorst and colleagues demonstrated acute improvements in the force-velocity relationship in grip strength with increasing muscle temperature (muscle temperature in the arm ~ 38°C) compared to a resting temperature of 32.8°C (Binkhorst et al., 1977). In addition, the relationship between acute performance improvements and increasing muscle temperature was further substantiated by the observations of Sargeant, who reported an ~11% increase in maximal peak power and force during a 20-second maximal cycle ergometer sprint completed after 45 minutes of hot water immersion at 44ºC (Sargeant, 1987). In a cohort of highly trained power athletes, Casadio and colleagues observed improvements in upper body power (medicine ball throw) in females (3.4%, 90% CL −1.5, 8.6) and males (3.3%, −0.1, 6.9) and an improvement in lower body power (vertical jump) only in males (3.2%, −0.4, 6.9) in an environmental temperature of ~30°C, 40-60% RH compared to the control conditions (~20°C) (Casadio et al., 2017). However, Davies and colleagues saw no positive relationship between the force generating capacity of *triceps surae* and muscle temperature (~39°C) compared cool conditions (~29°C) (Davies and Young, 1983). However, collectively, this evidence suggests that acute localised and full body HS may benefit performance post intervention.

Investigating the impact of chronic HS on performance aspects, Goto and colleagues reported a mean increase in isometric knee-extension torque of 5.8 ± 2.5% in the heat treated legs compared

to non-significant 3.7 ± 6.9% increase in the control legs, following localised heating of the *vastus lateralis* for eight hours a day, four days a week for ten weeks (Goto et al., 2011). These findings were strengthened by Racinais and colleagues where they found significant improvements (ES $\gamma^2 = 0.484$, $p < 0.004$) in isometric plantarflexion torque following 11 consecutive days of full body HS (rectal temperature ~39°C), in the form of heat acclimation (Racinais et al., 2017). Similarly, Kim and colleagues observed that after localised heat application to the quadriceps for 90 minutes per day, five days a week for eight weeks, isokinetic peak torque at $180°s^{-1}$ improved by 6% and 5% at weeks four and eight respectively (Kim et al., 2020). They reported no difference in fatigability (measured via isokinetic dynamometer) between the heat and control groups at the end of the intervention, indicating no cumulative HS to fatigue resistance (Kim et al., 2020). Controverting these findings, Labidi and colleagues reported no improvements in isometric, concentric or eccentric torque following six weeks of localised HS to the *gastrocenimus* muscle for five consecutive days a week, (Labidi et al., 2020). While equivocal, these results indicate that HS does have the ability to improve skeletal muscle performance.

Meline and colleagues reported significant improvements (precise results not reported) in maximal isometric strength in knee flexion and extension ($p < 0.0001$ and $p < 0.02$ respectively) at four weeks in a cohort of ($n = 6$) short-track ice skaters who underwent a ten minute hot water bath (40.3 ± 0.6 °C) post training (two consecutive days a week) compared to the untreated group. However, neither the exact structure of training nor the type of training were reported (Méline et al., 2021). These findings support claims by Stadnyk and colleagues, who reported no differences in the improvements in peak isokinetic torque at 90 deg/s or 3-RM strength between a control and heat intervention group following 30 sessions of unilateral knee extensions over 12 weeks. The study was a contralateral limb-control design where one limb received ~38°C localised heating to the thigh concurrently to the RE bout (four sets of eight knee extensions at 70% 1RM) in a mixed cohort ($n = 10$). Goto and colleagues saw that after ten weeks of localised HS before exercise to the *biceps brachii* improved maximum isometric torque of flexion, compared to the non-heated contralateral arm at a muscle temperature of ~38°C (Goto et al., 2007).

While it could be suggested that acute and chronic HS does contribute to improvements in performance, the evidence is slim with number of contributing factors such as varying muscle, core and environmental temperatures (Girard et al., 2013, Racinais et al., 2017, Thomas et al., 2006), differences in training modalities (some may not have been suitable for the targeted

adaptations) (Lorenzo et al., 2010, Labidi et al., 2020), acute neuro-muscular adaptations (Racinais and Oksa, 2010) such as variability in motor unit recruitment, as well as the variability of heating modalities that could explain the observed responses. However, there is sufficient evidence to suggest that HS is potentially beneficial in improving performance aspects. Furthermore, it has not been investigated whether chronic, full body HS applied concurrently with high intensity, progressive RE further improves the performance aspects of strength, force, speed and agility. In previous investigations, full body and localised HS methods have been tested, however, they have been equivocal in raising CMT to ~38.5-39°C which has been suggested a threshold for performance improvements. Furthermore, there is a lack of evidence supporting the concurrent application of HS with ecologically valid RE protocols that might aid in increasing athletic performance as well as muscular wellbeing.

We hypothesised that application of chronic full body HS with concurrently full body RE may increase the muscle temperature past the threshold of 38.5-39°C, as justified in Chapter Two. Thereby, improving upon RE driven performance gains in strength, force, speed and agility. In the present investigation, range of motion (ROM) is preserved and homogeneity of heat application is ensured. CMT will measured to verify muscle temperature.

Based on the above hypothesis, the aim of this study was to investigate the effects of an ecologically valid, concurrent, full body, chronic HS on performance adaptations induced by RE.

3.2 Methods

The tested participant subset consisted of males between aged between 18-45 years, devoid of health conditions (cardiovascular decreases asthma, lung disease, epilepsy), musculoskeletal injuries. The cohort was deemed recreationally active which was defined as not regularly undertaking a high RE volume (exclusion threshold set at >2 days a week of training) within three months leading up to the day of recruitment. Furthermore, potential participants who had suffered from dizziness, nausea, fainting, palpitations, breathing difficulties or heat exhaustion during or post exercise, were also excluded. This was verified via a pre- recruitment screening questionnaire. The participation was entirely voluntary with informed consent.

The study was approved by the Victoria University Human ethics committee (HRE15-302) and was conducted fully in accordance with the approved stipulations of the Declaration of Helsinki.

3.2.1 Overview

A cohort (n = 18) of recreationally active, males were assigned to two height and body mass (BM) matched groups (Table 3.1). Each group undertook an identical, ten week, full body RE program comprising of three sessions per week, each separated by a full rest day in between. The control group (CON) trained in thermoneutral conditions (23°C, 20% RH) while the test group (HEAT) trained in a climate chamber (40°C, RH 30%) where the specified conditions were maintained rigorously. Each participant first underwent a pre-experimental familiarisation for the performance testing. Performance tests were taken pre-, mid- (post week 5) and post-intervention. Core and muscle temperature were also measured during session two (week one). Furthermore, they underwent three dual x-ray densitometry scans (DXA) pre-, mid- and post intervention in order to determine their lean muscle mass (LMM) through the intervention (Figure 3.1).

Table 3.1. Participant anthropometrics. All values expressed as mean ± SD

	Group size	Age (years)	Body mass (kg)	Height (cm)
CON	10	21.0 ±.2.7	76.0 ± 11.3	177.2 ± 9.6
HEAT	8	23.3 ±.3.1	75.6 ± 14.5	175.6 ± 8.8

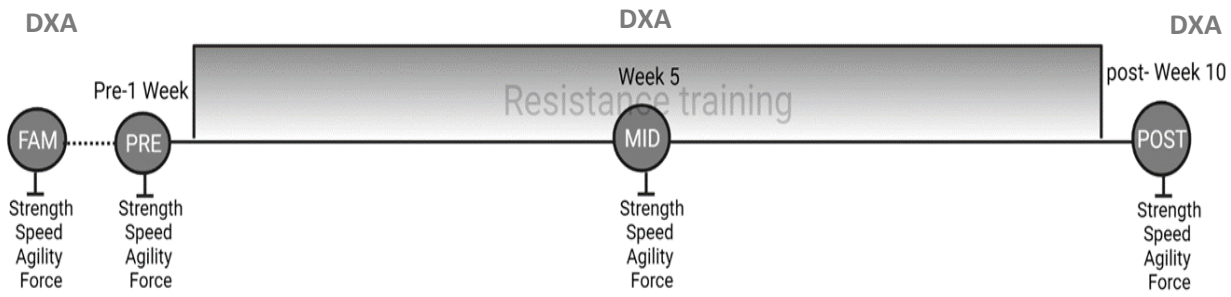


Figure 3.1. Study overview

3.2.2 Pre- experimental familiarization and group allocation

Each participant visited the Exercise Physiology Laboratory, Footscray Park campus a week prior to the first experimental session for a complete familiarisation of the training and the testing process. Their technique in performing the component exercises examined and corrected where necessary by highly trained exercise physiologists.

Participants were also familiarized with the components of the performance tests for strength, power, speed and agility. Basic anthropometrics (height and body weight) were also obtained and the participants were anthropometrically matched and assigned to either the test or the control group.

3.2.3 Body Composition

Three DXA scans were performed pre-, mid- and post intervention to determine upper body, lower body, appendicular and whole body lean muscle mass (LMM). The associated methodology is further expanded upon in chapter four.

3.2.4 Resistance exercise

A ten-week full body, progressive RE program split into two five-week meso-cycles and utilizing a linear periodization of load and volume was prescribed to both groups. Each session was comprised of single and multi-joint bilateral and unilateral free weight (barbell and dumbbell) primary exercises, and dumbbell and body weight supplementary exercises. Given the limited RE experience of the cohort, the repetition range was set at eight to twelve and the exercises were performed at a moderate-fast contraction velocity (1-3s for eccentric/concentric phase) (ACSM, 2009)

The RE program consisted of upper body exercises; (primary exercises) barbell bench press, incline barbell bench press, barbell bent-over row, dumbbell chest press, dumbbell one arm row, bodyweight horizontal pull, (supplementary exercises) dumbbell curl, barbell overhead press, tricep prone overhead extension, dumb-bell rear deltoid fly; and lower body exercises (primary) high-hip deadlift, sumo deadlift, barbell front squat, barbell zercher squat. Additional supplementary trunk exercises were crunches, reverse crunches, and isometric trunk holds (prone and side bridges).

Volume-load $= External\ load\ (Kg)\ x\ number\ of\ repetitions$ (Peterson et al., 2011)

Exercise tonnage $= (Volume - \ load)\ x\ number\ of\ sets$ (Schoenfeld et al., 2016)

Session tonnage = $\sum_{first\ exercie\ of\ session}^{final\ exercise\ of\ session} exercises\ (Exercise\ tonnage)$

Total tonnage = $\sum_{session\ 1}^{session\ 30} Session\ \ (Session\ tonnage)$

Tonnage was recorded as a cumulative marker for each session and was calculated excluding supplementary isometric trunk holds.

Training volume was constant across each session in a given week and was maintained within a similar range throughout the week. Training volume increased across the first four weeks of each meso-cycle. Week five and ten were scheduled de-load weeks to allow adequate recovery prior to the performance testing sessions (Figure 3.2). The training volumes were greater in the second meso-cycle with week nine being the highest. The primary exercises were arranged within each

session in the order of lower body pull/push followed by upper body pull/push. On weeks three, four, seven, eight and nine, all exercises following the primary lower pull/push were combined into “super-sets” engaging agonist and antagonist muscle groups in immediate sequence with no rest in between.

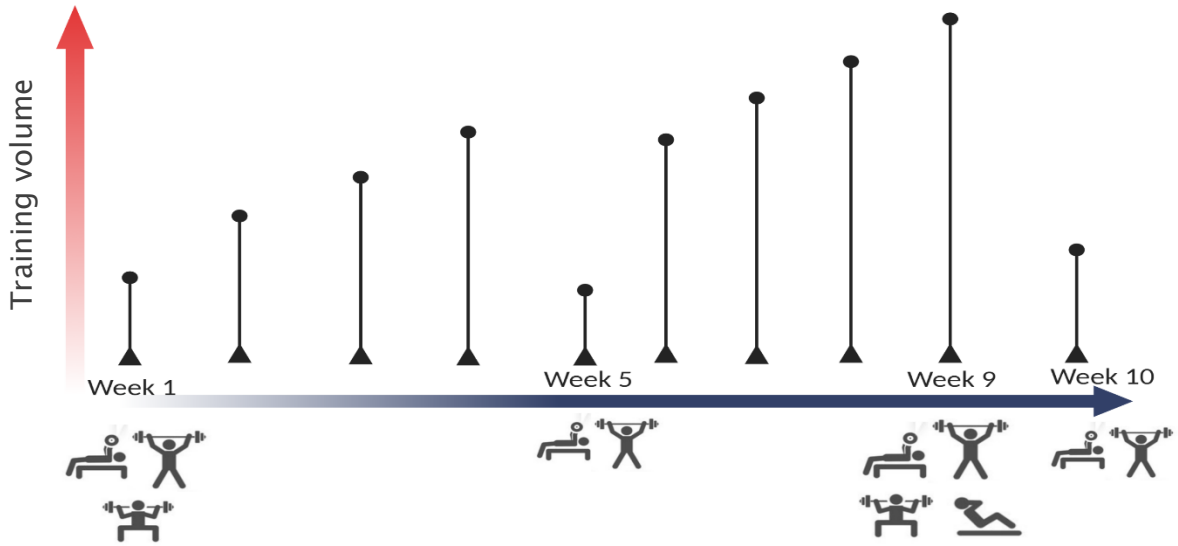


Figure 3.2. Ten week resistance program with increasing total volume and load intensity. Highest load in week nine. De-loading in week five and ten.

Session structure

Prior to the commencement of each session the body weight of the participant was recorded followed by a 30 minute seated rest with a standardised hydration protocol of 250 ml of water (*ad libidum* intake was allowed during the session). Each session commenced with a primary lower body pull variant (high hip or sumo deadlift). In the first week, the initial load was set low (less than 40% of the body weight) and arbitrarily (with a target RPE). At the completion of the set (12 reps) the reported RPE (explained below) was recorded and the load for the next set was adjusted accordingly to achieve the target RPE. Maximal exertion (RPE 20) was targeted for each movement at the completion of the prescribed number of sets for every session. For each movement, the time under tension/ tempo was standardised via a metronome, with adherence regulated by close supervision from a trained research team member. To control for intensity of effort, a target rating of perceived exertion (RPE) was established for each set throughout the training regimen, and repeated load adjustments guided by post-set RPE measurement was used to ensure participants worked within the prescribed effort ranges for each set (Borg, 1982). When a participant was unable to maintain tempo due to exhaustion or otherwise physical discomfort, the set was truncated and recorded as maximal exertion. The rest time between each set and between exercises were precisely timed and closely monitored. Verbal encouragement was

Session 1								(in seconds)						(in kilograms)	
Category	Notes	Exercise	Reps	Load	Tempo	RPE target	RPE reported	Rest	Time per rep	Duration per set	Elapsed time	Elapsed time (min)	Movement	Tonnage per movement	
Primary		High hip deadlift	10	35	2:2	13-15	12	90	4	40	130	2.2	Lower Pull	350	
			10	38	2:2	15-16	13	90	4	40	260	4.3	Lower Pull	380	
			10	41	2:2	16-18	15	90	4	40	390	6.5	Lower Pull	410	
			10	47	2:2	18-20	16	90	4	40	520	8.7	Lower Pull	470	1610
Primary		Barbell front squat	10	33	2:2	13-15	14	90	4	40	650	10.8	Lower Push	330	
			10	36	2:2	15-16	16	90	4	40	780	13.0	Lower Push	360	
			10	39	2:2	16-18	18	90	4	40	910	15.2	Lower Push	390	
			10	41	2:2	18-20	19	90	4	40	1040	17.3	Lower Push	410	1490
Primary		Barbell benchpress	10	24	2:2	13-15	15	90	4	40	1170	19.5	Upper Push	240	
			10	28	2:2	15-16	16	90	4	40	1300	21.7	Upper Push	280	
			10	30	2:2	16-18	17	90	4	40	1430	23.8	Upper Push	300	
			10	31	2:2	18-20	19	90	4	40	1560	26.0	Upper Push	310	1130
Primary		Barbell bent-over row	10	24	2:2	13-15	15	90	4	40	1690	28.2	Upper Pull	240	
			10	26	2:2	15-16	16	90	4	40	1820	30.3	Upper Pull	260	
			10	27	2:2	16-18	17	90	4	40	1950	32.5	Upper Pull	270	
			10	28	2:2	18-20	19	90	4	40	2080	34.7	Upper Pull	280	1050
Supplementary		DB curl	10	20	2:1	13-15	17	60	3	30	2170	36.2	Supplementary	200	
		(use BB curl for > 10kg per arm)	10	18	2:1	15-16	18	60	3	30	2260	37.7	Supplementary	180	
			10	18	2:1	16-18	19	60	3	30	2350	39.2	Supplementary	180	560
Supplementary		Tricep prone overhead extension	10	10	2:1	13-15	14	60	3	30	2440	40.7	Supplementary	100	
		(use Ezcurl bar for heavy loads)	10	12.5	2:1	15-16	17	60	3	30	2530	42.2	Supplementary	125	
			10	15	2:1	16-18	18	60	3	30	2620	43.7	Supplementary	150	375
		Totals	220					1800		820				6215	
								(Total rest)		(Time under tension)				(Total tonnage)	
Session 1 RPE (1-10)		**9**								Work:rest for primary exercises =	0.44				
										Work:rest for secondary exercises =	0.50				
										Work:rest (mean) =	0.46				

Figure 3.3. Sample session structure.

offered intermittently and particularly towards the end of the session to aid and motivate participants to complete the set or session, while maintaining appropriate exercise technique.

In this investigation, Borg's 15 point (6-20) RPE scale was employed (Figure 3.4a) to monitor and prescribe exercise intensity. The scale integrates psychophysical signals from musculoskeletal, cardiovascular and the respiratory as well the central and peripheral nervous system to generate a scaled, normalised numeric output of the perceived intensity of the activity (Borg, 1982). Given the varied nature of the exercises, the physical capabilities of the participants as well as the two different training conditions, a more expansive, graded scale such as Borg's provides more reliable outputs over more condensed ratio scales (Pageaux, 2016). Furthermore, the verbal anchors on the scale provide a positional guide to the participants on real time intensity of the exercise performed (Borg, 1982). Lagally and Costigan reported correlation coefficients of 0.7-0.8 and percent agreements of 60%-90% between RPE and RE intensity, deeming the reliability acceptable (Lagally and Costigan, 2004). Each participant was provided with an extensive explanation on the reporting of the exertion values. The intra-subject and inter-subject variability were taken into account and adjusted for on a session to session and a subject to subject basis guided by the targeted RPEs for each set in the previous sessions.

Session RPE

At the end of each session, a session RPE was also extracted. A 1-10 scale with ratio properties was adopted (Figure 3.4b) to allow for reporting of the cumulative intensity of the session and was deemed more suited over the 15 point scale, given the fact that a 1-10 scale incorporates both ratio and scale properties (Borg, 1982). Bove and colleagues established the test- retest reliability of the 10 point scale as excellent (threshold coefficient = 0.75) with a correlation coefficient of 0.895 (95% confidence 0.866-0.918) during RE (Bove et al., 2016).

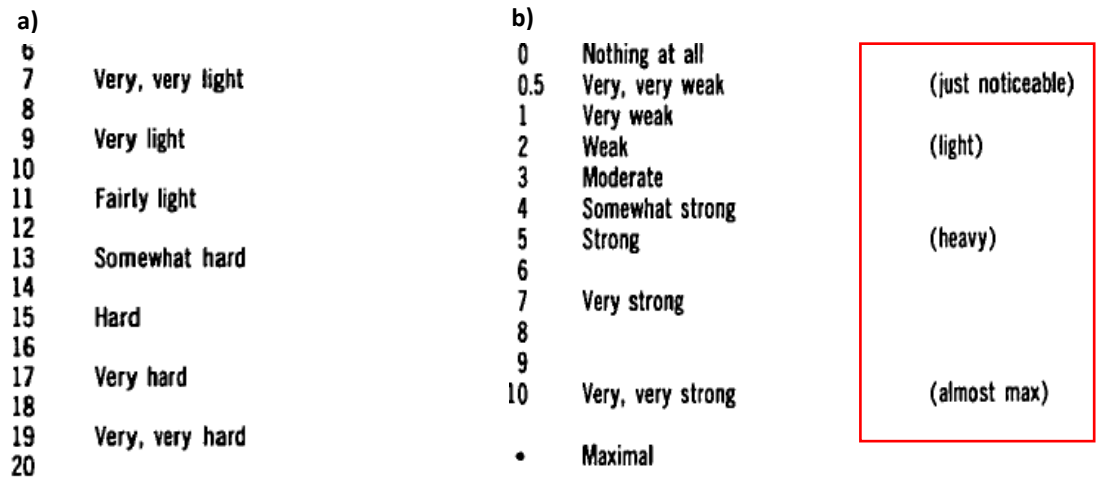


Figure 3.4. a) Borg's 15- grade scale for ratings of perceived exertion during a session. b) Borg's 10 – grade ratio scale. The four categorisations (noted in red outline) were used as anchors for total session intensity.

Resistance Volume and Volume-Load

The total session, weekly and whole-program loads (tonnage) were calculated along with total upper body and lower body tonnage and total leg press and bench press tonnage

3.2.5 Performance testing

Each participant underwent four identical performance testing sessions assessing strength, force, speed, power and agility. An initial familiarization of the testing was followed by the pre-intervention baseline testing one week later (one to two days prior to training commencement), mid-intervention testing at week five, and post-intervention test at week eleven.

Maximal strength

Absolute upper body and lower body strength were measured via one repetition maximum (1RM) bench press and leg press, respectively. Relative strength was determined by calculating upper and lower body strength per one kilo of LMM.

The 1RM testing is the established gold standard method of quantifying absolute muscular strength, defined as the load at which only one controlled repetition of a chosen movement is possible without suffering technical shortcomings specific to the exercise performed (Levinger et al., 2009, Reynolds et al., 2006).

In determining the bench press 1RM at the familiarization session, each participant warmed up with ten repetitions of either a 20 kg barbell or at 40% (the greater of the two) of the predicted 1RM (70% of the body mass) followed by five repetitions at 60% of the predicted 1RM with a 60 second seated rest in between. Additionally, the participants were inquired about their previous bench press maximum in order to aid with the 1 RM prediction and load increments.

After a 60 second rest, one repetition (i.e. the first working set) was performed at 85% of the predicted 1RM. The second and third working sets were a single repetition at 95% and 100% of the predicted 1RM (predicted RPE at set 3 was 19-20); the rest period was 180 seconds between each working set. If a participant was not at maximal exertion (RPE 19-20), the load was increased by increments of 2.5-5kg until they reported maximal exertion (Figure 3.5). Pre-, mid- and post intervention 1RM test procedures were identical

Leg press 1RM was predicted as 350% of the body mass based on previous experience in our lab with similar participants (Fyfe et al., 2019). The exercise was performed on a 45° inclined leg press with a 53 kg non-loaded sled (Hammer strength ®, Schiller Park, IL, USA). Each participant was re-familiarised with the machine on the days of testing and technique and form were adjusted where necessary. Warm up was identical to the bench press test in repetitions and rests. The first warm up set was prescribed as ten repetitions of the non-loaded sled. Similar to the bench press, maximal exertion was predicted at test repetition three and the load was increased by increments of 10-20 kg progressively.

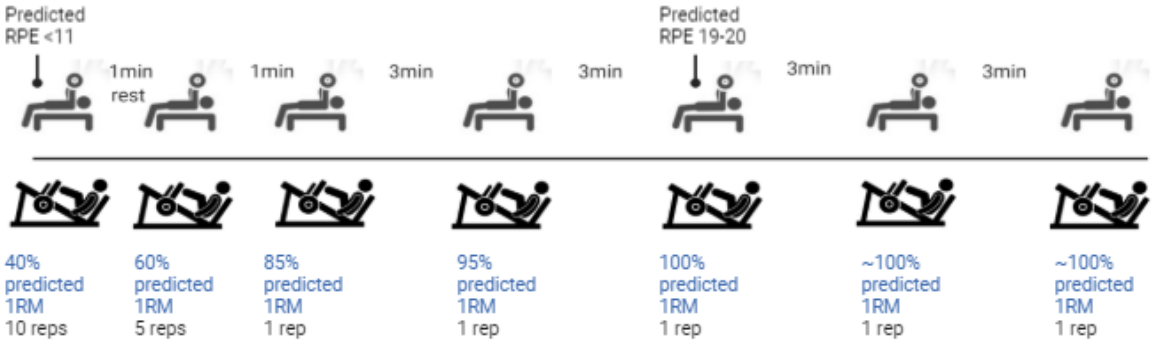


Figure 3.5. 1 RM test for leg press and bench press.

Force

Upper body and lower body force were measured respectively by repeated measurements of the vertical force exerted when performing ballistic push ups and squat jumps.

Ballistic push up

Each participant warmed up with five minutes on a cycle ergometer (Sporte Excalibur, Lode ®, Groningen, Netherlands) at $1W \cdot kg^{-1}$. They were re-familiarised with the movement with three practice/warm up push-ups and were instructed to assume a prone push-up position with their hands on a force plate (Fitness Technologies ®, Skye, Australia) following a three minute rest. They were then instructed to lower themselves into the starting position with their palms firm and shoulder width apart in line with the elbows (similar to a bench press) until their elbows were at 90° flexion while maintaining rigidity of their trunk and legs. At this point, they were

advised to pause to obtain a stable baseline force. Participants were then instructed to launch as explosively as possible, achieving full extension of the arms and as much height off the platform and land with elbows slightly bent and close to the launching position. Each test bout consisted of three ballistic push-ups with a 90 second rest between; the highest peak force of these three tests was taken as the vertical concentric force. Failed attempts which were defined as technique failures, false peaks before launch or failure to clear or land on the platform, were discarded.

Squat jump

The squat jump was performed in the same session after the ballistic push-ups following a 15 minute rest. Following three practise jumps, participants were instructed to position themselves at the centre of the platform (marked with a cross) with feet flat and shoulder width apart and hands placed at the waist with elbows extending perpendicular to the trunk. Following that, they were advised to lower themselves into a squatting position with hips hinging to maintain a straight spine and quadriceps parallel to the platform. The participants were then required to pause to achieve stability and launch off as explosively as possible achieving complete extension of the legs with toes pointing down at peak height and land with knees slightly bent. The peak vertical force of three jumps was recorded as final output. Instances in which participants did not achieve complete extension of the legs or dipped below parallel plane before the jump were deemed unsuccessful.

Speed

Speed was measured via 5 and 10 m sprint time from a standing start. Five and ten meter sprint were performed on the same surface for all participants. Each participant was asked to ensure that they wore the same footwear for all three tests in order to minimise any confounding effect. Laser timing gates (SMARTSPEED PRO®, SMARTSPEED, New Zealand) were placed at zero, five and ten metres (Figure 3.6). The timing gates were wirelessly paired with a control console and triggered the timer automatically as the participants crossed the first gate and stopped as they crossed the last gate. Participants performed two warm up sprints from a standing start three minutes apart followed by five minute rest, then performed three test sprints three minutes apart. The lowest of these three tests was taken as the fastest time for both 5 m and 10 m times.

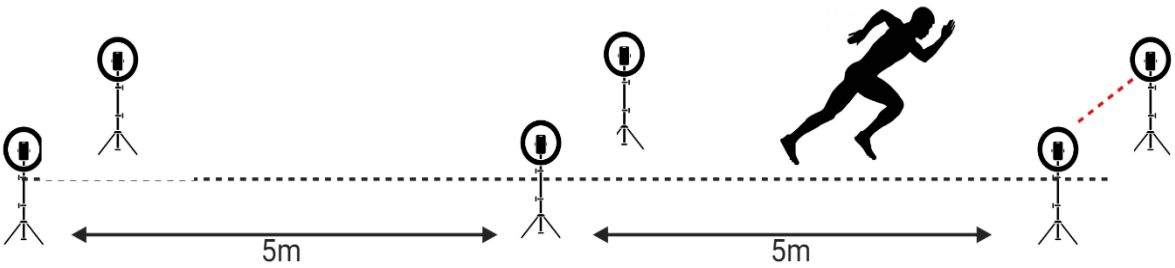


Figure 3.6. Sprint speed test

Agility

Agility was measured via a T-drill test (40m) timed from a standing start. T- drill test combines the movements of sprint, shuffle and back pedalling as well as change of direction and was conducted as previously described (Stewart et al., 2014).

The T-drill test was performed after the sprint test with a 3 minute rest in between the tests. A 10 m distance was marked with cones perpendicular to the previously marked 10 m (sprints) (Figure 3.7). Participants were instructed to sprint forward 10 m, acutely change direction and shuffle left for 5 m, shuffle right for 10 m, shuffle left for 5 m and then backpedal to the start line as fast as possible. Participants were instructed to maintain the flow of movement and transition as smoothly as possible from sprint to the shuffle to the back pedal in order to maintain maximum speed. A warm up drill was performed followed by three test drills with three minutes rest between trials. Laser timing gates were placed at the starting line and the total time was recorded. Verbal encouragement was offered during each of the tests. The best time was recorded as final output**.**

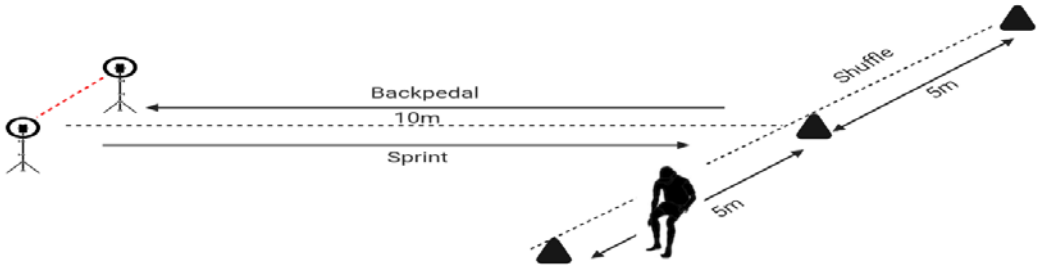


Figure 3.7. T-drill for agility

3.2.6 Core and muscle temperature

Each participant's core and muscle temperature were measured continuously for the duration of the session in the second session of week four of the intervention. Core temperature was measured via a telemetric capsule (CorTemp ®, Respironics Tnc,PA,USA) that each participant ingested the night prior. An intramuscular temperature probe (T204E, Physitemp Instruments Inc., USA) was inserted in to the *vastus lateralis* to a depth of 3.5 cm in a randomly selected leg

using an 18 mm gauge cannula. The cannula was withdrawn slightly while maintaining the positon of the probe, to negate any insulation effects that may be caused by the cannula to the probe sensor. The lead was heavily anchored near the insertion site and was further anchored at two different points on the quadriceps and the waist to ensure stability. Additionally, a waterproof sealing membrane (Tegaderm^{TM} Film, 3M, MN,USA) was placed over the insertion site (Figure 3.8). Data was collected at a rate of 0.2 Hz via a data logger and monitored in real time.

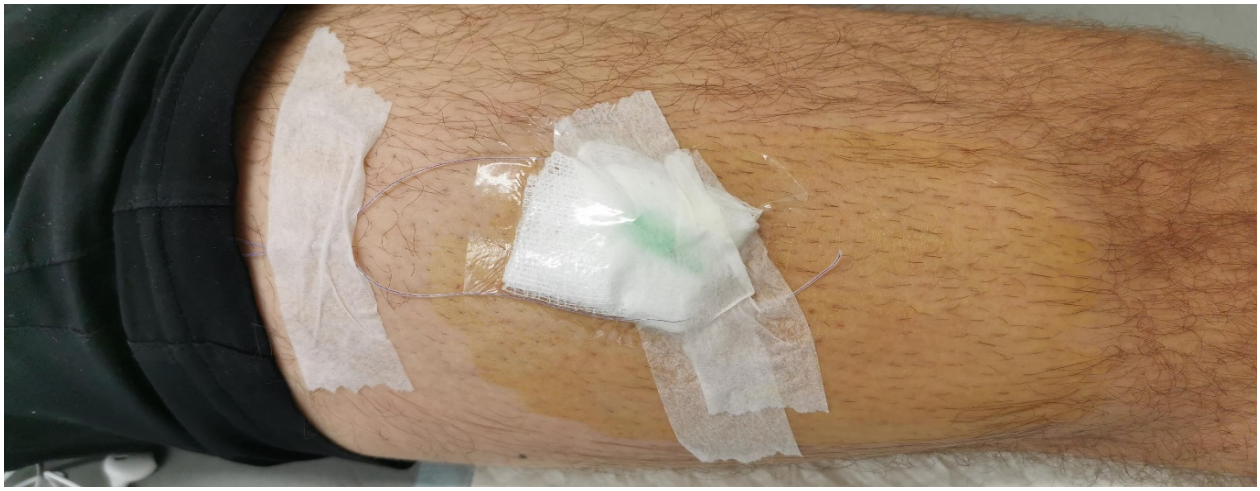


Figure 3.8. Insertion and securing of the temperature probe ensuring minimal movement of it during exercise movements

3.2.7 Statistical analysis

The analyses were conducted using Graphpad Prism (version 9.2.0, San Diego, CA). The effects of the intervention responses were analysed via mixed effects analysis (mixed models) “time” (repeated measures across all time points), “group” and “group x time” were identified as fixed factors and “subject” identified as random factor. Greenhouse-Geisser corrections were applied in case of non-sphericity of the data (Mauchly’s test).Bonferroni’s post hoc analysis was performed where significant main effects were evident. One way repeated measures ANOVA analyses were performed where significant time effects were present with Bonferroni’s post hoc analysis. Unpaired t-tests (Welch’s t-test) were performed to compare the total tonnage between the groups. Data are reported as mean ± SEM. Pearson’s correlation coefficient matrix analysis was performed to generate multiple correlations between performance variables (Supplementary material). Statistical significance was set at $p < 0.05$.

3.3 Results

In reporting results, all significant time and group effects are reported. Where time effects are present, the difference between the times are reported for CON and HEAT groups separately.

3.3.1 Training compliance

Training compliance was not different between the groups with CON 91.1 % ± 5.7% and HEAT 91.7% ± 4.8% (p = 0.321).

3.3.2 Strength

Absolute strength

No significant group or time x group interactions were observed for 1RM bench press or leg press strength. No time effect was seen for bench press 1RM. Leg press 1RM increased from Pre to Post in CON by 50.25 ± 13.99 kg (p = 0.0116) and HEAT by 32.75 ± 5.89 kg (p = 0.0017) (Figure 3.9).

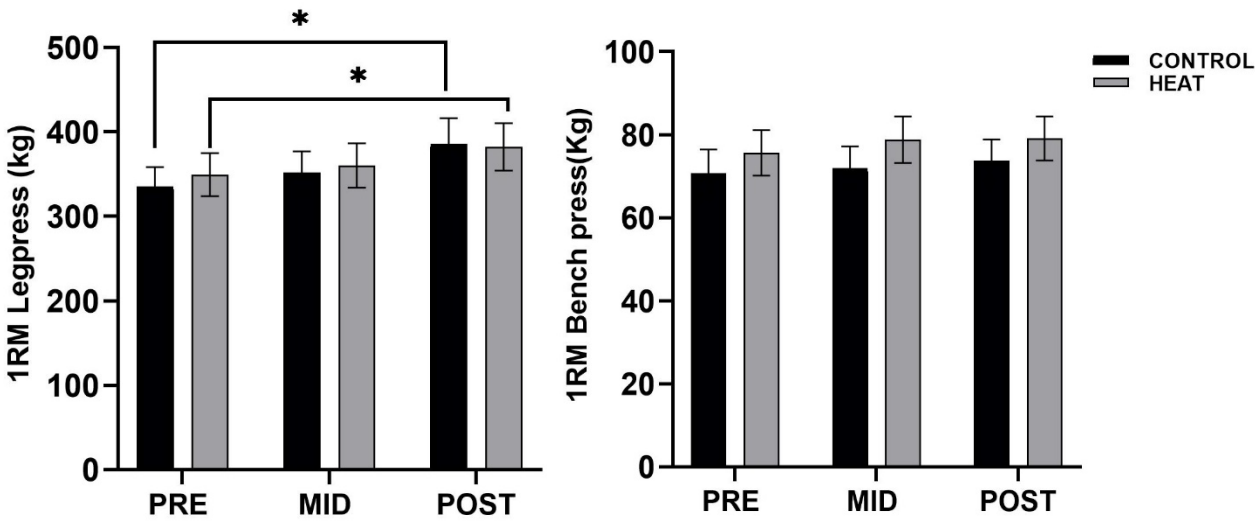


Figure 3.9. Absolute lower body and upper body strength. * p<0.05

Relative Strength

No significant group or time x group interactions were observed for 1RM bench press or leg press relative strength (1RM divided by upper or lower body LMM, respectively). No time effect was seen in relative leg press strength. Relative bench press strength improved Pre to Post in CON by 0.12 ± 0.009 kg.kg MM^{-1} (p = 0.0088) and in HEAT by 0.06 ± 0.008 kg.kg MM^{-1} (p = 0.0009) (Figure 3.10).

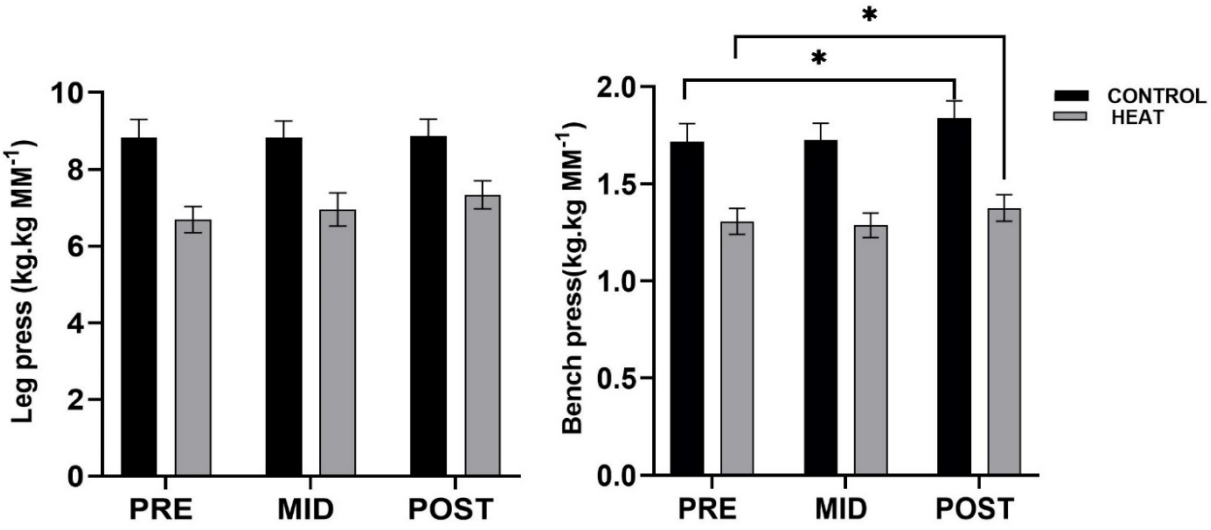


*Figure 3.10. Relative lower and upper body strength * p < 0.05*

3.3.3 Speed

No significant time, group or time x group interactions were observed for 5m or 10m sprint speed (Figure 3.11).

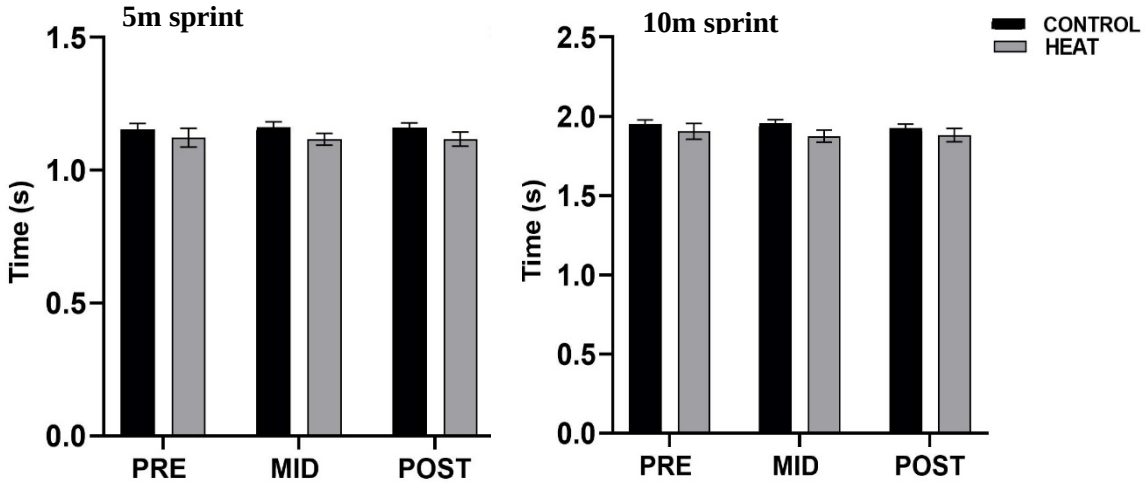


Figure 3.11. 5m and 10m sprint speed

3.3.4 Agility

No significant group or time x group interactions were observed for agility. No time effect was seen in the CON group. From Pre to Mid, agility improved in the HEAT group by 0.3 ± 0.16 s ($p = 0.0274$) but at Post was not different from Pre (Figure 3.12).

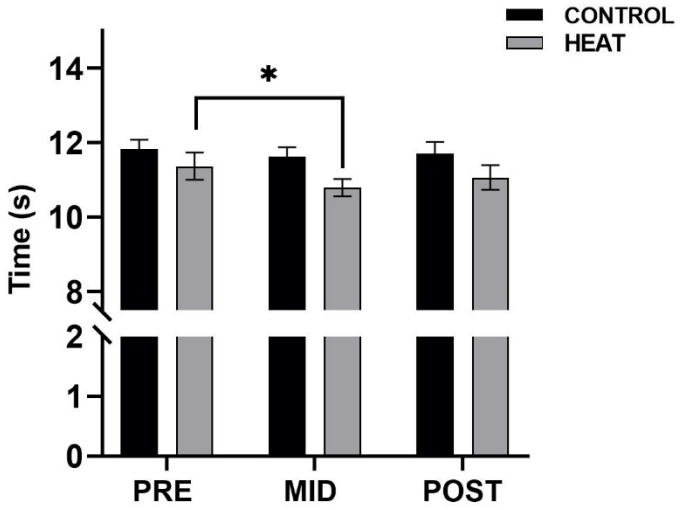


Figure 3.12. Agility *p<0.05

3.3.5 Force

No significant time, group or time x group interactions were observed for ballistic push-up (upper body) or squat jump (lower body) force (Figure 3.13).

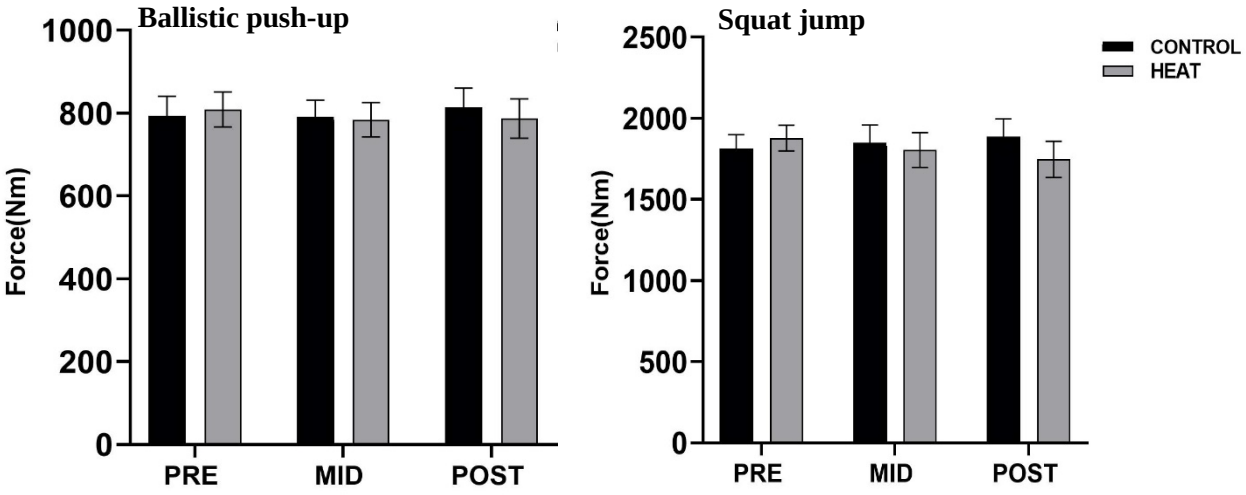


Figure 3.13. Upper body and lower body force

3.3.6 Session RPE

No significant time, group or time x group interactions were observed for average session RPE at any of the weekly sessions (Figure 3.14).

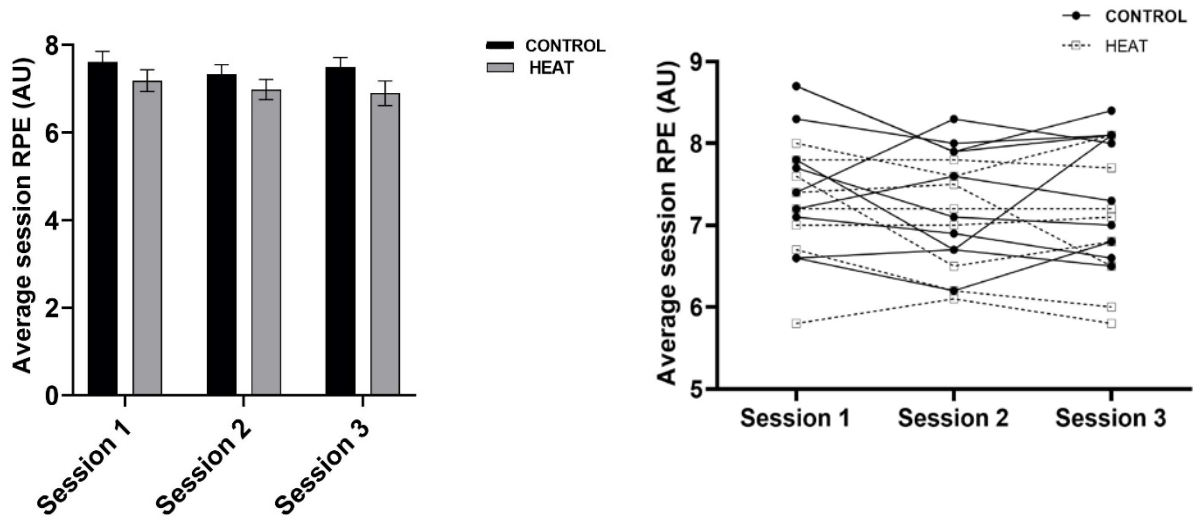


Figure 3.14. a) Intergroup comparison of the average session RPE feedback over the 10 week training period. Session 1(Monday), Session 2 (Wednesday) and Session 3 (Friday). Represented as mean ± SEM b) Average session RPE for each participant over 10 weeks.

3.3.7 Total Tonnage

Total tonnage, upper body or lower body tonnage was not different between the groups post intervention (Figure 3.15).

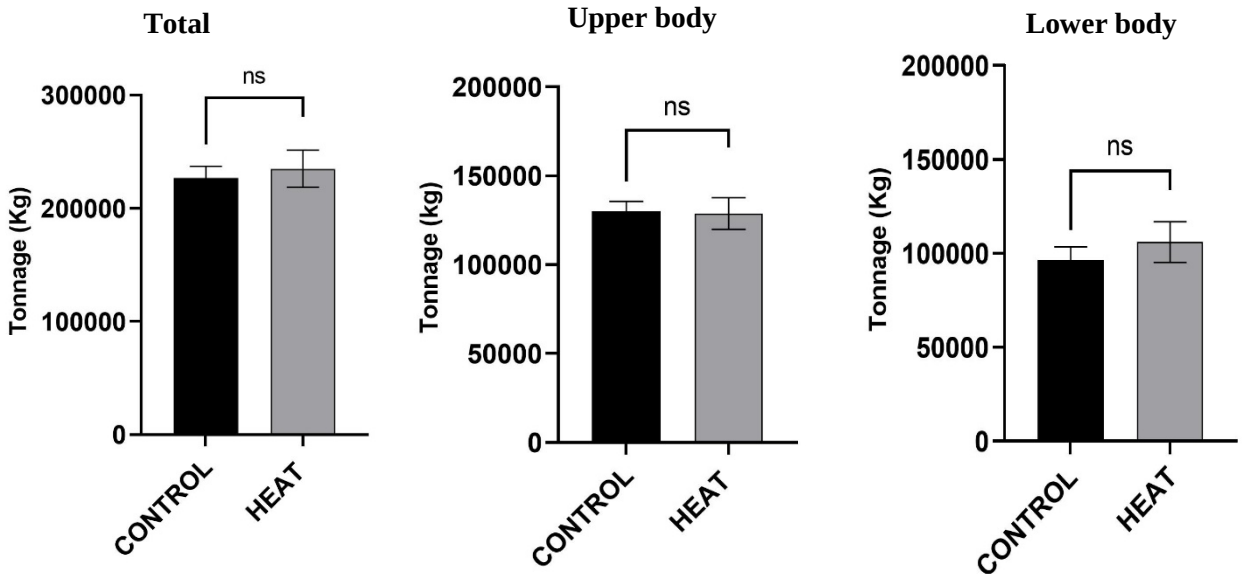


Figure 3.15. Total, upper body and lower body tonnage

3.3.8 Core and muscle Temperature

Peak CMT (36.79 ± 0.58°C) and core body (38.18 ± 0.13°C) temperatures trended higher in HEAT compared to CON (muscle = 35.95 ± 0.53°C; and core = 37.97 ± 0.16°C). However, these differences were not significant. CMT (p = 0.303) Core body (p = 0.363) (Figure 3.16).

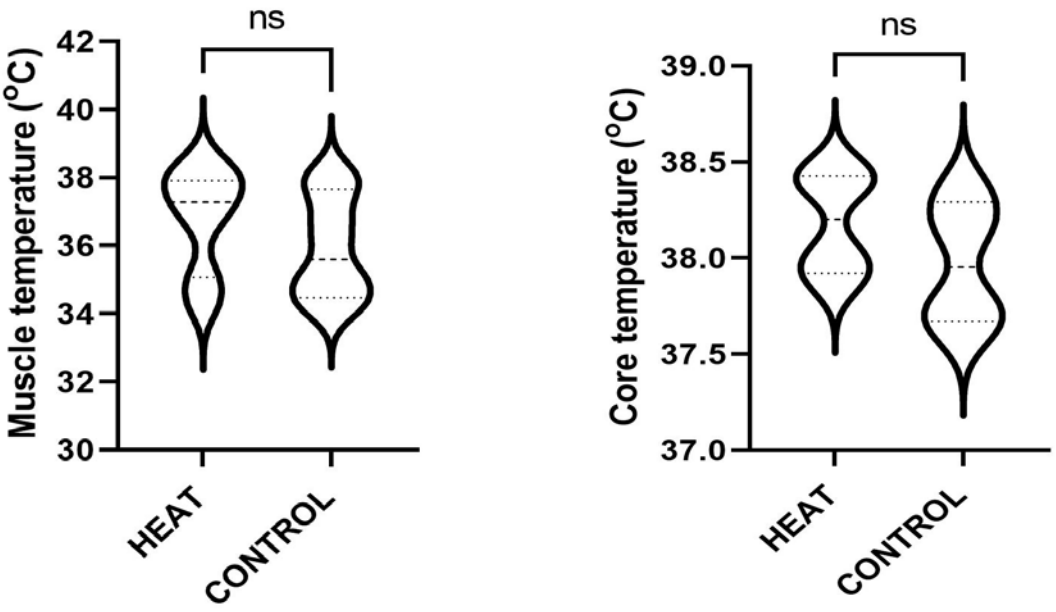


Figure 3.16. Violin plots of muscle and core temperatures for HEAT and CON groups (mean ±SEM p<0.05). The lateral width represents the frequency of individual values at a given temperature. (‑ ‑) = mean, (…)= SD.

3.4 Discussion

The aim of this study was to invetigate the effects of long term, concurrent full body HS on performance adaptations to RE. While absolute lower body and realtive upper body strength imporoved in both groups in response to RE, we observed no impact of HS on strength, speed, agility or force over a period of ten weeks except for transient improvement in agilty at five weeks.

Within the limited body of work available for combined HS and RE interventions examing performance enhancements in the skeletal muscle, a number of studies have used localised heating before a bout of RE in order to measure performance outcomes such as muscle contractility, peak torque, maximal dymanic strength, maximal peak power, and force and have reporetd improvements (Asmussen et al., 1976, Bergh and Ekblom, 1979, Binkhorst et al., 1977, Sargeant, 1987). Another subset have reported improvements in isometric and peak torque after chronic (> 4 weeks), localised HS (Goto et al., 2011, Kim et al., 2020). Supported by reports of improvements in isometric torque via improved contractilty after multiday passive heat exposure (Racinais et al., 2017). Moreover, improvements in upper body and lower body power following acute, full body HS has been observed in higly trained athletic cohorts (Casadio et al., 2017). However, these findings have been contradicted by other studies reporting no improvements in isometric, eccentric or concentric torque, contractilty or strength after smilar interventions (Labidi et al., 2020, Stadnyk et al., 2017).

3.4.1 Performance adaptations

Absolute lower body strength and relative upper body strength improved significantly from pre to post intervention in both CON and HEAT groups. However, there was no difference between groups indicating that heat does not provide an additive effect upon performance gains by RE when the muscle temperatures are not different. While no other study published to date has investigated the effects of concurrent full body HS on strength adaptations to long term, progressive RE, the findings are in alignment with reports by Labidi as well as Stadnyk and colleagues who reported no improvements in strength after chronic concurrent localised HS (Stadnyk et al., 2017, Labidi et al., 2020). In this investigation, the CMTs in the HEAT group trended higher than the CON group. However, the core or CMT were not significantly different between the groups (Figure 3.16). Therefore, the lack of effect by HS is justified. Peak CMTs achieved by Labidi (37.6 ± 1.0°C) (Labidi et al., 2020) and Stadnyk (~38°C) (Stadnyk et al., 2017) were higher than what we observed (36.79 ± 0.58°C) with application of heavy RE in tandem with full body HS at 40°C (30% RH). Goto and colleagues saw the torque of flexion improve in the *biceps brachii* after ten week of localised HS followed by low intensity RE,

compared to the non-heated contralateral arm at a muscle temperature of ~38°C. However, heat was applied to the non-dominant arm for all participants. Therefore, raises the question whether the performance improvements were solely due to HS, as non-dominant limb could have responded to the low intensity RE intervention at larger magnitude. Overall, our results support the notion that regardless of the magnitude of the load-stimulus, absolute strength is not impacted by HS, when the muscle temperature is below 38.5-39°C.

Relative strength only improved significantly in the upper body in both groups. However, no effect of HS was observed. As with absolute strength, the lack of difference in muscle temperature justifies the lack of difference between the groups. This is the first investigation to investigate chronic relative strength (occasionally reported as muscle quality) response to RE in combination with concurrent full body HS. In the only other chronic study we are aware of, Stadnyk and colleagues reported no difference between muscle quality (peak torque per kg of thigh muscle mass) after 12 weeks of localised HS to the *vastus lateralis* concurrently with RE. As will be discussed in Chapter Five of this thesis, overall, upper body and lower body LMM improvements were similar between the two groups. This further explains the lack of difference in relative strength between the groups.

The intervention did not improve upper or lower body peak force. There was no observable additive effect of HS compared to the CON group. As outlined above the evidence for and against HS improving force is limited and contradictory. The findings of this study aligns with the reportings by Labidi and others (Labidi et al., 2020) while contradicting the findings of Racinais and colleagues (Racinais et al., 2017). However, it is key to note that the methods of heat application in the aforementioned investigations were localised and full body respectively and the latter applied full body HS at 45°C rasing while the the localsied heat application of Labidi only saw a muscle temperature increase to a peak of ~38°C. Our peak CMT was ~37°C, which possibly explians the lack of improvements in response to HS our test cohort as it improvements in force appear to be occuring at higher muslce temperatures. Furthermore, measurement of force was conducted via measuring contractility in an isolated movent (knee flexion) whereas in our investigation, it was evaluted via ballistic push ups for the upper body and squat jumps for the lower body, which are both complex, full body ballistic movenents. Therefore, in a prevoiously untrained cohort such as ours, it is possible that the performance of the movements, while meeting the test criteria, were not performed with the same fluidity of a trained athlete. However, there are no previous studies which have investigated the effect of full body HS on force adaptations/improvements (upper or lower body) to long term RE.

As was expanded on ealier in the introduction, the improvements in performance aspects such as force are predicated upon the type of training (Stone et al., 2002). Increased absolute

strength (leg press) and relative strength (bench press 1RM), as observed in our study would be expected to lead to improvements in absolute force production (Suchomel and Comfort, 2017). There is also evidence to suggest that a progressive, hypertrophy driven RE regimen, similar to the intervention of this study, will improve the force and power production capacity of muscles, mainly via the improvements in muscle (fibre) CSA (Minetti, 2002, Zamparo et al., 2002, Häkkinen and Keskinen, 1989). Additionally, improvements/adaptations in force depend on chronic changes such as the fibre type ratio and motor unit recruitment as well as the technique of the participant (Cormie et al., 2008, Van Cutsem et al., 1998, Narici et al., 1989). However, the lack of improvements in force seen in the current investigation could arugubly be due to the facts that training intervention was not long enough to reach the threshold where the improvements in strength and hypertrophy translates in to signifcant improvements in force or the RE was lacking in specificty to improve force (Kraemer and Ratamess, 2004) . As well as the fact that, to improve explosive (ballistic) performance significanly, a periodisation of four to six days a week is recommended with integrated explosive ploymetric training as opposed to current intervention's three days a week (Garhammer and Gregor, 1992, Adams et al., 1992). While these reasons could be contributors to the lack of improvements seen in force, they do not rationalise it fully, as a combination of improvements in strength and muscle mass were observed. A potential reason could be that the two movemetnts utilised to measure force (ballistic push-up and squat jump) are complex and require high degree of familiarisation to be perfomed correctly, and the single falimlirisation session prior to testing was not adequete, especailly in the case of the ballistic push up. While they were peformed the movements with correct tecqnique to qualify as a valid attempt, the possiblity exits that there was a degree of inter-subject and intra-subject variability in form over the three test milestones, for the results to be statistically non-signifcant post intervention. Sprint speed measured from a standing start at 5m and 10m did not improve from pre to post intervention in either HEAT or CON group. Similar to force, effects of HS in any manner of application on speed adaptations to long term RE has not been reported previously. Imporvements in sprint speed is strongly linked to improvements in force generation, specifically in the lower body (Alexander, 1989). As discussed above, this study did not produce lower body force improvements post intervention. Therefore, the lack of improvments in sprint speed is partially explicable. Contradictorily however, Griffiths as well as Chelly and colleagues reported significant improvements in squat/countermovent jump as well as sprint speed (10 m and 40 m) respectively in recreational cohorts (soccer players) anthrophometrically similar to this invetigation, after a six week (two sessions per week) RE intervention. Griffiths and colleague reported improvements in both test groups, following

both explosive RE and traditional RE (Griffiths et al., 2019, Chelly et al., 2009). Criticially, the intervention was focussed on lower body (predominatly hip flexion) exercises, which could potentially explain the difference in findings (Deane et al., 2005). Interestingly, Cronin and colleagues oberseved a high (r = 0.88) correlation between relative strength to body mass and sprint speed (and jump height relating to force) (Cronin and Hansen, 2005). In this study however, the relative lower body strength did not improve post intervention.This further explains the lack of imporvements in short distance sprint speed. Which further highlights lack of traning specificity, perhaps, in this investigation for speed. Maximal (strength driven) and rapid (neuromuscular) force proudtion are two distinictly different perfromance aspects. Explosive movements such as sprinting from standing start require more targted, explosive movement traning (Izquierdo et al., 1999).

Agility imporved in the HEAT group at five weeks, however, showed no imporovements post intervention from CON group. It higly likely that the improvement seen in the HEAT group was transeient and an outcome of individual varaiblity within the group and not an effect of HS. This is the first investigation where the effects of HS on agility adaptations following long term RE have been evaluated. Smilar to strength, force and speed, it can be posited that the dose of HS applied was not enough in magnitude to elict a significant effect on RE driven adaptations in agility as the CMT were not different between the groups. However, a more likely rationale for the lack improvements is that the RE intervention in this investigaiton was not designed to improve agility. While it could be argued that strength gains thorough performing structured RE at 70-80% 1RM for > ten weeks may lead to improvements in coordinated movement, the lack of explosive, power driven elements in the progamme does impact the agility improvements (Fleck, 2011). Supporting these findings, Barbalho and colleagues reported no improvements in agility (measured via T-test) following a 15 week RE intervention (three sessions a week) in a cohort of recreational soccer players, where, similar to this investigation, did not specifically train agility (Barbalho et al., 2018). Hojka and colleagues, in their extensive review, suggested a weak correlation between strength gains and agiltiy (Hojka et al., 2016).The available body of literature supports this suggestion that traditional, progressive or non-progressive RE does not improve or change agility, especially post 10-20 week interventions (Cronin and Hansen, 2005, Cressey et al., 2007, Barbalho et al., 2018).

There was no significant difference observed between the groups in total tonnage, total upper body or total lower body tonnage, indicating that the RE training stimulus was not different. The absolute strength improvements, as discussed above, were not siginificantly different

between the groups and given the lack of difference in total load (volume) is this observation is expected.

3.4.2 Perceived exertion

An interesting outcome of this investigation is the lack of difference observed in the average exercise specific and sessional RPE. Given the HEAT group trained at 40°C (30% RH) compared to the CON group who trained at a thermoneutral 23°C, an observable difference between the groups (average rating being higher in the HEAT group) would have been anticipated given the sensory displeasure of performing heavy RE in a hot environment. Furthermore, the reported maximal exertion (19-20) for a given exercise, high hip dead lift for instance, was consistently reached at the same set number or total tonnage for the movement for both groups. Therefore, HS does not appear to have an impact on the perceived exertion to RE. It could be argued that since there was no cross-over between groups, participants in each group had no reference to the contrast in conditions, therefore, reported the intensity purely pertaining to the difficulty of the exercises.

Borg's 15 point scale (6-20) employed in this study denotes a heart rate range from 60 to 200 beats·min^{-1} (Borg, 1982). While RPE does vary depending on other contributors such as age, environment and levels of anxiety, heart rate is a consistent central indicator (Borg, 1982). A limitation of this study is the lack of in-session heart rate data to be compared between the two groups. However, as reported by Bove as well as Lagally and colleagues, the re-test reliability of Borg's 15 (and 10) point scale has been verified against RE (Bove et al., 2016, Lagally and Costigan, 2004, Eston and Williams, 1988, Day et al., 2004, Gearhart JR et al., 2002).

It is key to note that, there exists a potential limitation when using RPE to inform load prescription and selection in untrained cohorts. It is a possibility that untrained participants were over-reporting (or under reporting) their perceived exertion influencing the load selection in the subsequent sets and sessions. Helms and colleagues have observed that auto-regulation and RPE, there exists discrepancy in consistency of reports between trained athletes and recreationally active untrained cohorts where the true levels of exertion is not reported. Given the cohort in this study was untrained and previously unfamiliar with the RPE scale, it is possible that the values were over or under reported, leading to inconsistencies in load settings over the course of the intervention affecting potential performance gains.

It could be suggested that maybe percentage based prescription predicated upon pre-interventions 1RMs of the participants would have provided a more effective load control over the period of the intervention. However, given the multiple primary and supplementary elements involved in the RE program, auto-regulation via self-reported perceived exertion was deemed more appropriate.

3.4.3 Conclusion and significance of findings

HS, applied concurrently with long term RE (ten weeks) does not improve upon the strength gains. Furthermore, it does not appear to have an effect on peak force, speed or agility. With muscle temperatures not significantly different between the CON and HEAT groups, the lack of effect of HS on performance gains by RE is justified.

While there is limited evidence to suggest a positive effect of HS on performance enhancement, there also exists a number of reports that found no impact in either acute or chronic settings. It is crucial to note that the studies reporting a positive effect did so with an acute bout of RE with the exception with Goto (Goto et al., 2007), who tested the effect of HS only on the non-dominant limb, in contrast to this investigation's long-term full body approach. Therefore, a longer RE invention may have yielded a clear, significant effect, at least in some performance aspects such as strength and force.

Limitations

The full body heating protocol was not effective in raising core muscle temperatures to the desired levels. Another one of the limitations of this study is the reporting of RPE values. While the participants were familiarised with the ten and 15 point scales prior to intervention, they were previously untrained. Therefore, had no previous point of reference to compare to, which may have had an impact on their reporting.

Future directions

HS has been shown to increase muscle contractility and improve aerobic performance. The impact of HS on the skeletal muscle appears to be predicated upon the muscle temperature, especially during chronic application. Whether there is a threshold muscle temperature above which HS can positively add to performance gains by RE has significant clinical and performance implications.

Chapter 4: Methods for muscle hypertrophy, protein content, fibre type, capillarisation, mitochondrial and satellite cell content analysis

4.1 Overview

This chapter outlines all the molecular methods used in chapters five, six and seven in order to minimize repetition of methods.

Parallel to the performance measurements, the muscle adaptive response to resistance exercise (RE) and RE combined with full body heat stress (HS) at a phenotypic and molecular level, was also measured. Lean muscle mass (LMM) was measured at pre-, mid- and post-intervention via dual x-ray densitometry scanning (DXA). Muscle samples extracted via biopsies, pre and post intervention were analyzed via western blotting and immunohistochemistry for muscle molecular response to RE and HS.

A panel of muscle biopsies were extracted from the *vastus lateralis*, immediately pre (Pre 0), one hour (Pre 1) and 48 hours (Pre 48) following an RE session that was identical to the first RE session of the ten week training intervention 72-96 hours before the commencement of the intervention (Figure 4.1). As outlined in Chapter 3, a progressive ten week RE program was then undertaken. Three further biopsies, Post 0, Pos1 and Post 48, were extracted in identical time course to the first three biopsies, 72-96 hours after the final session of the RE intervention following an exercise session identical to the first session of the intervention (Figure 4.1).

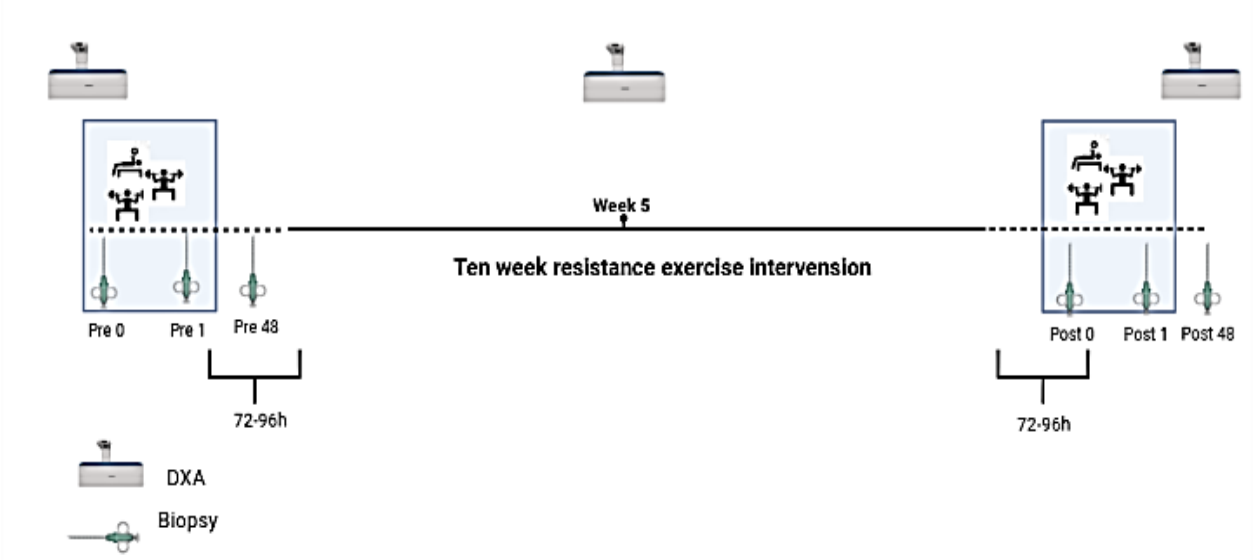


Figure 4.1. The DXA and biopsy trial schematic during the RE intervention

4.2 Procedures

4.2.1 DXA analysis

DXA scans (Lunar iDXA, GE Healthcare, Australia) were performed at one week before pre-, mid (5 weeks) and one week post intervention (Figure 4.1). For each scan, the participants were asked to refrain from any exercise 12 hours leading up to the scan and report fasted (12 hours) on the day of the scan. Their height and body mass (Proscale, Accurate Technologies, USA) were obtained prior to the scan. Each scan was performed by the same practitioner in order to alleviate operator induced variability. LMM as was quantified at each time point for the whole body and for the legs, arms, and the trunk separately.

4.2.2 Muscle biopsy trials

Each participant was provided with a standard meal determined by their body mass to be consumed the night prior (between 7.30-8 pm) to each biopsy trial (Table 4.1). For all pre and post exercise intervention biopsies, participants were instructed to report to the laboratory between 7.30-8.30 am in the morning, fasted. They were asked to abstain from alcohol consumption and exercise in the 24 hours preceding the trial. Biopsies were taken fasted immediately prior to the first RE session and one hour post completion of the session. Upon completion of first biopsy, participants were provided with breakfast and proceeded to undertake the training session. Upon completion of the session the remained in the laboratory in thermoneutral conditions (23°C, 24% RH) until the second biopsy was conducted. The third biopsy was conducted 48 hours post the initial training session. The muscle was extracted from the same site as the previous two biopsies, ~ 1-2 cm distal from the last incision. An identical panel of biopsies were performed ten weeks later, pre and post an RE session identical to the first session, 72-96 hours from the last session of the RE intervention (Figure 4.1). During the ten week intervention, participants were provided with a snack (Table 4.2) after each training session.

Table 4.1. Macronutrient composition and total kilojoules for 1kg of body mass in the standard pre biopsy meal provided to participants

Body mass (kg)	Kilojoules per kg of BM (kJ)			
	Protein	**Carbohydrate**	**Fat**	**Total**
<70	8.033	11.046	5.468	24.727
71-80	9.54-8.535	12.384-11.046	5.648-4.895	27.570-24.476
81-90	9.54-8.525	13.054-11.715	6.025-5.272	28.620-25.52

Table 4.2. Macronutrient composition and total kilojoules for 1kg of body mass in the standard snack provided to participants after training sessions

Body mass (kg)	Kilojoules per kg of BM (kJ)			
	Protein	**Carbohydrate**	**Fat**	**Total**
<70	3.012	12.887	6.025	21.924
71-80	3.012-2.678	14.393-12.72	6.778-6.025	24.184-21.420
81-90	2.678-2.5104	13.556-12.217	7.908-7.155	24.140-21.882

Muscle biopsy procedure

Upon arrival, the lateral aspect (*vastus lateralis*) of the dominant leg was anaesthetised sub-dermally (1% Xylocaine) by a trained medical professional. Once the anesthetics were in effect, two separate, consecutive incisions (~1-2 cm apart) were made. The locale for the extraction was defined as one third of the distance between the top of the knee and the hip. The muscle biopsies were taken from the belly of the *vastus lateralis*, with the use of a 5 mm Bergström needle, modified for suction (Evans et al., 1982). After each biopsy, the incisions were closed using a reinforced skin closure (Steri-Strip, 3M, North Ryde, NSW, Australia) and the area was covered using a waterproof dressing (Tegaderm; 3M, North Ryde, NSW, Australia).

The muscle samples were processed immediately after each biopsy. The tissue were blotted on filter paper to remove excess blood. The majority of the sample was snap frozen in liquid nitrogen for later analysis. A smaller portion (~20 mg) was embedded in O.C.T compound (Tissue-Tek®, Sakura Finetek, Tokyo, Japan) and snap frozen for later immunohistochemical analysis. Both samples were stored at -80 ºC.

4.2.3 Muscle analysis

Preparation of muscle lysates

Muscle samples were withdrawn from long term storage (-80 ºC) to liquid nitrogen. A section sizing from 10-25 mg was then separated from the main sample within a cold chamber (-20 ºC). The separated muscle samples were then suspended in ice cold lysis buffer (20 µl/mg of tissue) (AUWERX buffer; Table 4.3). A protease/phosphatase inhibitor cocktail (product no: 5872S, Cell signaling technology, MA, USA) was added at a ratio of 1:100 µl of lysis buffer. A cool, sterile metal bead (Tugsten carbide beads, Quiagen, Hilden, Germany) was then added to each suspension and was sealed in extensively. The samples were then homogenized mechanically (TissueLyserII, Qiagen, Hilden, Germany) for four minutes at a rate of 30Hz at room temperature. The whole lysate was used for western blotting without centrifugation. Centrifuging

was not performed as it has been shown that discarding of the "debris" after spinning, could lead to losing ~50% of total cellular protein (Murphy and Lamb, 2013). The protein concentration of the lysate was then determined via protein concentration assay. Five µl of each lysate was diluted with 45 µl milliQ® water. Bovine serum albumin (BSA) was used as the standard against which the sample concentrations were measured (5 µl of lysis buffer in 45 µl milliQ as the blank standard). A 96 well plate was then loaded with the standards and samples in triplicates. Each well was standardised (total well volume) to 5µl of sample or standard and 200µl of commercially available colourimetric assay (Bradford assay, Bio-Rad, Hercules, USA). After a ten minute incubation (in dark) at room temperature, the palate was read at 595nm (SpectraMax® i3, Molecular devices, San Jose, California, USA).

Once the concentration was determined, varying amounts of the lysates were further diluted with 75µl 4x Laemmli buffer, 250 mmol/l of Tris·Hcl, pH 6.8, 8% SDS, 40% Glycerol, 0.030% bromophenol blue, 20% β-mecercaptoethanol in order to achieve a standard protein concentration of 2.5µg/µl across all samples.

Table 4.3. AUWERX buffer composition

Component	**Concentration**
Tris·HCl	50mM
NaCl	150mM
EDTA	1M
$Na_2P_2O_7$	5mM
$NaVO_4$	1M

Western blot analysis

Optimisation of blotting conditions

A pooled sample internal standard was prepared by combining 20 µl of lysate from each sample. A series of condition optimisation gels were then run for all targets in order to determine the most effective set of conditions for blot detection for each target. A series of heating conditions (unboiled, boiled 70 ºC and boiled 90 ºC) as well as loading amounts (10 and 20µg) were tested using fractions of the pooled sample to determine the optimal loading volume and the heat status.

Lysates were loaded and resolved in 26-well 4-20% gradient pre-cast, pre stained gels (Bio-Rad. Mississauga, ON, Canada), by means of gel electrophoresis (1h at 80V followed by 1h at 120V) in running buffer (25mM Tris, 190mM Glycine, 0.1% SDS) against a 10kDa-250kDa pre-stained molecular weight ladder (Product no:26619, Page Ruler^{TM} Plus, Thermo Scientific,MA,

USA). Gels were then dry-transferred on to low fluorescence polyvinylidene fluoride membranes (MIDI-size LF-PVDF, Bio-RAD) in transfer buffer (1.68mM Tris, 1.36M Glycine, 1.75% SDS, 20% Ethanol) at 25V for 10mins via a preset transfer system (TransBlot Turbo, Bio-Rad, USA). After transfer, the membrane was extracted on to Tris buffered saline-Tween (TBST; 150mM NaCl. 20mM Tris, 0.1% Tween 20, pH 7.56) and imaged (ChemiDoc ^{TM} MP imaging system, Bio-Rad, Mississauga, ON, Canada) to confirm positive transfer as well as to serve as the total protein control the target proteins are normalised against. As the gels are pre-stained the imaging process can be conducted directly using above referred compatible imaging system. The membranes were then blocked with 3% skim milk in TBST for one hour at room temperature and subsequently washed (3 x 5 minutes) in TBST to remove residual milk from the membrane. The membranes were then cut at specific molecular weight ranges to include the target proteins and incubated in primary antibodies (Table 4.4) diluted at 1:1000 µl in 5% BSA treated for bacterial growth with sodium aside (NaN_3; 0.01%) overnight at 4ºC with gentle rocking. The following morning, the primary antibody solution was removed and the membranes were washed in TBST (3 x 5 minutes). Membranes were then incubated in appropriate species specific, horse radish peroxidase-conjugated secondary antibodies (product no: 7074, anti- rabbit IgG HRP-linked antibody, product no:7076S anti-mouse IgG HRP-linked antibody, Cell Signaling Technology) diluted in 5% skim milk (1:10000) at room temperature with gentle rocking for 90 minutes. They were then washed again in TBST (3 x 5 minutes).

Subsequently, the washed membranes were exposed to appropriate chemiluminescent substrates (Clarity Western ECL substrate, Bio-Rad, Hercules, CA, USA or Super Signal^{TM} West Femto Maximum sensitivity substrate; Thermo Scientific, Rockford, IL, USA) for times varying from ~30 seconds to two minutes in order to identify the optimal visualisation substrate for each target. The membranes were then visualised and imaged using an automated imaging system (ChemiDoc^{TM} MP; Bio-Rad). The antibody specificity, optimal heating status as well as the adequacy of the primary antibody concentration were verified. Targets that did not respond optimally to the provided set of conditions were re-optimised by altering the heating status or primary antibody concentration.

Blotting and quantification of target proteins

It was established via serial optimisation that all targets of interest were best visualised by boiling the lysate at 90 ºC. Furthermore, the optimal lysate load amount was recognised to be 10µg (4µl). All samples from a single participant (six time points pre and post RE intervention) were loaded on to the same gel. Each gel comprised of a combination of participants from CON and HEAT groups to account for intra-gel variability. Four lanes on each gel were allocated for a calibration

curve of internal standards (5µg- 20µg) to account for inter-gel variability and ensure that blot was within the linear range of detection (Murphy and Lamb 2013). All gels were run at the above stipulated specifications and identical blocking and washing protocols were also followed. Primary antibody dilution ratios were within a range of 1:200- 1:4000 (Table 4.4) post optimisation for all targets. Secondary antibody dilution was maintained at 1:10000 for both species (rabbit and mouse). Some targets were visualised using ECL substrate while others were visualised using West Femto Maximum sensitivity substrate per each target's optimisation specifics. Once imaged, the raw blot density was normalised against the internal standard calibration curve and normalized against total protein content from the stain free image, using Image laboratory 6.0 software (Bio-Rad) and each band was quantified accordingly.

Citrate synthase activity

Maximal citrate synthase (CS) activity was measured using a portion of the same, undiluted lysate prepared for western blots. CS activity was investigated at two time points, pre and post intervention ten weeks apart (Pre 0 and Post 0). Each time point was run in triplicate. The lysate amount for each series of triplicates was calculated using the protein concentration analysis for western blots. Each well contained 3mM of acetyl CoA, 1mM of 5'5-dithiobis -2- nitrobenzene (DTNB), 10mM of oxaloacetate (OAA) and 100mM Tris buffer and a 50-fold dilution of the lysate. Reactions were started by the addition of OAA as the final component of the mix. The plate was then analyzed in a spectrophotometer (SpectraMax I3x, Molecular Devices, CA, USA) over a three minute period, with absorbance readings (412 nm) being extracted at every 15 seconds at a set constant temperature of 30ºC.

Table 4.4. Primary antibody information. Cell signalling technology (CST), Enzo life Sciences (ELS), antibody (Ab), Bovine serum albumin (BSA)

Antibody	**Supplier (catalog number)**	**Dilution (μl) (Ab: BSA)**
mTOR	CST (#2972S)	1:1000
Phospho-mTOR (Ser 2448)	CST (#5536S)	1:1000
Akt	CST (#2531)	1:1000
Phospho-Akt (Thr308)	CST (#4058S)	1:1000
AMPK	CST (#8325S)	1:1000
Phospho-AMPK (Thr 172)	CST (#2531)	1:1500
4E-BP1	CST (#9644S)	1:1000
Phospho-4E-BP1 (Thr37/46)	CST (#2855L)	1:1000
rpS6	CST(#2217)	1:1000
Phospho-rpS6 (Ser235/236)	CST (#2211S)	1:1000
P70S6K	CST (#2708S)	1:1000
Phospho-P70S6K (Thr389)	CST (#9234S)	1:1000
TSC2 (Tuberin)	CST (#3653S)	1:1000
αB-crystalline	ELS #ADI-SPA-222)	1:1000
Phospho-αB-crystalline (Ser59)	ELS (#ADI-SPA-227)	1:1000
HSP27	ELS (#ADI-SPA-800)	1:4000
Phospho-HSP27 (Ser15)	ELS (#ADI-SPA-525)	1:1000
HSP60	ELS (#ADI-SPA-806-D)	1:1000
HSP70	ELS (#ADI-SPA-810)	1:4000
HSP90	ELS (#ADI-SPA-830-F)	1:2000
HSF-1	ELS (#ADI-SPA-901-D)	1:1000
Phospho-HSF-1 (Ser303/307)	Abcam (#ab81281)	1:1000
Phospho-HSF-1 (Ser325/326)	Abcam (#ab76076)	1:1000
VEGFA	Abcam (#ab46154)	1:1000
Angiopoetin-1	Abcam (#ab94684)	1:1000
CyclinD1	Santa Cruz Biotechnology (#SC-8396)	1:200
eNOS	BD Biosciences (#612664)	1:500
TRPV1 (VR1)	Alomone Labs (#ACC-030)	1:1000
PGC-1α	Sigma-Aldrich; 4C1.3 (#ST1202)	1:1000

4.2.4 Immunohistochemistry (fibre type, capillarisation and satellite cell content analysis)

Sectioning

Unfixed muscle samples embedded in O.C.T were withdrawn from long term storage (-80ºC) on to dry ice. The samples were then further mounted in O.C.T within the cold chamber (-20ºC) of the cryostat (CM1520, Lecia Biosystems, Nussloch, Germany) on a metal mounting base. They were then sectioned using the cutting machinery of the cryostat (~8µm) and mounted on polarized glass slides (7-8 sections per slide) ensuring the cross sectional alignment of the samples. Consecutive sections were mounted alternatively on two slides for fibre typing and capillarisation analysis. The sections were isolated from each other via hydrophobic boundaries (ID300, Liquid blocker super pap pen, Proscitech). One slide was utilised for fibre type analysis while the other for capillarisation related quantification. The slides were then stored at -80ºC for later analysis. A separate batch of slides were prepared in identical manner for SC content analysis.

Fibre type staining

Slides were withdrawn from long term storage (-80ºC) and left to air dry for 20-30 minutes. Once completely dry, the sections were incubated (blocked) with 10% goat serum (product no: 50197Z, ThermoFisher Scientific) for one hour. The excess goat serum was then removed, and sections were incubated with primary fibre type antibodies (Table 4.5) over night at 4ºC. The following morning, the primary antibodies were washed off in milliQ water (3 x 5 minutes). Once the slides were fully dry, they were incubated with fluoro-conjugated secondary antibodies (Table 4.5) for two hours. Slides were washed further with milliQ water (3 x 5 minutes) post incubation. Subsequently, slides were incubated for ten minutes in fluoro-conjugated wheat germ agglutinin (WGA; product no: W11262, Alexa Fluor™ 594 conjugate, Invitrogen) diluted in phosphate buffered saline (PBS; 2µg/1ml). Slides were washed again (3 x 5 minutes) and dried before adding ~15µl of PBS per section and mounting with cover slips. The slides were then viewed and imaged in high resolution using an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) and Cell F software (Olympus). All images were obtained with the x10 objective and ≥200 fibres were included in the analysis per subject for each time point. CSA, fibre periphery measurement and fibre type distribution was conducted using Image J software (National Institutes of Health, Maryland, USA)

Capillarisation

The slides were treated identically to fibre typing and were incubated with primary antibodies overnight and then incubated with secondary antibodies for two hours (Table 4.5) and treated imaged identically to fibre typing. The quantification of capillarisation via capillary density, capillary contacts, capillary to fibre ratio, capillary fibre perimeter index (CFPE), was conducted according to the methodology by Hepple and colleagues (Hepple et al., 1997) with 50 fibres per participant per time point.

Satellite cell content

Slides were withdrawn from -80ºC and air dried for 20-30 minutes. The sections were then fixed in 4% paraformaldehyde for ten minutes. They were then washed with PBS (3 x 5 minutes) and blocked for one hour with a blocking cocktail (5% goat serum, 2.5% BSA, 0.1% Triton-X, 0.005% NaN_3). The slides were then incubated overnight at 4ºC with Pax7 antibodies (Table 4.5). The following morning, the slides were washed with PBS (3 x 5 minutes) and incubated with secondary antibodies for two hours (Table 4.5). Subsequently washed again with PBS (3 x 5 minutes). The sections were then incubated with 4'6-diamidino-2-phenylindole (DAPI; 1:15000 Sigma-Aldrich, Oakland. ON, Canada) for ten minutes followed by wash with PBS (2 x 5 minutes). The slides were then incubated with fluoro-conjugated WGA (2µg/ml; product no: W11262, Alexa Fluor™ 594 conjugate, Invitrogen, MA, USA), washed further with PBS (2 x 5 minutes) and dried for ~ five minutes and mounted with PBS and imaged as per fibre typing and capillarisation. The activation and identification of SC was confirmed via the co-localization of $Pax7^+/DAPI^+$ and quantified as $Pax7^+/DAPI^+$ per 100 fibres. Counting was conducted using Image J software.

Table 4.5. Antibody information for immunohistochemical analysis (Ab: antibody, GS: Goat serum)

Antibody (primary)	Supplier (catalog number)	Primary ab dilution (Ab: GS)	Antibody (Secondary)	Secondary ab dilution (Ab: GS)
Fibre type				
Anti- MHC I	DSHB (BA-F8)	1:25	Alexa Fluor (AF) 350 -Anti Mouse (IgG2b)	1:500
Anti- MHC IIa	DSHB (BF-35)	1:25	AF 488- Anti mouse (IgG1)	1:500
Anti- MHC IIx	DSHB (6H1)	1:25	AF 555- Anti mouse (IgM)	1:500
Capillarisation				
Anti-CD 31	Abcam (ab28364)	1: 50	AF 488-Anti rabbit (IgM)	1:500
Anti- MHC I	DSHB (BA-F8)	1:100	AF 350- Anti mouse (IgG2b)	1:500
Satellite cell content				
Anti-Pax-7	DSHB (Pax-7)	1:10	AF 488-Anti rabbit (IgM)	1:500

4.3 Statistical analysis

The analyses were conducted using Graphpad Prism (version 9.2.0, San Diego, CA). The effects of the intervention responses were analysed via mixed effects analysis (mixed models) “time” (repeated measures across all time points), “group” and “group x time” were identified as fixed factors and “subject” identified as random factor. Greenhouse-Geisser corrections were applied in case of non-sphericity of the data (Mauchly’s test). One way repeated measures ANOVA analyses were performed where significant time effects were present with Bonferroni’s post hoc analysis. Where appropriate parametric unpaired t-tests were performed. Data are reported as mean ± SEM. Statistical significance was set at $p < 0.05$. All protein content data were normalised against internal standard controls. P values that are 0.05 -0.09 have been defined as tendencies. While defining p <0.05 as the threshold for significance, in a moderate sample size, p values within the defined range could carry physiologically meaningful information while not reaching significance (Caldwell and Cheuvront, 2019).

Chapter 5: Effects of chronic concurrent heat stress on resistance exercise induced anabolic synthesis and muscle hypertrophy

5.1 Introduction

The key morphological muscle adaptation to chronic RE is muscle hypertrophy and other adaptions include hyperplasia, changes in overall muscle architecture and fibre type transitions (Kraemer et al., 2002, Staron et al., 1994). Muscle hypertrophy is defined as an increase in CSA of individual muscle fibres which results in an overall increase in the entire muscle's CSA via an accumulation of proteins overtime (Folland and Williams, 2007, Kraemer et al., 2002). In already well resistance trained cohorts, training induced hypertrophy improvements are markedly limited (Hakkinen, 1994, Ahtiainen et al., 2003). However, it has been reported that untrained individuals undergoing standard RE improved muscle size in a relatively linear manner over a six month period (Narici et al., 1996). Increased muscle mass is important for its positive relationship with increase in lean muscle mass (LMM), which has shown to have consequent improvements in muscle power and force. Moreover, muscle hypertrophy is beneficial in attenuation of atrophy or sarcopenia in ageing and immobilization, as well as improvements of physical aesthetics. HS also shown the ability to improve muscle hypertrophy, albeit at a smaller magnitude compared to RE, upon chronic application (Goto et al., 2011). Therefore, whether the chronic application HS concurrently with heavy progressive RE would improve or expedite upon the hypertrophic response to RE is of great interest.

At a molecular level, hypertrophy occurs due to a net positive balance between protein synthesis and protein degradation (Brook et al., 2015). At the onset of RE, the mechanical stress of work against load compromises the existing muscle homeostasis. This is identified as muscle damage or disturbances in the muscle cell ultra-structure in the form of protein degradation (Beaton et al., 2002, Damas et al., 2016). The damage in turn triggers the consequent increase of muscle protein synthesis (MPS). However, the initial increase in MPS (after a single bout of RE) has not been found to correlate with chronic hypertrophy in some studies but more with the rapid attenuation of muscle damage via the repeated bout effect (Mitchell et al., 2014, Damas et al., 2016). With the progression of the RE intervention, the muscle protein allocation to negotiate muscle damage decreases and is primarily diverted towards improving the adaptive compensatory growth response of the skeletal muscle (Damas et al., 2015, Damas et al., 2018). It has been established that both type I and type II muscle fibres increase in size significantly in

response to chronic RE (Snijders et al., 2016). Furthermore, it has been observed that type II muscle fibres also respond acutely to RE via an increase in CSA (≥ 2-4 weeks). Conversely, type I fibres have not been observed to respond as rapidly, however, over an extended period may contribute to an overall increase in muscle CSA (Snijders et al., 2016).

The molecular response that drives hypertrophy predominantly functions via the mTOR pathway and has been identified to be sufficient and necessary for RE induced anabolic synthesis (Wang and Proud, 2006, Goodman et al., 2011). The critical nature of mTOR for anabolic synthesis and by extension MPS has been verified via the inhibition of this molecular pathway via rapamycin (highly specific mTOR inhibitor), where MPS response to load induced growth was attenuated significantly (Goodman et al., 2011, Bodine et al., 2001). Goodman and colleagues, in their seminal investigation, demonstrated that mTOR is in fact the rapamycin sensitive element centrally driving load induced muscle growth specifically within skeletal muscle. This specific attenuation has been identified as the loss of ability of mTOR as a kinase to phosphorylate the key and immediate downstream modulator P70S6K (Goodman et al., 2011). Along with tandem key modulator 4E-BP1, P70S6K activation has been found to initiate mRNA translation by mTOR via phosphorylation (Sarbassov et al., 2005). mTOR activation is mainly driven by key upstream regulators Akt, TSC2 and AMPK (Laplante and Sabatini, 2009).

While the effect of RE on anabolic MPS and hypertrophy is clear (Song et al., 2017, Dreyer et al., 2010), the effects of HS on anabolic responses and subsequent hypertrophy is not well investigated in skeletal muscle. Previous investigations have shown that, HS has the potential to induce hypertrophy *in vitro*, facilitates the recovery of atrophied muscle as well as improve proliferative potential (Kobayashi, 2003, Goto et al., 2004, Uehara et al., 2004). Conversely, Frier and Locke reported that HS prior to overload may attenuate overload induced hypertrophy in rats (Frier and Locke, 2007). In humans, the data is limited. Goto and colleagues observed improvements in overall muscle CSA after ten weeks of localised heat treatment (Goto et al., 2011). In addition, HS attenuated muscle atrophy during a ten day immobilization intervention (Goto et al., 2011, Hafen et al., 2019). In contrast, Hesketh and colleagues observed that fibre CSA of type I and II fibres did not improve from pre intervention after six weeks of full body HS (Hesketh et al., 2019). However, Labidi and colleagues saw no improvements in the overall muscle CSA after six weeks of localised HS (Labidi et al., 2020). Collectively, there is evidence to suggest that HS may induce anabolic synthesis thereby improving hypertrophy or attenuating muscle degradation. However, the impact may depend on the overall temperature and the modality of HS.

The molecular response that triggers aforementioned hypertrophy following HS is not well defined in comparison to RE. However, Goto and colleagues reported that rat myoblasts subjected to HS showed improved total protein content over the untreated cells. While they did not test for mTOR (or associated upstream or downstream modulators), the findings do suggest that HS does have the ability to drive protein synthesis (Goto et al., 2004). Uehara and colleagues further reported that levels of phosphorylated P70S6K increased in response to HS 24 hours following heat shock (Uehara et al., 2004) which suggests increased upstream activation of mTOR. However, Yoshihara and colleagues reported that there was no improvement in total protein levels of Akt, mTOR, P70S6K or 4E-BP1 after 30 minutes of HS at temperatures ranging from 37-41ºC (Yoshihara et al., 2013), but reported improved levels of phosphorylation of Akt and P70S6K at 40 and 41 ºC respectively indicating activation. They did not however, report improvements in phosphorylation levels of mTOR or 4E-BP1. This suggests that the activation of some markers in the mTOR pathway appear to occur in a dose-response manner in response to HS in the skeletal muscle (Yoshihara et al., 2013). Ihsan and colleagues observed that a bout of full body HS was able to increase the levels of phosphorylated Akt and mTOR as well as P70S6K, while not improving phospho-4E-BP1 in the same manner. However, the total protein levels of 4E-BP1 showed a significant improvement (Ihsan et al., 2020). Moreover, they did not see similar results with localised HS, indicating that full body HS potentially might be the more effective method of HS.

A very limited number of studies have investigated the effect of HS as a supplementary stressor combined with RE for its impact on anabolic synthesis. *In vitro*, Goto and colleagues observed a significant improvement in total protein content in myoblasts that were subjected to mechanical stretch in conjunction with HS compared to myoblasts that were only subjected to either HS or mechanical stretch alone (Goto et al., 2003). It has further been reported that mTOR signaling improved significantly when HS was combined with RE compared to RE alone, when the *vastus lateralis* was heated during the RE bout (Kakigi et al., 2011). The authors reported significantly greater improvements in the phosphorylation levels of Akt and mTOR in the HS and RE leg when compared to the RE intervention alone. However, phosphorylation of both P70S6K increased and 4E-BP1 decreased significantly in both groups at one hour and there were no between group differences. However, Fuchs and colleagues saw no acute improvements in total protein levels when RE was combined with post exercise HS (Fuchs et al., 2020). Moreover, HS attenuated the overload induced hypertrophy when applied prior to overload, however they did not investigate the mTOR pathway (Frier and Locke, 2007). Stadnyk and colleagues reported that 12 weeks of localised HS did not improve LMM in the quadriceps when applied concurrently

with RE (Stadnyk et al., 2017). With the limited and somewhat contradictory research on the impact of HS as combinatory stressor on hypertrophy, the potential of HS a combinatory stressor on improving mTOR driven anabolic response to RE remains uninvestigated in the skeletal muscle. Previous chronic concurrent application of HS and RE (Stadnyk et al., 2017) no clear benefit on hypertrophy were seen, but it could have been due to muscle temperature, impediment of range of motion due to the heat application method, or the lack of load stimulus. However, full body HS combined with long term full body RE may address these limitations to garner potential muscle adaptive advantages of HS.

Ribosomes are a key component in the protein synthesis pathway. The synthesis of ribosomes is a highly regulated and specific process within cells (Figueiredo et al., 2015). While some rodent studies have established the involvement of ribosome biogenesis in muscle hypertrophy, there is limited information pertaining to how ribosome biogenesis is modulated by active mechanical loading in the human skeletal muscle (von Walden et al., 2012). Key molecular markers that have been investigated to quantify the effect RE on ribosomal biogenesis are ribosomal protein S6 (rpS6 or S6rp), phospho-rpS6 (Ser235/236) as well as cell cycle regulating cyclins (Cyclin D1) (Voit et al., 1999, Wilson and Cate, 2012). After a bout of full body HS, Ihsan and colleagues saw a rapid activation of ribosomal biogenesis markers in the *vastus lateralis* (Ihsan et al., 2020). Kakigi and colleagues reported that acutely, HS combined with RE improved acute phospho-rpS6 (Ser235/236) significantly compared to RE alone (Kakigi et al., 2011). While limited, the evidence suggest that HS may improve ribosomal biogenesis acutely. Moreover, HS appears to have an additive effect on ribosomal biogenesis when combined concurrently with RE.

The activation of satellite cells (SC) (Nederveen et al., 2017, Bellamy et al., 2014) and the myonuclear proliferation and accretion response to RE has been well established (Kadi et al., 2005). SC are categorised as myogenic precursor cells that are yet to be differentiated. From a non-activated (quiescent) state, they have the ability to transform in to either new muscle cells (hyperplasia) or to fuse with existing muscle fibres to increase the overall muscle volume (MacDougall et al., 1984, Zammit et al., 2006). The evidence for contribution of newly generated muscle fibres in improving muscle size in response to long term RE is sparse and not well supported, especially in untrained cohorts (Rehfeldt, 2007). The SC response to RE via their fusion with existing fibres is more established (MacDougall et al., 1984, Kadi et al., 2005). Myonuclei also fuse with existing fibres thereby increasing the size of the skeletal muscle under chronic mechanical stress (Bruusgaard et al., 2010, Petrella et al., 2008). The activation of a SC is marked by the co-localisation of the key SC maker $Pax7^{+}$ with a myonuclei in the skeletal muscle (Nederveen et al., 2017). The evidence of the impact of HS or HS combined with RE is

extremely limited. Studies have reported an increase in the number of $Pax7^{+}$ positive cells after an acute bout of HS in rats promoting muscle regeneration after crush injuries in the skeletal muscle (Kojima et al., 2007, Takeuchi et al., 2014, Oishi et al., 2009). In humans, after a chronic HS intervention, Goto and colleagues saw an increase in the myonuclear density (Goto et al., 2011). Therefore, HS may have the potential to activate SC in the muscle.

Finally, transient receptor potential (TRP) channels (ion channels) will be another focus of this study. This family of channels are responsible for sensing physiological changes in the tissues such as pH and temperature and triggering intracellular pathways such as mTOR, by allowing ions, predominantly calcium (Ca^{2+}) into the cell (Hudson et al., 2016, Hilton et al., 2015). It has been found that eleven TRP channels are highly sensitive to temperature. Studies have demonstrated that cellular temperature sensing is mediated by the activation of TRP channels (Wang and Siemens, 2015). Importantly, these receptors are polymodal, and are therefore activated by other stimuli besides heat, such as mechanical loading (Hudson et al., 2016, Hilton et al., 2015). Transient receptor potential vanilloid type (TRPV1) is one such polymodal receptor that carries significance in the context of this study given it has been shown to modulate the cellular-physiological response to exercise as well as HS (Hudson et al., 2016). Furthermore, TRPV1is found in skeletal muscles (in mice) and has been linked with triggering the acute angiogenic responses to stress (Lotteau et al., 2013). Ito and colleagues showed that the load induced activation of TRPV1 activated mTOR via increased $Ca^{2+}$and promoted hypertrophy (Ito et al., 2013a, Ito et al., 2013b). It has also been shown that inhibition of TRPV1 mouse myoblasts subjected to HS diminished the mTOR pathway activation response (Obi et al., 2019). This evidence indicates TRPV1 could be key in RE as well HS driven anabolic synthesis. However, the acute and chronic effect heavy RE on the TRPV1 channel in the human skeletal muscle has not been investigated before. Moreover, the impact of full body HS on the potential TRPV1 response to RE is yet to be tested.

The evidence of improved hypertrophy driven by RE, especially in untrained cohorts is well established (Hakkinen, 1994). While less in magnitude, there is adequate evidence to suggest that HS has the ability to induce MPS and hypertrophy in humans (Goto et al., 2011, Kakigi et al., 2011). Moreover, HS does appear to have a cumulative positive effect on MPS when applied in tandem with RE (Kakigi et al., 2011). However, the acute and chronic effects of full body HS applied concurrently with full body progressive RE has not been investigated in the human skeletal muscle. We hypothesised that full body HS may improve upon the anabolic synthesis and overall muscle hypertrophy response to progressive RE when applied concurrently. If found

beneficial, the findings will be important to developing new strength and hypertrophy training modalities.

5.2 Methods

5.2.1 Biopsy sequence and western blots

mTOR signaling

Muscle biopsies were obtained at a depth of 3-3.5cm below the epidermis from the *vastus lateralis* pre-intervention at rest (Pre 0), one hour (Pre 1) and 48 hours (Pre 48) post a RE session that was identical to the first training session of the intervention 72-96h prior to the commencement of the intervention. Three further biopsies were extracted post-intervention at rest (Post 0), one hour (Post 1) and 48 hours (Post 48) after an identical RE session to the first session of the intervention, which was performed ~72-96h after the last training session of the intervention.Western blot analysis for of mTOR, phospho-mTOR (Ser2448), Akt, phospho-Akt (Thr308), P70S6K, phospho-P70S6K (Thr389), AMPK, phospho-AMPK (Thr172), 4E-BP1, phospho-4E-BP1(Thr37/46), TSC2 and TRPV1 via western blots at all six time points. Methods were as described in Chapter Four

Ribosomal biogenesis

Muscle samples were analysed for expression levels of rpS6, phospho-rpS6 and Cyclin D1 at all time points pre and post intervention as above. Methods as outlined in Chapter Four.

5.2.2 Immunohistochemical analysis

Fibre type analysis

Immunofluorescence was used to quantify the distribution of MHCI, MHCIIa and MHCIIx, and fibre CSA pre and post intervention. Methods were as described in Chapter Four

Satellite cell content and Myonuclear density

Immunofluorescence was used to quantify SC and myonuclear density. Indices of $Pax7^{+}/DAPI^{+}$ co-localisations per 100 fibres were quantified pre and post intervention for SC. Myonuclei per mm^2 of fibre cross section was determined for myonuclear density. Methods are as described in Chapter Four.

5.2.3 Body composition analysis

Total, lower body, upper body and appendicular LMM was measured pre-, mid- and post intervention via DXA scan. Methods are as described in Chapter Four.

5.3 Results

In reporting results, all significant time, group or time x group effects are reported. Where time effects are present, the difference between the times are reported for CON and HEAT groups separately.

5.3.1 mTOR anabolic synthesis signaling

There were no group or group x time effects observed for any of the mTOR signalling proteins measured. No time effects were observed for phospho-mTOR (Ser2448), P70S6K, rpS6, or TSC2. Strong tendencies were seen for total Akt ($p = 0.07$) and AMPK ($p = 0.09$). There were significant time effects for total mTOR ($p = 0.016$), phospho-Akt (Thr308) ($p = 0.017$), phospho-P70S6K (Thr389) ($p = 0.002$), phospho-AMPK (Thr172) ($p = 0.017$), 4E-BP1 ($p = 0.045$) and phospho-4E-BP1 (Thr37/46) ($p = 0.043$). In CON mTOR levels tended to decrease from Pre 0 to Pre 1 ($p = 0.206$). Post intervention mTOR decreased by 35% from Post 0 to Post 1 ($p = 0.048$). While not significant, Phospho-mTOR (Ser2448), levels trended up accordingly at the same time points (Figure 5.2). Akt decreased by 23.2% from Pre 0 to Pre 1 ($p = 0.027$) but were unchanged from Post 0 to Post 1 post intervention. Phospho-Akt (Thr308) increased by 194% from Pre 0 to Pre 1 ($p = 0.035$) and trended up Post 0 to Post 1 ($p = 0.252$). Phospho-AMPK (Thr172) levels tended to increase from Pre 0 to Pre 1 and increased by 97% Post 0 to Post 1 ($p = 0.003$). Phospho-P70S6K (Thr389) levels tended to increase from Pre 0 to Pre 1 and increased by 71% from Post 0 to Post 1 ($p = 0.025$). In the HEAT group AMPK levels decreased by 16% Pre 0 to Pre 1 ($p = 0.019$) and trended down Post 0 to Post 1 ($p= 0.05$). P70S6K levels decreased by 37% from Pre 0 to Pre 1 ($p = 0.034$) and trended down Post 0 to Post 1. Phospho-P70S6K (Thr389) levels increased by 134% Pre 0 to Pre 1 ($p = 0.048$). No differences were seen acutely at 48 hours for any of the markers pre or post intervention (Figure 5.2).

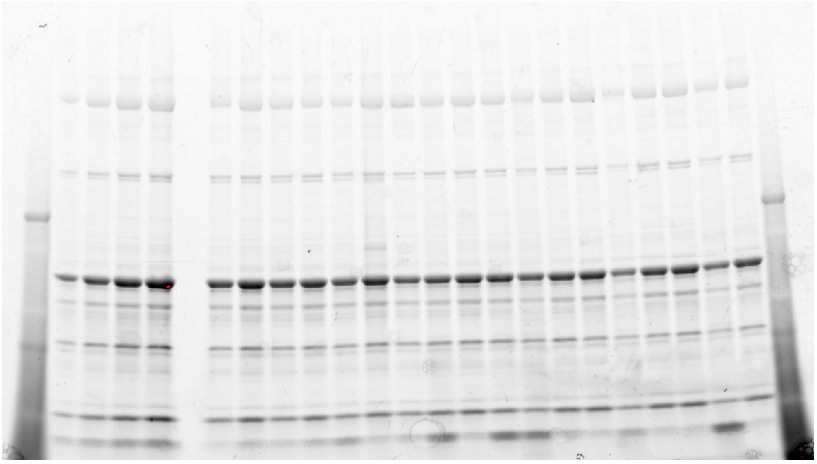


Figure 5.1. Representative stain free image

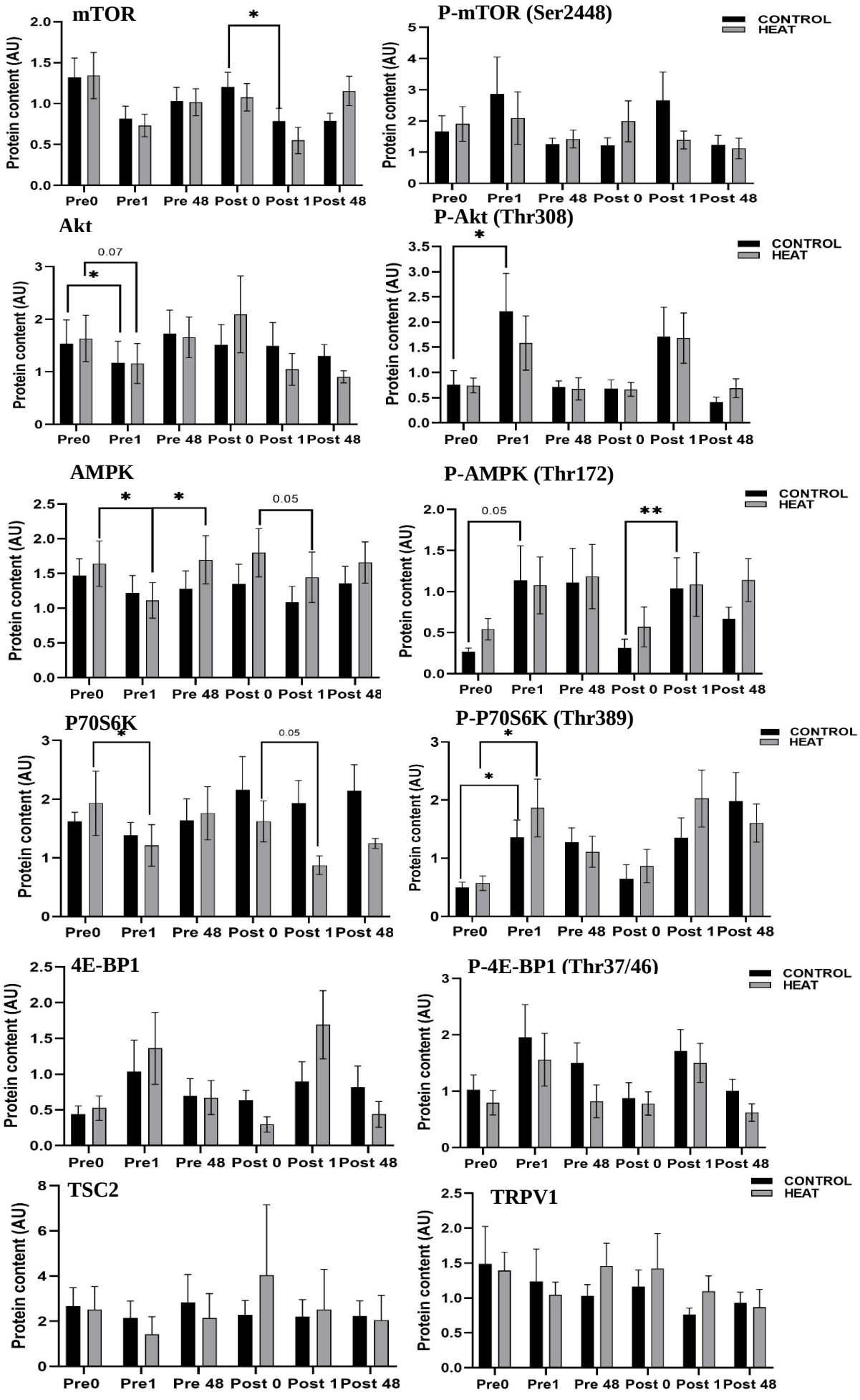


*Figure 5.2. Total expression levels of key markers in the mTOR pathway for anabolic synthesis. * p < 0.05 ** p < 0.005*

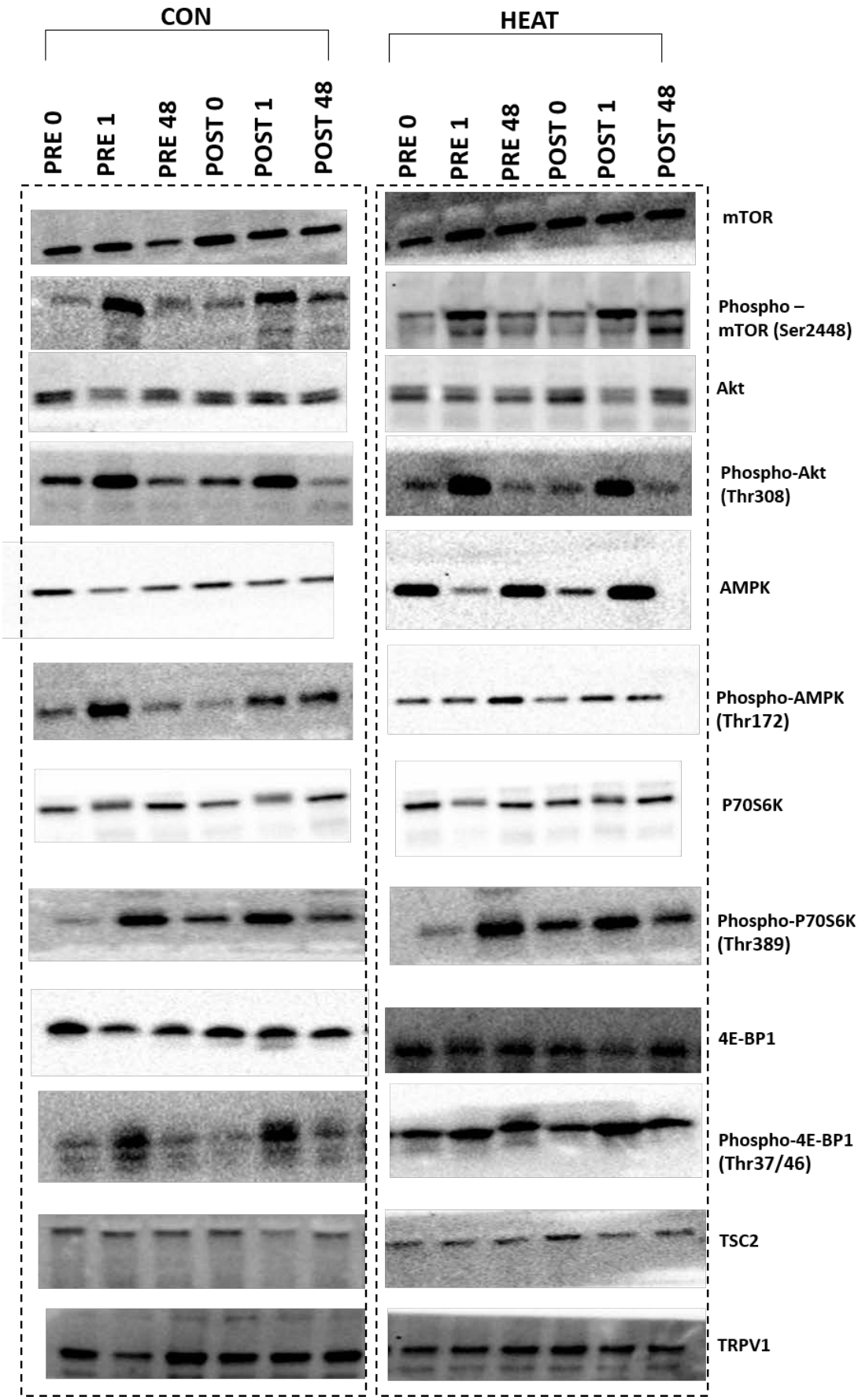


Figure 5.3. Representative western blots for all the marker quantified in the mTOR anabolic synthesis pathway. PRE; Pre intervention. Post; Post ten week RE or RE and HS intervention. Biopsies extracted acutely before, one and 48 hours after a single RE session pre and post the intervention

5.3.2 Ribosomal biogenesis

No time, group or time x group interactions were observed in the total expression levels of Cyclin D1 or rpS6 (Figure 5.4). A significant time effect was seen for phospho-rpS6 (Ser235/236) ($p = 0.0013$). In CON phospho-rpS6 levels increased by 2700% from Pre 0 to Pre 1 ($p = 0.020$) and by 6600% Post 0 to Post 1 ($p = 0.046$). In HEAT phospho- rpS6 levels increased by 7570% Pre 0 to Pre 1 ($p = 0.026$) and 8840% Post 0 to Post 1 ($p = 0.046$). No changes were observed at 48 hours in either group pre or post intervention.

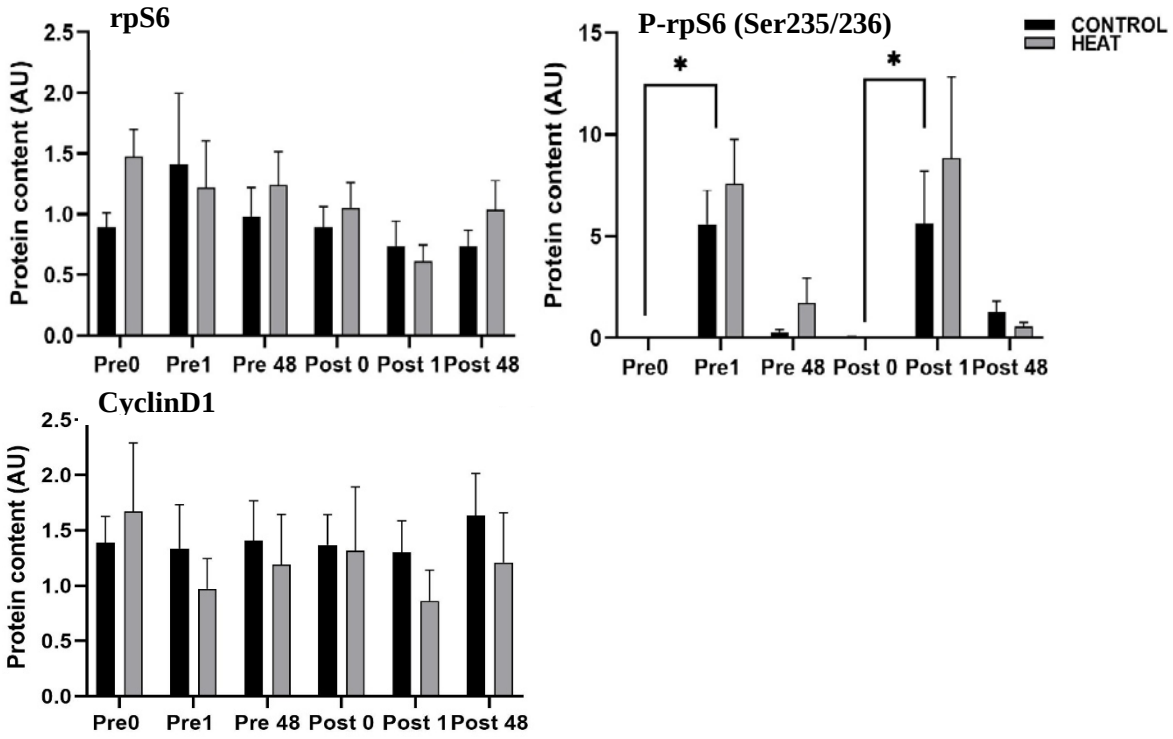


*Figure 5.4. Total expression levels of ribosomal protein S6 (rpS6), phospho-rpS6 and CyclinD1. * $p < 0.05$*

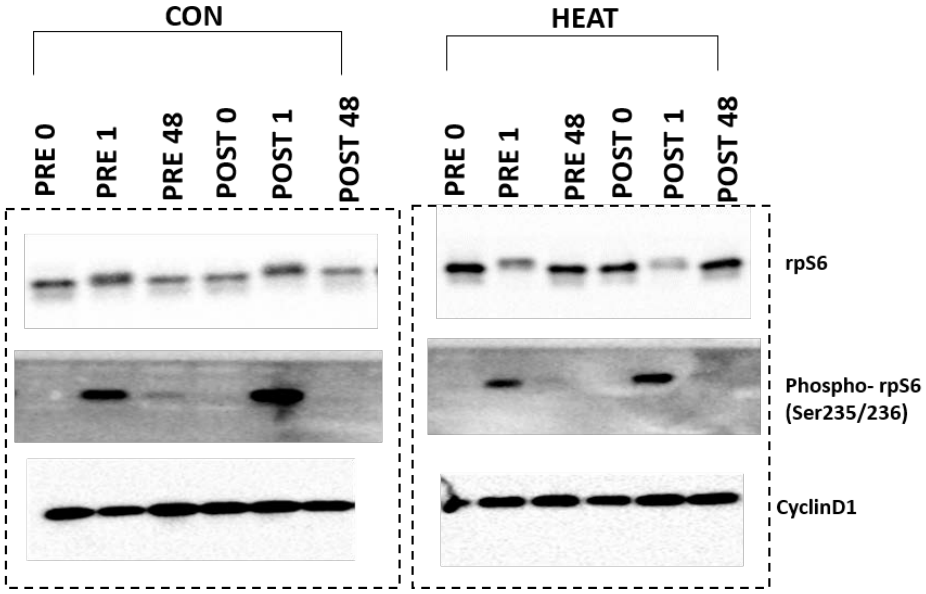


Figure 5.5. Representative western blots for all the marker quantified in ribosomal biogenesis. PRE; Pre intervention. Post; Post ten week RE or RE and HS intervention. Biopsies extracted before, one and 48 hours after a single RE session pre and post the intervention

5.3.3 Fibre type analysis

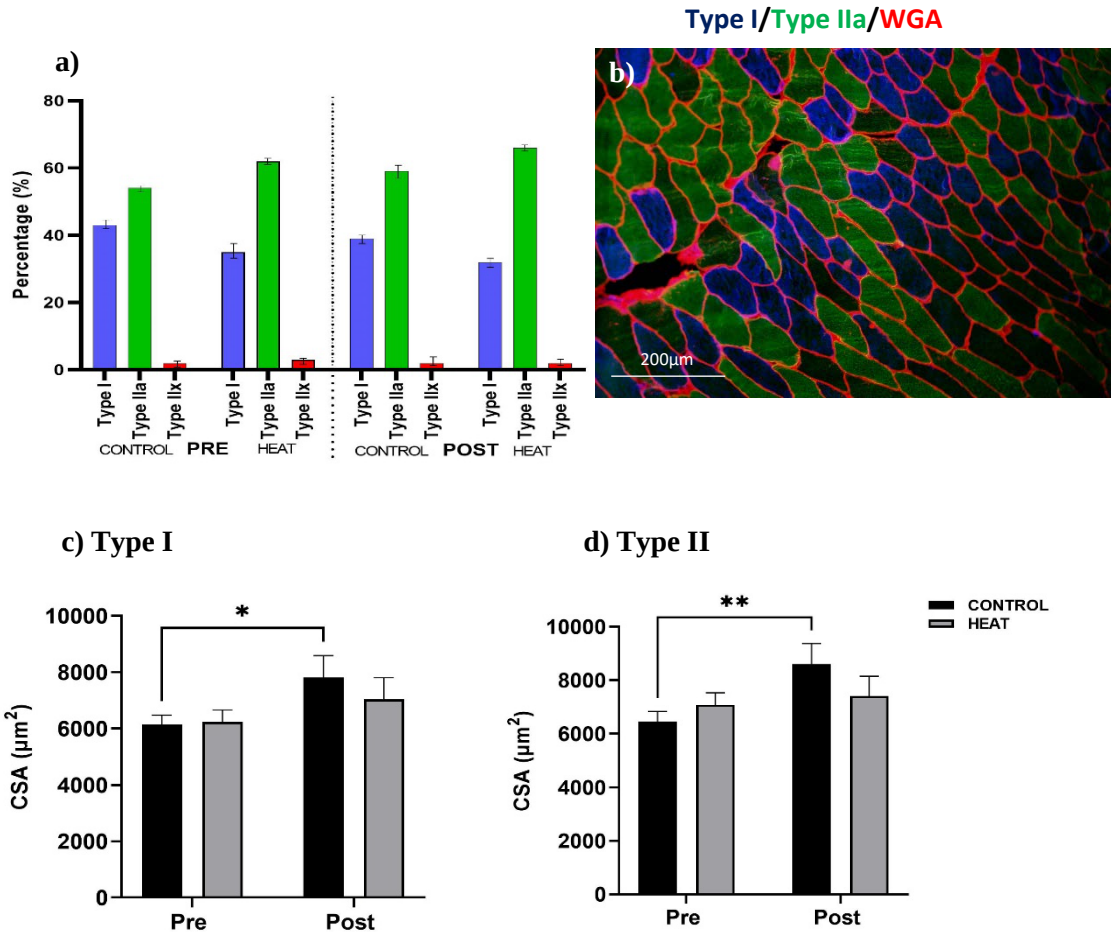


Figure 5.6. a) Quantification of fibre type distribution pre and post intervention in CON and HEAT groups b) histochemical stain of fibre cross section from vastus lateralis. c) and d) fibre cross sectional area pre and post intervention in CON and HEAT groups .* p <0.05 ** p < 0.005.

Immunofluorescence was used to quantify the percentage MHCI, MHCIIa and MHCIIx fibre distribution and CSA pre and post intervention. There were no time or time x group effects in the fibre type distributions pre or post intervention in any of the fibre types. Significant group effects were seen for percentage of Type I ($p = 0.025$) and, Type II ($p= 0.010$) but the differences were only seen pre intervention and were not different between groups post intervention. No group effects were seen for distribution of Type IIx. No group or time x group effects were observed for CSA. Time effects were seen in CSA pre to post intervention for Type I ($p= 0.025$) and Type II ($p = 0.019$). CSA improved significantly in the CON group in Type I ($p =0.038$) and II ($p = 0.005$) by 27% and 33% respectively. In the HEAT group by 13% and 5% (Figure 5.6).

5.3.4 Satellite cell content and myonuclear density

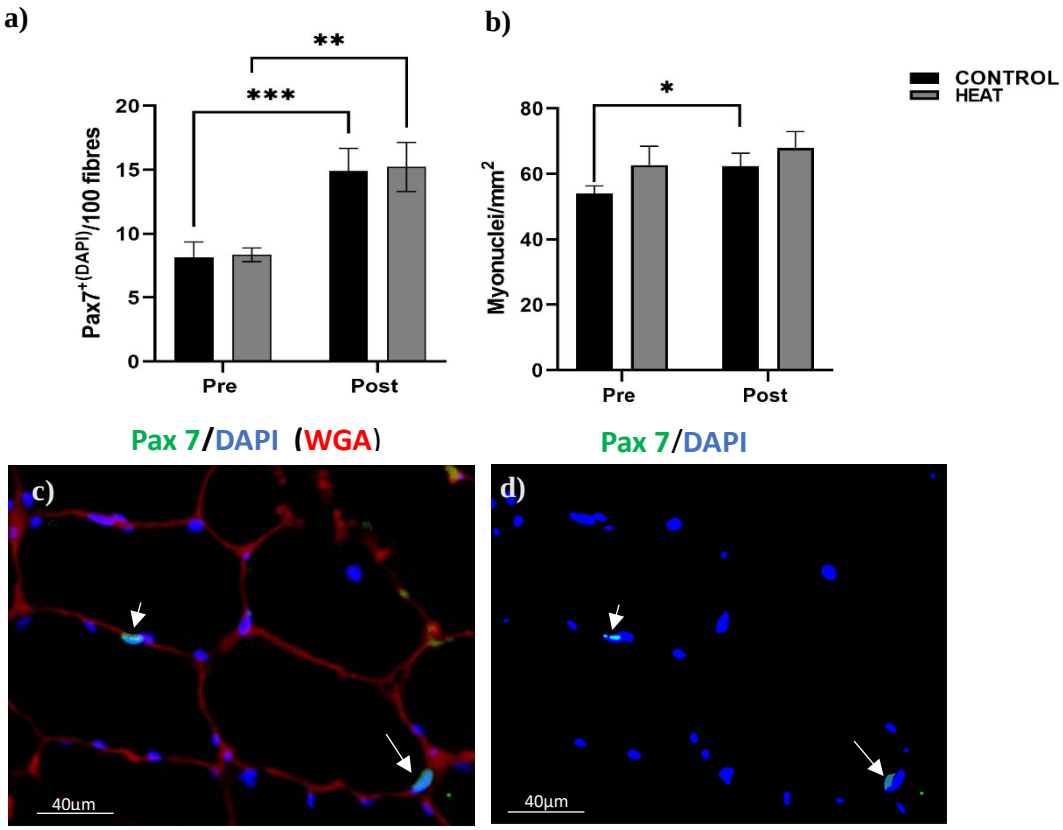


*Figure 5.7. a) Quantification of satellite cell content (SC) and b) myonuclear density pre and post intervention in the HEAT and CON c) and d) histochemical stain of fibre cross section from vastus lateralis depicting co-localized Pax 7/DAPI * < 0.05 ** p < 0.01 *** p < 0.005*

PAX-7/DAPI co-localisation immunofluorescence was used to quantify satellite cell (SC) numbers pre and post intervention. Myonuclear proliferation was quantified via myonuclear density (DAPI). There were no group or time x group effects in the SC content or myonuclear density pre or post intervention. Significant time effects were seen for SC ($p < 0.0001$) and myonuclear density ($p = 0.029$). SC content improved by 82% in CON ($p = 0.0003$) and by 82% in HEAT ($p = 0.0013$). Myonuclear density increased in CON by 11% ($p = 0.025$) (Figure 5.7).

5.3.5 Body composition analysis for lean muscle mass

Body composition was analysed via DXA. No group or time x group interactions were observed in upper, lower, appendicular or total body lean muscle mass (LMM). No time effect was observed in the lower body LMM. Significant time effects were observed in upper body LMM ($p < 0.0001$), appendicular LMM ($p = 0.0008$) and total body LMM ($p < 0.0001$). Upper body LMM increased by 4.0% ($p = 0.001$) in the CON group and by 2.3% in the HEAT group ($p = 0.020$). Appendicular LMM increased in the CON group by 2.8% ($p = 0.0002$). Total body LMM increased by 3.2% in the CON group ($p = 0.0008$) and tended to increase 1.7% in the HEAT group ($p = 0.060$) (Figure 5.8).

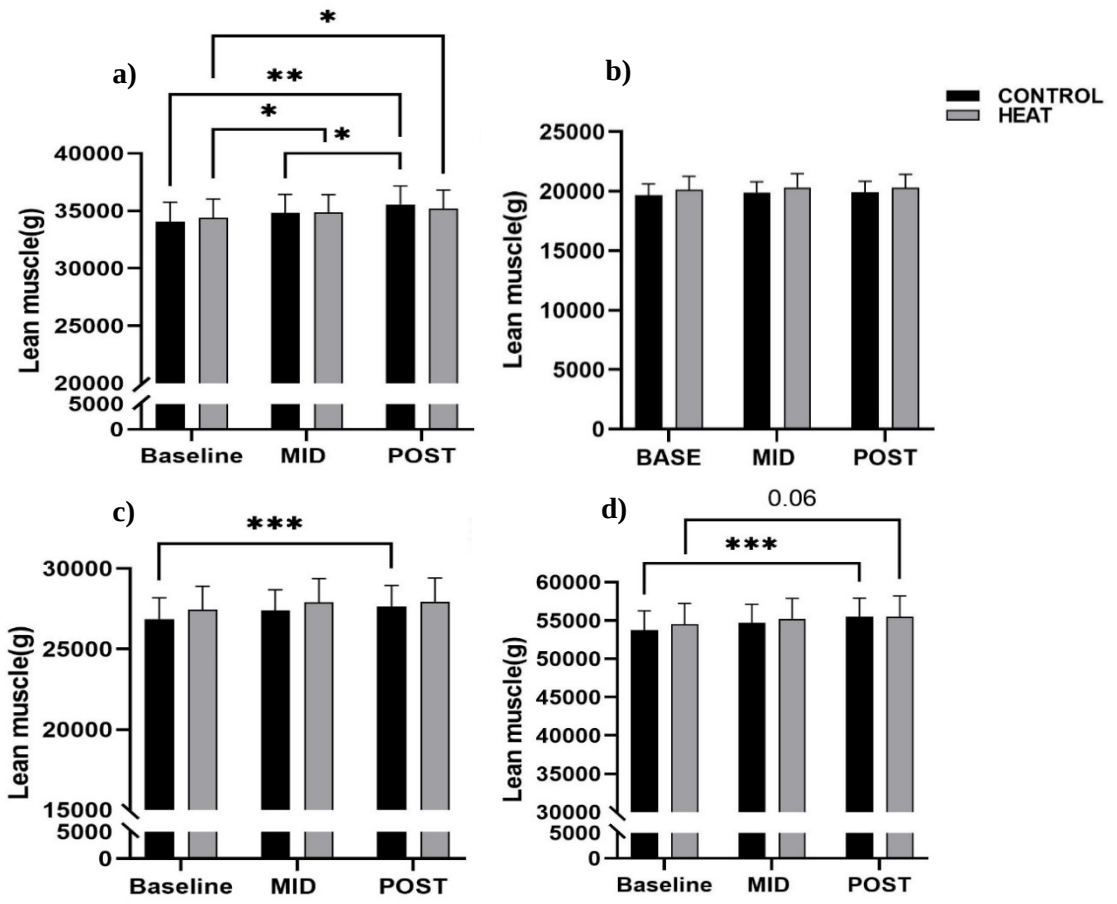


Figure 5.8. a) Lean muscle mass upper body b) lower body c) appendicular and d) total lean muscle mass pre-, mid- and post intervention in the CON and HEAT groups. * p <0.05, ** p < 0.01, *** p < 0.005

5.4 Discussion

5.4.1 Molecular response to RE and HS: anabolic synthesis

The aim of this investigation was to examine the impact of full body HS applied at 40°C (30% RH, climate chamber) on the phenotypic and muscle molecular response to acute and chronic RE over a ten week intervention. The anabolic synthesis response was investigated via quantifying key markers of the mTOR pathway as well as ribosomal biogenesis. Phenotypically, SC content, fibre type distribution and fibre CSA as well as myonuclear density were quantified. This data was further corroborated by the quantification of upper, lower, appendicular and total LMM pre-, mid- and post intervention.

We observed no differences between the CON and HEAT groups in any of the key markers we investigated acutely or chronically post RE at one hour or at 48 hours, alluding to the fact that HS applied at 40°C (30% RH) may not improve upon the acute anabolic synthesis response to RE and appears to have no cumulative effect chronically post ten weeks of hypertrophy-oriented RE.

mTOR

We observed a transient reduction in mTOR levels in CON group ($p < 0.05$) one hour after an RE session post the ten week intervention. While not reaching statistical significance, we observed the same trend of mTOR levels decreasing one hour post in both groups (Figure 5.2). Consistent with this observation, phospho-mTOR (Ser2448) levels were elevated at the same time point in both groups, however, did not reach statistical significance. These results support the previous findings of Kakigi and colleagues who reported no elevations in phospho-mTOR (Ser2448) at one hour post from baseline in the *vastus lateralis* following an acute bout of lower body RE (Kakigi et al., 2011). However, Kakigi further reported that with the concurrent localised application of HS, mTOR phosphorylation increased significantly from baseline, ($p<0.05$) at one hour post which contradicts our findings of no significant elevations at one hour post with the concurrent application of HS (Kakigi et al., 2011). It is worthy to note that the load-intensity-stimulus may have contributed to the divergent mTOR findings between the two studies. Kakigi ulitised maximal contraction, high intensity single leg bouts, whereas our intervention was full body RE. Fuchs and colleagues saw that after 45 minutes of RE-type exercise followed by 20 minutes of hot water immersion (46°C) increasing CMT to ~38°C did not improve upon post prandial MPS and mTOR response. No changes were seen immediately or two hours post HS, compared to a control leg at 30°C with a CMT of ~35°C (Fuchs et al., 2020).

To our knowledge, this is the first investigation to quantify the mTOR phosphorylation response to full body RE combined with HS. A significant difference to be highlighted here is that compared to our observations of peak CMT 36.79 ± 0.58°C in the HEAT group (at a depth of 3.5 cm), Kakigi reported a CMT of 41.1 ± 1.3°C (Kakigi et al., 2011). Ihsan and colleagues saw a 64% increase in the phospho-mTOR levels after one hour of whole body HS application at 50°C (50% RH) adjusted within a range of 44-50°C to maintain a steady core temperature of 39°C and a CMT of 38.8 ± 0.5°C, compared to core temperature of 38.18 ± 0.13°C and CMT of 36.79 ± 0.58°C that was observed in our study (Ihsan et al., 2020). Interestingly, Ihsan and colleagues did not observe elevations in the phospho-mTOR (Ser2448) levels at the same time point after 30 minutes of localised HS applied directly to the *vastus lateralis* increasing CMT to 38.1 ± 0.6 °C. While the combined stress load of full body HS combined with hypertrophy driven RE is likely higher than RE alone, the instigation of anabolic synthesis seems to be dependent on a CMT threshold of ~39°C in the *vastus lateralis.* The acute improvement of mTOR phosphorylation seems to occur at an expedited time frame compared to RE alone as seen by Kakigi (Kakigi et al., 2011), or even without RE if this threshold temperature is exceeded. In this

investigation the CMT were not different between the groups, therefore ten weeks of RE or RE combined with full body HS at 40°C does not have a cumulative impact on the acute mTOR response to RE. It is noteworthy that in the CON group mTOR kinase activity was more prominent compared to the HEAT group.

Akt, AMPK and TSC2

The key upstream regulators of mTOR activation, Akt and AMPK and co-activator TSC2 were also investigated. Total Akt levels trended down acutely one hour post RE pre and post intervention in both groups. Phospho-Akt (Thr473) levels trended up in both groups at the same time point, signifying activation. Phosphorylated Akt (Ser473) forms Akt/mTOR complex to drive anabolic synthesis under mechanical stress (Bodine et al., 2001). Following an acute bout of RE, phospho-Akt (Ser473) has been shown to increase by 130% at 30 minutes post-exercise and by 100-200% post a bout of endurance training which demonstrates its complex involvement in multiple acute cellular responses to exercise (Camera et al., 2010). However, in the only other concurrent RE and HS study, Kakigi and colleagues did not observe a significant elevation of phospho-Akt (Ser473) one hour post an acute RE session which contradicts our results as we observed a significant increase at one hour post RE. However, there appears to be an early time-course response in Akt phosphorylation following RE and it appears that the phosphorylation is intensity dependent. However, as mentioned in the mTOR discussion, Kakigi's total RE load-intensity is different to our current investigation and Camera's work given the fact that they used concentric only isokinetic intervention. However, the potential intensity or stress load dependent Akt activation is further made evident by the fact Kakigi observed a significant elevation of phospho-Akt (Ser473) levels one hour following acute RE and HS in combination, whereas we did not observe significant improvements at one hour post RE with the concurrent application of full body HS at 40°C (30% RH). However, this observation was most likely due to the difference in CMT where Kakigi raised CMT over 40°C compared to ~37°C in the current investigation.

Total AMPK levels decreased significantly in the HEAT group pre intervention, and tended to increase in the HEAT group post intervention. In the CON group the levels trended down while not being significant. Interestingly, phospho-AMPK (Thr172) increased significantly in the CON group pre and post intervention acutely one hour after the RE session but not in the HEAT group that trended up while not reaching statistically significant levels. Interpretation of these observations was approached with the notion that, phospho-AMPK levels increased in both groups pre and post intervention while not showing a significant effect of HS possibly due to sample size inadequacies in the HEAT group. Kakigi and colleagues did not observe elevations in the phospho-AMPK (Thr172) levels acutely one hour post RE or acutely one hour post RE

combined with HS. AMPK is a key metabolic regulator and an established energy sensing protein as well as supposed repressor of the anabolic synthesis (Camera et al., 2010, Dreyer et al., 2006b). It has been reported that parallel time course elevations of phospho-AMPK (Thr172) occur concomitantly to anabolic synthesis regulators such as activated Akt (Camera et al., 2010). Furthermore, it has also been shown that the AMPK response is also intensity dependent with regards to exercise (Dreyer et al., 2006b). Therefore, the speculation arises that phosphorylated AMPK acts as suppressor of synthesis, at least partially, while the contractile activity is still in progress and acutely after (Rasmussen and Phillips, 2003). However, this process still has not been fully investigated with a number of concurrent cellular process that occur during and acutely post exercise. Our observations support the notion that during an acute, but extended RE session, AMPKα would be activated through phosphorylation to suppress anabolic synthesis in order to maintain cellular energy expenditure economy during the RE session. Our findings showed that AMPKα still remained elevated acutely at one hour post returning to baseline at 48 hours post while anabolic synthesis response was being initiated via Akt phosphorylation. Assessing the observations by Kakigi and as well as ours, it could further be suggested that HS, regardless of the CMT (intensity) achieved, might not be a contributor to AMPK activation in the human skeletal muscle. A secondary reason could be that fact the AMPK response, as an energy gauge could be predicated upon the muscle target. A muscle with high oxidative potential may show an increased response to HS (Goto et al., 2015).

TSC2, which has been posited to be a key regulator of cellular energy management and an inhibitor in the mTOR pathway of anabolic synthesis (Huang and Manning, 2008) did not change significantly at any of the time points in CON or HEAT groups. TSC2 has been proposed to inhibit mTOR via phosphorylation (Philp et al., 2011), inhibiting the translation initiation (Inoki et al., 2003b). However, the evidence in human skeletal muscle is limited. In mice, it has been suggested that in response to mechanical stimulation, TSC2 is inactivated by Akt via hyper-phosphorylated thereby allowing mTOR activation (Jacobs et al., 2013). Song and colleagues saw that one hour after a bout high intensity exercise TSC2 dissociated strongly from Rheb, the direct upstream mTOR regulator, this was concomitant with the Akt phosphorylation at the same time point (Song et al., 2017). We did not observe evidence of this notion as we did not measure phosphorylated TSC2 or investigate cellular localisation of TSC2. Therefore, our results on TSC2 in this study are inclusive with regards to acute and chronic RE or RE combined with HS.

P70S6K and 4E-BP1

Downstream of mTOR, we investigated two key translation initiators P70S6K and 4E-BP1 and their phosphorylated levels. We observed that the total expression levels of P70S6K acutely

decreased in the HEAT group pre intervention ($p < 0.05$) and post intervention ($p = 0.05$). While a trend down was observed in the CON group, there was no significant reduction. Phospho-P70S6K (Thr 389) levels increased significantly pre intervention in the HEAT and CON groups ($p < 0.05$). However, the trend towards an increase observed post intervention was not statistically significant. There no were significant alterations observed in total expression levels of 4E-BP1or phospho-4E-BP1 (Thr37/46) levels at any of the time points for either group. While it has been relatively well established that P70S6K is involved in anabolic synthesis, there have been a number of contradictory reports. According to Terzis and colleagues, phospho-P70S6K (Thr 389) levels increased multifold 30 minutes after an acute bout of full body RE (Terzis et al., 2008). One hour after an acute bout of RE, Kakigi and colleague also saw a significant elevation in the Thr389 phosphorylation levels (Kakigi et al., 2011). However, 30 minutes after an acute bout of lower body RE, Mitchell and colleagues did not observe the same change in the phosphorylation levels in the *vastus lateralis* but saw elevations at five hours post RE (Mitchell et al., 2013). In combination with acute HS, Kakigi saw a similar improvements to RE alone in the phosphorylation levels one hour after RE, indicating no additive effects of HS, which agree with our observations. Fuchs and colleagues saw Phospho-P70S6K (Thr389) increase significantly at two hours and sustained at five hours following 20 minutes of HS. However, at two or five hours CMT in the HS leg was not different from the control (~36°C) (Fuchs et al., 2020). Ihsan and colleagues reported 174% increase in Phospho-P70S6K levels (Thr389) 30 minutes after one hour of whole body HS application maintaining a core temperature of 39°C and muscle temperature of 38.8 ± 0.5°C, but did not observe the same changes in the localised HS application for the same period after raising CMT to 38.1 ± 0.6 °C. The heat only response to full body HS appears to be a stress response to high core temperature, as localised HS, raising CMT to < 39°C failed to trigger a P70S6K phosphorylation at Thr389 (Ihsan et al., 2020). Overall, our results agree with previous observations that the phosphorylation of the key translator initiator P70S6K is upregulated acutely after RE, however, the response is not altered by full body HS applied at 40°C. The phosphorylation response appears independent of the CMT, however, seems responsive to an elevation of core temperature above 39°C. Which also poses the intriguing question of whether or not localised HS is a valid method in improving anabolic synthesis via the mTOR pathway.

Total 4E-BP1 displayed an interesting trend in both CON and HEAT groups where the levels elevated noticeably but did not reach statistical significance due to high variability within the data. Phospho-4E-BP1 showed a similar trend in both groups, while not reaching statistical significance. 4E-BP1 is another key translation regulation factor which suppresses translation

when not phosphorylated at multiple sites (Gingras et al., 1998). It has been reported to be directly targeted by mTOR/(p)Akt for phosphorylation, thereby initiating anabolic synthesis (Gingras et al., 1998).We investigated phospho-4E-BP1(Thr37/46). Kumar and colleagues reported a ~25% increase in phospho-4E-BP1 levels one hour post an acute RE session, however, they did not report the site of investigation (Kumar et al., 2009). Camera and colleagues reported no elevations of phospho-4E-BP1 (Thr37/46) (nor at Thr70) levels one hour post a bout of RE (Camera et al., 2010). Interestingly, Kakigi reported a significant decrease in phospho Thr37/46 levels immediately and one hour post an acute bout of RE (Kakigi et al., 2011). This observation is reasonable given they did not observe increasing levels of phospho-Akt (Thr308) or phospho-mTOR (Ser2448) at the same time point. It is possible that phospho-4E-BP1 levels were on the rise at the time of biopsy and would have theoretically continued to rise (Kakigi et al., 2011). Interestingly, they saw the phosporylated-4E-BP1 return to pre-exercise levels faster at the same time point when RE was combined with HS indicating elevated levels of phosphorylation by mTOR (Kakigi et al., 2011). Given that they saw a significant elevation of phospho-Akt (Thr308) levels at the same time point when RE was supplemented with HS, this observation further verifies the Akt/4E-BP1 "switch off" dynamic. 20 minutes o of hot water immersion following 45 minutes of RE did not improve phospho-4E-BP1(Thr37/46) levels at two or five hours post intervention at a CMT of ~36°C (Fuchs et al., 2020). After applying full body HS for an hour (core temperature 39°C) or localised HS for raising CMT to 38.1 ± 0.6, Ihsan and colleagues saw no improvements of phospho-4E-BP1(Thr37/46), 30 minutes post treatment (but saw 103% increase in total 4E-BP1 levels, along with ~50% in phospho-Akt (Thr308) (Ihsan et al., 2020). However, given the time-course and intensity dependent nature of these responses, it is possible they the upregulation may have occurred later than one hour. However, it is important to note that they saw no improvements at 3 hours post treatment either. Overall, however, HS does not appear to elicit a response effect on the phosphorylation of 4E-BP1, in skeletal muscle. We propose the possibility that the Akt/mTOR dependent 4E-BP1phosporylation at Thr37/46, might respond to a higher (> 39°C) muscular temperature. This notion is partially supported by the observations that phospho-4EBP1 levels returned pre-exercise levels faster when CMT was ~41°C, compared to our and other investigations who saw no changes at the same point at CMTs below ~39°C.

Ribosomal biogenesis

Another key process of the anabolic synthesis response we investigated was ribosomal biogenesis as its involvement in hypertrophy as a translational apparatus has been documented in rodents. However, its response to mechanical loading in humans is less well known and has

been proposed to be mTOR dependent (von Walden et al., 2012, Iadevaia et al., 2014). Furthermore, the ribosomal biogenesis response to HS or HS combined with RE has not been well defined. We investigated the key ribosomal biogenesis marker rpS6 and Cyclin D1 as well as phospho-rps6 (Ser235/236).

Acutely post a RE session, we saw no significant differences in the total expression levels of rpS6 pre and post intervention in either CON or HEAT groups. Phospho-rpS6 (Ser235/236) levels improved rapidly after RE, however, there were effects of HS. Our observations are in alignment with the reports by Figueiredo and colleagues where they observed a significant improvement in the phospho-rpS6 (Ser235/236) levels one hour after an acute bout of lower body oriented RE. Furthermore, Figueiredo and colleagues also reported no training effect on the total rpS6 content or their phosphorylation levels after eight weeks lower body only heavy RE which coincides with our observations (Figueiredo et al., 2015). Additionally, they reported an upregulation in the phosphorylation of the immediate upstream "switch" P70S6K. Although Figueiredo and colleagues did not quantify mTOR levels, there is strong evidence that acute heavy RE promotes the activation of rpS6 via phosphorylation in preparation for the anabolic synthesis response. These observations are further supported by Kakigi's findings where phospho-rpS6 (Ser235/236) levels were elevated one hour post an acute bout of RE. Interestingly, and contradicting our results, they reported that phosphorylation levels of rpS6 showed a significant up regulation when RE was supplemented with localised HS where CMT was raised to 41.1 ± 1.3°C (Kakigi et al., 2011). No changes in in the phospho-rpS6 (Ser235/236) were seen at two or five hours after 20 minutes of HS following 45 minutes of RE. CMT at the point of measurement was not different from contralateral control leg (Fuchs et al., 2020). Ihsan and colleagues saw a similar improvement in the phospho-rpS6 (Ser235/236) levels 30 minutes post a full body one hour bout of HS where CMT was raised to 38.8 ± 0.5°C in the *vastus lateralis* (Ihsan et al., 2020). However, they did not see any improvements in the phosphorylation levels after a bout of acute localised HS raising CMT to 38.1 ± 0.6 (Ihsan et al., 2020) . Given that we saw no effect of HS where we raised CMT to 36.79 ± 0.58°C, concurrently during RE, it appears that a supplementary effect of HS on the phosphorylation of rpS6 has a threshold effect at ~39°C.

To our knowledge, this is the first investigation to quantify total Cyclin D1 levels in response to RE and HS acutely pre and post a long term training intervention (Figure 5.4). Figueiredo and colleagues saw a significant improvement in the total expression levels of Cyclin D1 one hour post a lower body only RE session, but saw no training effect (Figueiredo et al., 2015). Similar to their investigation, we saw no training effect on the Cyclin D1 levels. In contradiction however, we saw no changes in the total expression levels of Cyclin D1 after an acute full body

RE session. We did not observe an additive effect by concurrent full body HS applied at 40°C either. Cyclin D1 is associated with the regulation of ribosomal biogenesis and has been suggested to have mTOR independent translational regulation (driven by eIF4E) (Rosenwald et al., 1993). However, Figueiredo did not observe phosphorylated activation of eIF4E at the same time point. They posited that they may have missed the spike in translation at the time of the biopsy (Figueiredo et al., 2015). It is possible however, given the lower body only nature of their RE bout, Cyclin D1 levels elevated quicker than ours given the biopsy target of vastus lateralis would have been consistently active during the work out as well as the higher load intensities at 70 and 90% 1RM used in training It is likely that the Cyclin D1 levels were increasing, but at the time of the biopsy, it was too premature to detect a significant elevation in the Cyclin D1 levels. Furthermore, HS applied at 40°C (30% RH), elevating CMT to 36.79 ± 0.58°, does not expedite the Cyclin D1 response in the *vastus lateralis*, but we speculate that at temperatures reaching 39°C might have an additive effect based on the fact that improved rpS6 phosphorylation is seen above this temperature.

In summary, RE acutely improved the upregulation of the key kinases in the mTOR pathway. However, the mTOR driven anabolic synthesis response to RE showed no training effect overall. Full body HS applied at 40°C (30% RH), does not seem to have an impact on the anabolic synthesis response to RE acutely or chronically. It is strongly suggestible that there might be threshold CMT in the skeletal muscle (> 39°C) above which HS may begin to improve upon the anabolic synthesis response to RE in a recreationally active cohort.

5.4.2 Phenotypic response to RE and HS

Along with the anabolic synthesis response, we investigated the effect of full body concurrent HS on the fibre typical response, SC content response as well as the overall chronic hypertrophic response to RE pre and post a ten week RE program.

The hypertrophic response to long term RE is well established. There exist only a very limited number of studies that have investigated the impact on HS on the phenotypic muscle response to RE. The number further diminishes when the length of the study is taken in to account.

Over a ten week period of RE we observed no significant difference in the distribution of Type I, Type IIa or Type II x (Figure 5.6a) between groups alluding to the fact that external full body HS (40°C, 30% RH) applied concurrently to RE does not drive the proliferation of one fibre type over the other. The number of Type IIa fibres were 10-20% higher than the number of Type I fibres in both CON and HEAT groups. This difference was maintained post intervention without significant deviations (Figure 5.6a). Type IIx percentage did not alter pre or post

intervention in either group. However, our results contradict reports by Nederveen and colleagues, who reported a significant reduction in the Type II fibres after 16 weeks of RE (67 ± 3 % to 62 ± 2%) (Nederveen et al., 2017). It is to be noted that we observed a tendency for a time effect of $p = 0.06$ between pre and post intervention albeit with increasing percentage over the intervention. This however maybe due to individual variability within the groups, rather than an effect of intervention. To our knowledge, this is the first investigation to examine fibre type distribution with respect to full body HS. Given that CMT was not raised significantly above that of the CON in the HEAT group, the lack of effect of HS is expected. Whether a significantly higher CMT would alter the overall distribution post a chronic RE intervention in the skeletal muscle remains to be investigated.

Muscle fibres

We observed overall CSA improvements in both Type I and Type IIa muscle fibres over the ten-week intervention. In the CON group, the CSA of Type I and Type IIa improved significantly. Intriguingly, the overall improvements in CSA in the CON group by 27% and 33% respectively in Type I and IIa respectively, were considerably higher compared to HEAT group's non-significant 13% and 5% increases (Figure 5.6c & d). The CSA improvements observed in our tests agree with previous reports of Nederveen who reported similar findings where after 16 weeks of RE CSA improved in Type I and II to similar levels with type II showing greater improvements over type I (Nederveen et al., 2017). Tang and colleagues observed significant improvements in both fibre types after 12 weeks of whole body high intensity RE, similar to our protocol, albeit lower in sessional training load (defined in Chapter 3) (Tang et al., 2006). Interestingly, Rupal and colleagues reported significant improvements in Type II but not Type I fibres after ten weeks of progressive RE (Ruple et al., 2021). Notwithstanding the response variability in the population however, the CSA improvement to chronic, progressive RE ≥ ten weeks, appears consistent over the available literature, that myofibrillar area improves in both fibre types via hypertrophy as observed in our CON group. However, we report that in the HEAT group, CSA, while trending up, did not improve significantly post the ten week intervention in the *vastus lateralis*. After ten weeks of localised HS (four days per week for eight hours a day) to the *vastus lateralis*, Goto and colleagues saw an overall significant increase in CSA the quadriceps of 2.7 ± 0.7% from pre intervention with a peak muscle temperature of 38.3 ± 0.1°C at a depth of 1.5cm (biopsy depth was ~2cm). They also reported that in a single chosen subject, muscle fibre CSA improved significantly post HS but the fibre type was not reported and the overall credibility of n=1 is low (Goto et al., 2011). The number of studies investigating muscle fibre CSA is extremely limited to. Hafen and colleagues showed the ability of localised, daily

HS to attenuate muscle atrophy via ten days of HS to the *vastus lateralis*, but reported that the changes in immobilisation atrophy were independent of the CSA changes of the fibre types (Hafen et al., 2019). Hesketh and colleagues saw no improvements in the fibre CSA of Type I or II after six weeks of full body HS at 40°C (Hesketh et al., 2019). Similarly, Kim and colleagues saw no improvements in type I or II or CSA after eight weeks of localised HS to the vastus lateralis at a CMT of ~38 (Kim et al., 2020) Indicating towards the fact that HS, independent of form of application or muscle temperature may not improve muscle fibre hypertrophy. Alluding to the fact that the reductions in muscle CSA observed in immobilisation occurs via non-myofibril oriented volume loss, for instance, sarcoplasmic atrophy (Roberts et al., 2020) and is halted by the application of HS. This supports the observations by Goto and colleagues that perhaps, CSA increases *vastus lateralis* post HS they observed was non-fibre driven as well. To our knowledge, this is the first investigation in humans to report that long term, full body concurrent HS may have a negative impact on muscle fibre growth. However, this is speculative given there was no significant difference between the CMT of the CON and the HEAT groups and requires further investigation with perhaps a larger sample size. It is noteworthy however, that CMT trended higher in the HEAT group compared to CON. At the inception of this investigation, we hypothesised based on limited evidence that HS drives mild hypertrophy and therefore might augment the muscular response to chronic RE. However, there was no HS related fibre specific data available.

Satellite cells and myonuclei

We further investigated the impact of HS on the SC response and myonuclear proliferation to long term RE. SC content/activation was quantified via the identification of Pax7/DAPI co-localisations and myonuclear density via identification of fibre associated myonuclei pre and post intervention (Figure 5.7c &d). Following the RE intervention, we observed a significant elevation in the levels of SC content in the HEAT and CON groups (Figure 5.7a). However, we did not conduct fibre specific SC quantitation, as it has been previously shown that type II has higher SC association compared to type I (Nederveen et al., 2017). Furthermore, myonuclear density increased significantly in the CON group and trended up without reaching statistical significance in the HEAT group. Our observations regarding SC content were strongly in agreement with that of Nederveen and colleagues post 16 weeks of RE in terms of the training effect as well as that of Bellamy and colleagues post the same training length, showing similarly increased SC content (Nederveen et al., 2017, Bellamy et al., 2014). Given the hypertrophic response observed in both fibre types in the CON and to a lesser extent HEAT groups, these observations are partially justified. However, a key point to note is that there still remains debate

on the level of contribution by the increasing SC pool to RE driven muscle fibre hypertrophy (Rehfeldt, 2007). To this point, while SC content was not different between the groups, myofibre CSA showed a marked difference between the groups where the CON group showed larger improvements. We are the first to investigate the effect of concurrent full body HS on SC content. We report that HS had no impact on SC content.

Previous studies have reported the chronic effect of RE on improving myonuclear density (Petrella et al., 2008). The efficacy of myonuclear addition has been suggested as a key factor in myofibre hypertrophy response to RE (Petrella et al., 2006). In this study, a significant improvement was observed in the myonuclear density in the CON group in accordance with the CSA improvements observed in previous studies. This observation is further validated by the fact in the HEAT group, no significant improvements were improved in the myonuclear density and the CSA improvements were considerably less compared to CON. To our knowledge we are the first to quantify the effect of HS on chronic myonuclear response to RE in the human skeletal muscle. It has been reported in previous rodent studies that HS may increase myonuclear numbers in damaged muscle but not in healthy muscle (Oishi et al., 2009). In line with this report, our findings strongly suggest that HS, even with a non-significant improvement in CMT over the CON group, may have a negative impact on chronic muscle fibre hypertrophy response to long term RE by attenuating the myonuclear proliferation response to chronic RE. However, it is critical to point out that our investigation is the first instance in which this observation has been made in the current body of work. As stated above, in the discussion of fibre CSA, this observation requires further investigation.

Lean muscle mass

In order to quantify the effect of full body HS to the overall phenotypic muscular response to RE, we examined the LMM response pre-, mid- and post intervention. Overall LMM mass improved significantly ($p < 0.05$) in the CON group but failed to reach significance in the HEAT group ($p = 0.06$).Upper body LMM improved significantly ($p < 0.05$) in both groups over the ten week intervention, with no differences observed between groups. Interestingly, the HEAT group improved significantly at mid intervention (within the first meso-cycle) and the CON group improved significantly from mid to post intervention (within the second meso-cycle) (Figure 5.8a). These results indicate a potential expedited LMM response in the upper body in the HEAT group reaching a slower response rate during the second meso-cycle plateauing. Conversely, it appears that the upper body response was slower in the CON group and reached a faster growth rate in the second meso-cycle, which is a commonly observed response pattern to progressive full body RE (Phillips, 2000). However, we saw no significant changes in the lower body LMM

in either group. Appendicular LMM only increased significantly ($p < 0.005$) in the in the CON group. The overall results agree with previous reports by Bellamy and colleagues who saw a significant improvement in the total LMM after 16 weeks of progressive RE as well as observations by Ruple and colleagues after ten weeks of progressive RE (Bellamy et al., 2014, Ruple et al., 2021). Given the overall CSA improvements observed in type I and II fibres in the *vastus lateralis*, especially in the CON group, the lack of overall improvements in the total lower body LMM is intriguing. However, it is possible that the lack of significant improvements in both groups (while trending up) and the lack of correlation between CSA improvements is due to the fact that LMM takes in account other forms of hypertrophy that are non-fibrillar and are not related to the long term hypertrophic response to training (Roberts et al., 2020, Mitchell et al., 2014). Furthermore, these varying observations illustrate the high degree of individual variability in response to long term RE. This variability has been credited to differences in gene expression (Roth et al., 2002), muscle protein synthesis signaling response (Damas et al., 2015), SC activation and content variation as well as undulations in hormonal response to exercise (Kraemer and Ratamess, 2005) Overall, we report that full body HS applied at 40°C (30%RH) does not improve upon the fat and bone free LMM in response to ten weeks of progressive RE. However, in the upper body, HS may have had in impact in expediting the hypertrophic response. Though this observation could also be due to the fact that the participants had comparatively faster hypertrophic response to RE compared to the CON group, rather than an intervention effect of HS. Lastly, the lack of significant improvements in overall LMM as well as appendicular LMM in the HEAT group appears to suggest a potential negative effect by HS on hypertrophy, that coincides with the myofibre CSA as well as myonuclear density observations.

In summary, within this investigation, we hypothesised that full body HS applied concurrently with progressive full body RE may improve upon the hypertrophy gains by RE chronically. Moreover, the combined stress may acutely improve upon the mTOR driven anabolic synthesis response to full body RE in the human skeletal muscle in a previously untrained cohort. We predicted that the concurrent stress of increased muscle temperature would combine with the mechanical stretch of full body RE to increase the magnitude of the MPS and overall hypertrophy response.

We observed the upregulation of the key kinases such as mTOR, AMPK, Akt as well as P70S6K acutely post heavy, full body RE in CON and HEAT groups in the *vastus lateralis*. However, full body HS applied at 40°C (30% RH) had no discernible impact on acute or chronic anabolic signaling responses to RE. However, it is noteworthy that the overall mTOR kinase response was more prominent in the CON group compared to HEAT, which might explain the reduced

hypertrophy response seen in the HEAT group fibre CSA. Muscle fibre CSA in Type I and Type II fibres improved significantly in the CON group post intervention. The HEAT group trended up in CSA however, did not reach statistical significance. While there was no group effect seen between fibre type CSAs in the CON and HEAT groups, the improvements in the CSA respective fibre types I and II were markedly lower in the HEAT group (13% & 5%) compared to CON (27% and 33%). This suggests that full body HS may attenuate myofibre hypertrophy when applied concurrently with heavy RE.

SC content improved significantly in both the CON and HEAT groups, however, myonuclear density only improved in the CON group. These observations align with the myofibre CSA differences observed between the two groups.

Total LMM improved significantly in the CON group post intervention, while reaching $p = 0.06$ ($p < 0.05$) in the HEAT group. In the upper body, the HEAT group showed an expedited improvement, where LMM improved at five weeks (Figure 5.8) over the CON group. However, CON improved significantly from mid to post intervention over HEAT group. Appendicular LMM only improved in the CON group. While an overall positive effect of HS cannot be discerned, it appears that full body HS applied concurrently with heavy full body RE maybe negatively affecting overall LMM hypertrophy.

As reported in chapter three of this work, during RE, peak CMT (36.79 ± 0.58°C) and core body temperature (38.18 ± 0.13°C) trended higher in HEAT group, compared to the CON (CMT =35.95 ± 0.53°C; and core = 37.97 ± 0.16°C) group. However, neither measurement were significantly higher in the HEAT ($p < 0.05$). However, we suggest that regardless of the lack temperature difference, the presence of chronic concurrent HS during heavy progressive RE may attenuate myofibre growth in both type I and type II possibly by attenuating myonuclear proliferation response to RE.

5.4.3 Conclusion and significance of findings

In conclusion, full body HS applied at 40°C (30% RH) does not appear to improve upon acute anabolic synthesis response to RE. Furthermore, there was no training effects on the acute anabolic synthesis response to RE. Phenotypically, the CON group displayed significant myofibre responses compared to the HEAT group, in Type I, Type IIa CSA as well myonuclear density. We suggest that to generate a prominent molecular and phenotypic response improvement in the skeletal muscle with HS, CMT may have to be raised above 39°C. Finally, concurrent HS applied at 40°C with RE may attenuate the myofibre growth response to RE.

Limitations

A limitation of this study was the biopsy time frame. We may have missed the peak mTOR pathway response at one hour post RE. It would have been beneficial to have had a biopsy at 3-4 hours post RE. Moreover, our findings do not elucidate on the transcription magnitude of the markers we investigated. Therefore, a panel of mRNA quantification at the same time points would have provided a further complete analysis of the acute time course response to RE combined with HS. Even though HS did not improve muscle temperature from the CON group, the overall presence of a secondary stress may have improved transcription, but not translation at the point of measurement. Additionally, a fibre specific immunohistochemical (type I and II) protein content analysis acutely in both groups would have provided more clarity on the potential attenuation of myofibre hypertrophy due to HS. Finally, there have been a limited amount of work investigating rapamycin-insensitive mTOR signaling (Ogasawara and Suginohara, 2018). However, its contribution to RE driven hypertrophy has not been investigated and is not discussed in this thesis. Finally, we did not investigate the markers of protein breakdown. Hypertrophy is a positive balance between protein synthesis and protein breakdown, therefore, protein breakdown is critical component of the muscle adaptations to RE and HS.

Future directions

We propose further investigation of the muscle temperature threshold in the skeletal muscle. At 40°C full body HS, we were unable to raise CMT significantly in the *vastus lateralis* However, a higher full body temperature intervention may potentially achieve a significantly higher muscle temperature. However, we propose incremental testing where two to three multiset primary movement exercises are tested to ensure the ecological validity and practicality of training in high temperature.

Furthermore, we propose the testing of chronic power and force focused full body RE intervention of the same length (ten weeks) at the same temperature conditions (40°C, 30%RH) instead of hypertrophy based training to test for the potential benefits in the skeletal muscle particularly in an untrained cohort. We showed in chapter three of this thesis that the RPE values were not significantly different in the HEAT group compared to CON. Therefore, the exertion effort is likely to not be affected by the additional stress.

Finally, we propose the investigation of the HS driven hypertrophy to further examine the mechanism of overall muscle hypertrophy against muscle fibre hypertrophy. We showed for the first time that when combined with chronic RE, HS may attenuate fibre hypertrophy. Previously a rodent study by Frier and Locke (Frier and Locke, 2007) reported HS may attenuate type I

hypertrophy acutely. But we show that chronically, HS impacted type I and II CSA in human skeletal muscle, therefore to investigate this further by examining protein breakdown markers would be an impactful area of research.

Chapter 6: The heat shock protein response to acute and chronic resistance exercise with concurrent application of full body heat stress

6.1 Introduction

Heat shock protein (HSP) response is a key compensatory and cyto-protective response that hold a multitude of stress management and mitigation roles within the cell. Predominantly, HSPs act as chaperones for other key proteins during periods of cellular stress caused by exercise, HS as well as oxidants, increases and decreases in pH, glycogen depletion, heavy metals, among others and are expressed in all cell types (Morimoto, 1993). Furthermore, they have been identified as a vital component in the MPS, aiding in protein assembly, folding and translocation (Morimoto, 1993, Morimoto, 1998, Becker and Craig, 1994, Kim et al., 2006). Therefore, HSPs may be important for skeletal muscle adaptations to exercise. The combination of HS with RE, two known instigators of HSP response, might elicit a further improved HSP response compared to RE alone. Thereby, improving upon the muscle adaptions to RE.

While the exact mechanism by which the cell senses stress in order to instigate the HSP response is yet unclear, a significant amount of literature has established the fact that translation of HSPs are under the regulatory and differential control of DNA binding heat shock transcription factors (HSF) (Lis and Wu, 1993). While HSF-1, 2, and 4 are found ubiquitously among vertebrates, HSF-1 has been identified as key in mammalian cells in the HSP gene activation and transcription cascade, playing a dual role as a regulator as well as a modulator of non-native proteins within the stressed cell (Ahn and Thiele, 2003).

The activation and deactivation of HSF-1 is understood to be achieved via hyper-phosphorylation upon cellular stress (Zuo et al., 1995, Xia and Voellmy, 1997). While many phosphorylation sites have been investigated as potential activation sites, it appears that phosphorylation of serine 326 is strongly correlated with HS driven transcriptional capabilities of HSF-1 (Guettouche et al., 2005). Furthermore, it has been identified that HSF-1 is negatively regulated via the phosphorylation of sites serine 303/307, whereby the transcriptional competency is attenuated (Knauf et al., 1996, Chu et al., 1998). In an *in vitro* study (HeLa cells), Kline and colleagues demonstrated that substitution of serine residues 303 and 307 via mutation increased the transcriptional activity of HSF-1(Kline and Morimoto, 1997).

Within skeletal muscle, a number of rodents studies have indicated that the absence of HSF-1 attenuates overloading associated hypertrophy, injured muscle regrowth and muscle recovery (Nishizawa et al., 2013, Koya et al., 2013, Yasuhara et al., 2011, Ohno et al., 2015), indicating a key role of HSF-1 in skeletal muscle growth and regeneration. It has been shown that after 15 minutes of isometric contractions, HSF-1 DNA binding activity increased acutely at 4, 12, and 24 hours post exercise in rodent skeletal muscle (Vasilaki et al., 2006, Vasilaki et al., 2003). This suggests that HSF-1 may be involved in skeletal muscle adaptation to RE, however no human studies have yet investigated the impact of RE on HSF-1 in human skeletal muscle.

In culmination, it is evident that HSF-1 expression and activation is responsive to HS as well as mechanical stress in the skeletal muscle. Moreover, in rodents it has been shown that HSF-1 may be a key regulating factor in muscle growth. Overall, HSF-1 is a critical regulator of the HSP response to skeletal muscle stress.

HSPs are classified in two major categories, small and large heat shock proteins. The most cyto-abundant large HSPs have been identified as HSP60, HSP72 (HSP70) and HSP90 (Becker and Craig, 1994, Morton et al., 2009). Among these, HSP72 is the most widely investigated. It has been reported that HSP72 binds unfolded proteins with a high affinity, especially in the endoplasmic reticulum, aiding in nascent protein translocation during cellular stress (Haas and Wabl, 1983). Moreover, it has been well established that intracellular HSP72 in the skeletal muscle upregulates to mitigate muscle damage and preserves muscle fibre size against atrophy (Senf et al., 2008).

Conversely *in vitro*, silencing of the HSP72 in rat L6 myoblasts lead to muscle fibre atrophy (Gwag et al., 2015). In rats, it has been shown that the absence of HSP72 severely impairs regeneration of the skeletal muscle (Senf et al., 2013). Moreover, over expression of HSP70 in the mouse skeletal muscle protects against muscle damage and dysfunction (McArdle et al., 2004a, Miyabara et al., 2006, Miyabara et al., 2012, Liu et al., 2013). Additionally, it has also been seen that activating HSP72 in the rodent skeletal muscle improved mitochondrial number and oxidative potential (Henstridge et al., 2014). In humans, it has been seen that protein expression of HSP72 increases acutely after RE and sustained chronically after RE in human skeletal muscle is well established (Liu et al., 1999, Liu et al., 2000, Tupling et al., 2007, Thompson et al., 2001, Fyfe et al., 2019, Gjøvaag et al., 2006, Paulsen et al., 2012).

HS induces HSP72 in the skeletal muscle. In humans, Ihsan and colleagues showed that a bout of full body HS upregulated the transcription of HSP72 in humans at a peak CMT of 38.1 ± 0.6 °C) (Ihsan et al., 2020). Furthermore, six days of repeated bouts of localised HS improved

HSP72 protein content in the *vastus lateralis* (Hafen et al., 2018). Similarly, ten day of localised HS, where muscle temperature was also increased by ~4°C, also improved HSP72 content in immobilised skeletal muscle (Hafen et al., 2019). Collectively, it appears that increases in HSP72 require a sustained HS stimulus as a single bout of HS did not improve the HSP72 protein levels (Hafen et al., 2018). Moreover, HSP72 upregulation after HS is strongly associated with HSF-1 activation (Locke and Tanguay, 1996). Therefore, it is clear that HSP72 is highly induced by RE and HS and plays an important role in muscle plasticity. However, whether the concurrent application of HS during RE amplifies the HSP72 response to RE in the skeletal muscle is yet to be investigated.

HSP60 is another key chaperone responding to skeletal muscle under stress (Bukau and Horwich, 1998). HSP60 is found predominantly in the mitochondrial reticulum (approximately a third have been reported to be found in extra-mitochondrial locales) (Marino Gammazza et al., 2018). HSP60 has been defined as an intra-mitochondrial protein and a critical component in cell viability, aiding protein translocation across cellular membranes in the skeletal muscle under stress (Marino Gammazza et al., 2018, Morton et al., 2009). Intriguingly, HSP60 has been linked to the induction of mitochondrial maker PGC-1α in cultured myoblasts (McArdle et al., 2004b). However, the number of studies that have investigated HSP60 activity in response to exercise in human skeletal muscle is very limited. After bout of non-damaging treadmill running, HSP60 levels did not increase at 24, 48 or 72 hours in the *vastus lateralis*. However, a peak significant increase in content was seen. They reported a peak muscle temperature of 40 ± 0.3°C immediately at the conclusion of the session (Morton et al., 2006a). Moreover, 72 hours after 45 minutes of ergometer cycling HSP60 protein content has been seen to increase significantly (Khassaf et al., 2001). Both studies inducing oxidative stress. However, the HSP60 response to RE is not yet known and the HSP60 response to HS in the skeletal muscle is extremely limited. In rodent skeletal muscle, HSP60 content improved significantly after raising muscle temperature to 42°C (Oishi et al., 2002). In the only human studies we are aware of, Morton and colleagues saw no elevations of HSP60 content after one hour of localised HS at 48 hours (Morton et al., 2006a). Furthermore, Hafen and colleagues saw no increase in HSP60 levels in the *vastus lateralis* after single or repeated bouts of HS after raising muscle temperature by ~4°C (Hafen et al., 2018).

It has been well established that HSP90 plays a chaperone role in binding and folding nascent proteins in to their native conformation particularly actin and tubulin in cells under stress (Becker and Craig, 1994). It has been also reported that inhibition of HSP90 destabilises the

actin cytoskeleton in cancer cells (Chaturvedi and Sreedhar, 2010, Becker and Craig, 1994). Moreover, HSP90 has been shown to bind and repress HSF-1 in the non-stressed cell (Zou et al., 1998). This above evidence indicates that HSP90 plays a key role in the stress response in the skeletal muscle. At present, here are no studies that have investigated the changes in HSP90 levels acutely or chronically in the human skeletal muscle in response to exercise. Kowalchuck reported that in rat skeletal myofibres no changes were seen in HSP90 protein levels 24 hours after high or low intensity treadmill running (Kowalchuk, 2013). . After a single bout of full body HS, HSP90 gene expression improved in the skeletal muscle in humans (Ihsan et al., 2020). After repeated bouts of HS, Hafen and colleagues saw HSP90 levels elevate in the *vastus lateralis* (Hafen et al., 2018). However, Kim and colleagues did not see changes after four or eight weeks of repeated HS (Kim et al., 2020). This limited and contradictory body of evidence does not provide a conclusive picture of HSP90 regulation by RE or HS in the skeletal muscle.

HSP27 and αB-crystalline have been found to be expressed at high levels in the skeletal muscle under stress (Morton et al., 2009). An important role has been assigned to HSP27 and αB-crystalline in short term cyto-protection from exercise induced skeletal muscle damage via microfilament stabilisation (Koh, 2002, Morton et al., 2009) which might improve the muscle tolerance to mechanical stress. A single bout of high intensity eccentric RE has been shown to increase the synthesis of HSP27. Moreover, HSP27 has been seen to translocate between cytosolic and cytoskeletal compartments following RE (Thompson et al., 2001, Thompson et al., 2002, Thompson et al., 2003, Paulsen et al., 2012, Gjøvaag et al., 2006, Folkesson et al., 2008). αB-crystalline has also been shown to upregulate post exercise (Folkesson et al., 2013, Folkesson et al., 2008). HSP27 and αB-crystalline have been reported to localise around the z-disks after mechanical stress and interact with actin and desmin, protecting the cytoskeleton and remodelling the contractile components during and after high-stress eccentric overloading (Koh, 2002, Mounier and Arrigo, 2002, Feasson et al., 2002). This evidence supports the role of HSP27 and αB-crystalline in mitigating acute muscle damage by RE. HS has been shown to be upregulate both HSP27 and αB-crystalline in the human skeletal muscle. While some studies have shown improvements in the HSP27 and αB-crystalline content after HS (Kim et al., 2020), some studies have not seen improvements with increasing muscle temperature (Morton et al., 2007, Hafen et al., 2018).

It has been posited that under stress, small HSPs such as HSP27 and αB-crystalline are phosphorylated and form oligomers, subsequently binding actin microfilaments and preventing the disruption of the cytoskeletal structure (Mounier and Arrigo, 2002). Furthermore, it has

been reported that mechanical loading may induce phosphorylation of small HSPs (Mounier and Arrigo, 2002). Phosphorylation is potentially the modification that aids HSP27 (Ser15) and αB- crystalline to interact directly with the actin cytoskeleton (Mounier and Arrigo, 2002). Critically, it has also been suggested that a small HSP response may be the earliest response to cellular stress, especially mechanical or heat (Mounier and Arrigo, 2002). Furthermore, there is some evidence to suggest that the small HSP phosphorylation response might be differentially modulated depending on the type of RE or the intensity (Morton et al., 2006a). Implying that the phosphorylated small HSP response may be correlated to the level of muscle damage. There is limited evidence on the effect of HS on the phosphorylation of HSP27 and αB- crystalline in the human skeletal muscle. It has been seen that a single bout of HS reduced the levels of phosphorylation in the *vastus lateralis*, but was unchanged from baseline after repeated bouts (Hafen et al., 2018). The indications of this observation are unclear and could be a response to acute altering of the redox balance to increasing muscle temperature (Montilla et al., 2014, Hafen et al., 2018). Thus, the adaptive phosphorylation of small HSPs in the skeletal muscle under stress remain largely unexplored.

The HSP response in the context of long term RE combined with concurrent full body HS has not been previously investigated in the human skeletal muscle. The available body of evidence on the HSP response to exercise suggests that they are acutely upregulated after exercise. The BHSP response is regulated by HSF-1. There is evidence to show that HSF-1 is upregulated by RE in the muscle. Under the direct regulatory control of HSF-1, HSP72 has been shown to be upregulated by RE. The evidence is limited and contradictory for HSP60 and HSP90 in the skeletal muscle and has not been investigated in humans with regards to heavy RE. Furthermore, the evidence is substantial for small HSP response to RE in the skeletal muscle. They contribute to stabilising the cytoskeleton and mitigate muscle damage during mechanical stress acutely post stress. This highlights the importance of the HSP response to RE and skeletal muscle plasticity. HS has also shown the ability to induce the activation of HSF-1 as well as upregulate large and small HSPs in the skeletal muscle

Overall, above evidence shows the importance of HSPs for skeletal muscle plasticity and that RE and HS have the ability to independently induce the HSP response. Therefore, we hypothesised that application of HS may augment the adaptive HSP response to RE in the skeletal muscle improving upon performance adaptations to RE by enhancing muscle plasticity.

Therefore, we investigated the acute and chronic impact of full body HS applied concurrently at 40°C on the HSP response to RE in the human skeletal muscle.

6.2 Methods

6.2.1 Biopsy sequence and western blots

Muscle biopsies were obtained at a depth of 3-3.5cm below the epidermis from the *vastus lateralis* pre-intervention at rest (Pre 0), one hour (Pre 1) and 48 hours (Pre 48) post a RE session that was identical to the first training session of the intervention, 72-96h prior to the commencement of the intervention. Three further biopsies were extracted post-intervention at rest (Post 0), one hour (Post 1) and 48 hours (Post 48) after an identical RE session to the first session of the intervention, which was performed ~72-96h after the last training session of the intervention. Muscle samples were analysed for expression levels of HSF-1, phospho-HSF-1 (Ser326), phospho-HSF-1 (Ser303/307), αB-crystalline, phospho-αB-crystalline (Ser59), HSP27, phospho-HSP27 (Ser15), HSP60, HSP72 and HSP90 via western blots at all six time points. Full methods are as reported in Chapter Four.

6.3 Results

In reporting results, all significant time and group effects are reported. Where time effects are present, the difference between the times are reported for CON and HEAT groups separately.

6.3.1 Heat shock factor-1

No significant time, group or time x group interactions were observed in the expression levels of HSF-1 or phospho HSF-1 (Ser326) (Figure 6.1). A significant time effect was observed for phospho-HSF-1 (Ser303/307) ($p = 0.007$). In the CON group, phospho-HSF-1 (Ser303/307) levels increased at Post 48 from Post 0 by 303% ($p = 0.015$) but the same was not observed in the HEAT group (Figure 6.2).

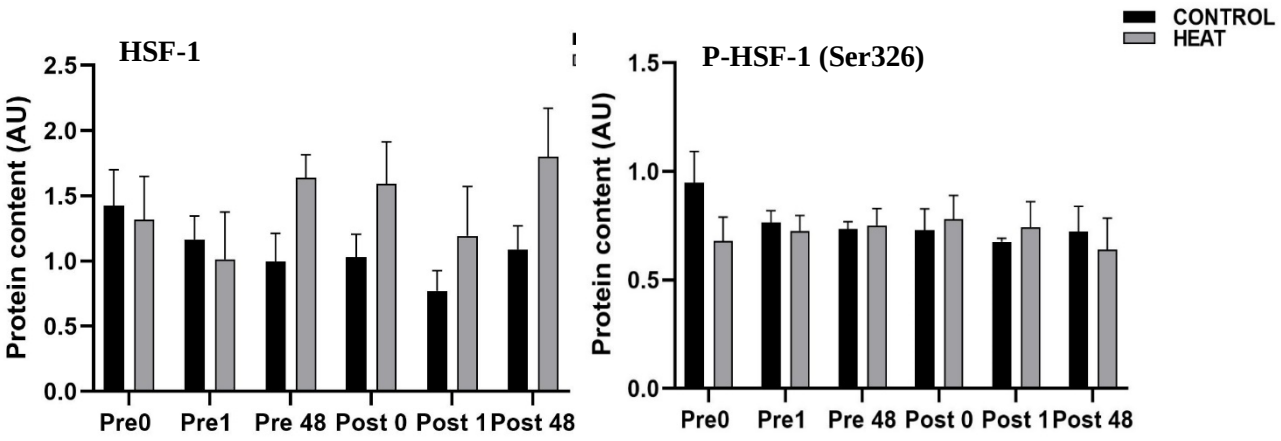


Figure 6.1. Expression levels of total HSF-1 and phospho- HSF -1(Ser326) pre and post ten week intervention

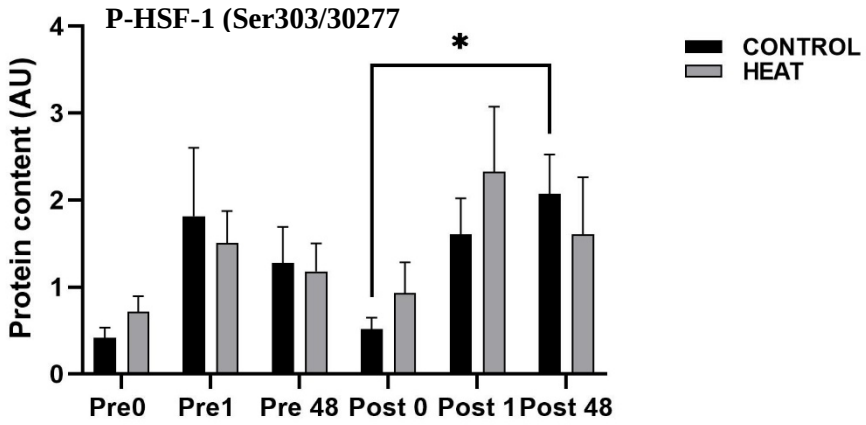


*Figure 6.2. Expression levels of total HSF-1(Ser303/307) pre and post ten week intervention * p < 0.05*

6.3.2 Small heat shock proteins

6.3.2.1 HSP 27

No significant time, group or time x group interactions were observed in the expression levels of total HSP27 (Figure 6.3). While no group or time x group effects were observed in phospho-HSP27 (Ser15) levels, significant time effects ($p < 0.0001$) were observed pre and post intervention (Figure 6.4). In the CON group phospho-HSP27 increased by 505% Pre 0 to Pre 1 ($p = 0.0007$) and by 508% Post 0 to Post 1 ($p < 0.0001$). In the HEAT group by 381% Pre 0 to Pre 1 and by 376% Post 0 to Post 1 ($p = 0.016$) (Figure 6.4).

6.3.2.2 αB-crystalline

No significant time, group or time x group interactions were observed in the expression levels of total αB-crystalline. While no significant group or time x group interactions were observed in phospho-αB-crystalline (Ser59) levels, significant time effects ($p = 0.004$) were observed

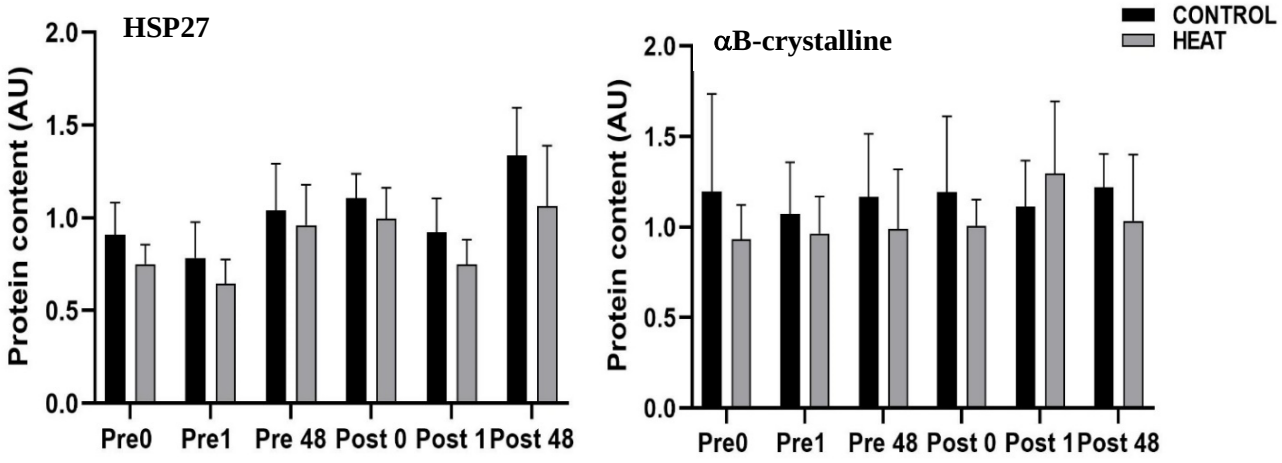


Figure 6.3. Expression levels of total HSP 27 and αB-Crystalline pre and post ten week intervention

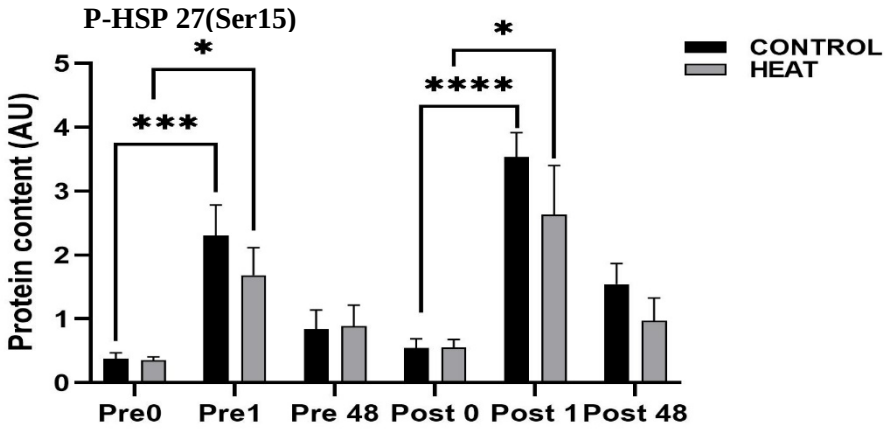


*Figure 6.4. Expression levels of total phospho-HSP 27(Ser15) pre and ten week post intervention. * p <0.05, *** p < 0.005, **** P < 0.0001*

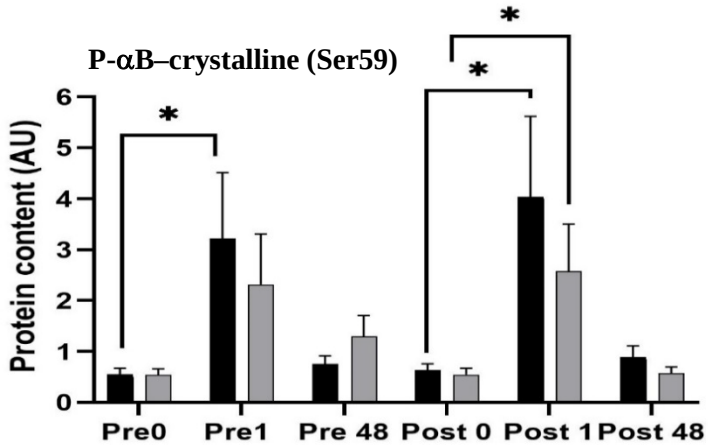


*Figure 6.5. Expression levels of total phospho-αB–crystalline (Ser59) pre and post intervention. * p<0.05*

6.3.3 Large heat shock proteins

6.3.3.1 HSP60

No significant time, group or time x group interactions were observed in the total expression levels of HSP60 (Figure 6.6).

6.3.3.2 HSP72

No significant time, group or time x group interactions were observed in the total expression levels of HSP72 (Figure 6.6).

6.3.3.3 HSP90

No significant time or time x group interactions were observed in the total expression levels of HSP90. A significant group effect ($p < 0.0001$) was observed pre intervention between CON and HEAT groups for HSP90 (control; 0.405 ± 0.111 AU, heat; 1.966 ± 0.671 AU), however, this effect was not present post intervention (Figure 6.6).

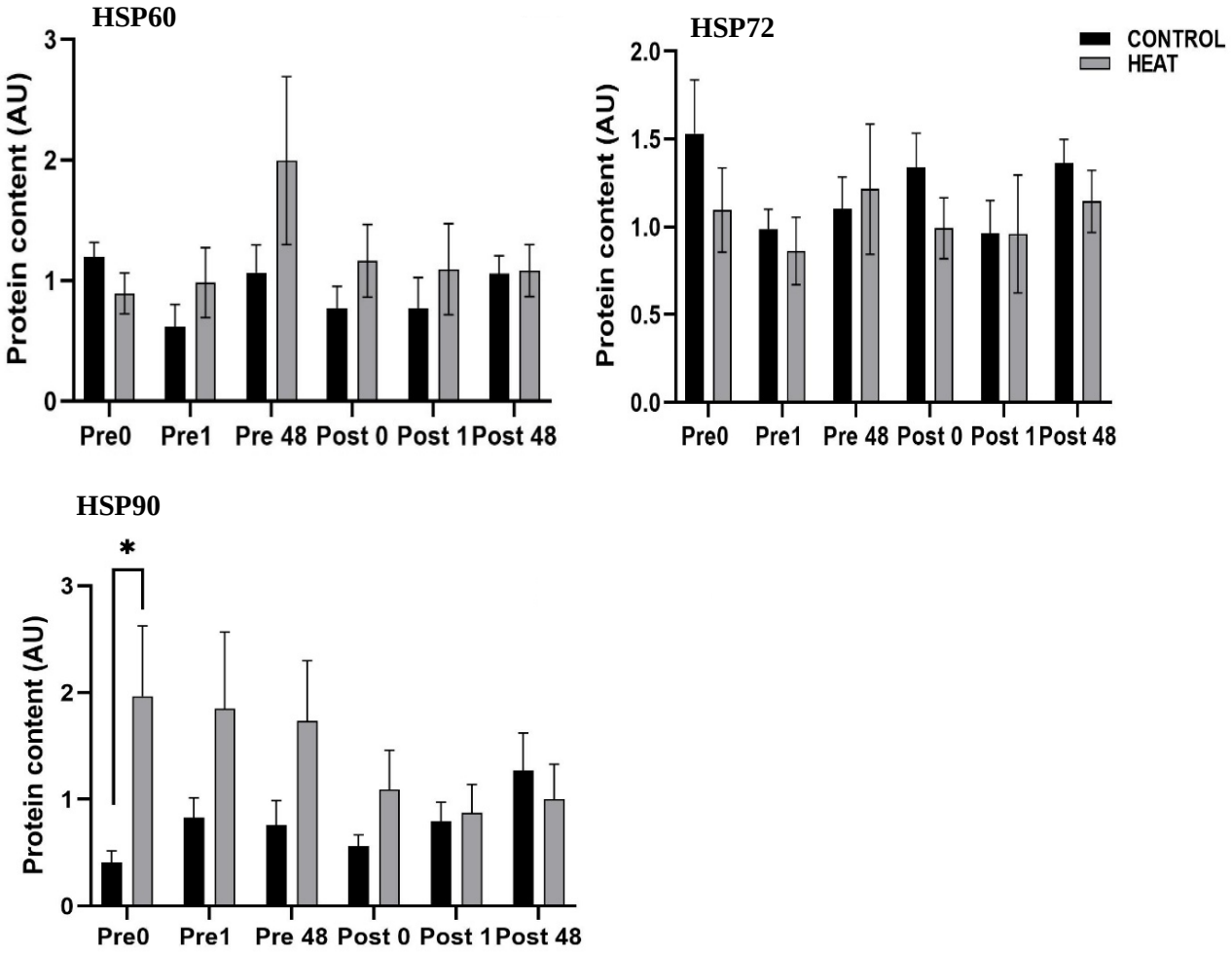


Figure 6.6. Expression levels of total HSP60, HSP72 and HSP90 pre and post ten week intervention. * p <0.05

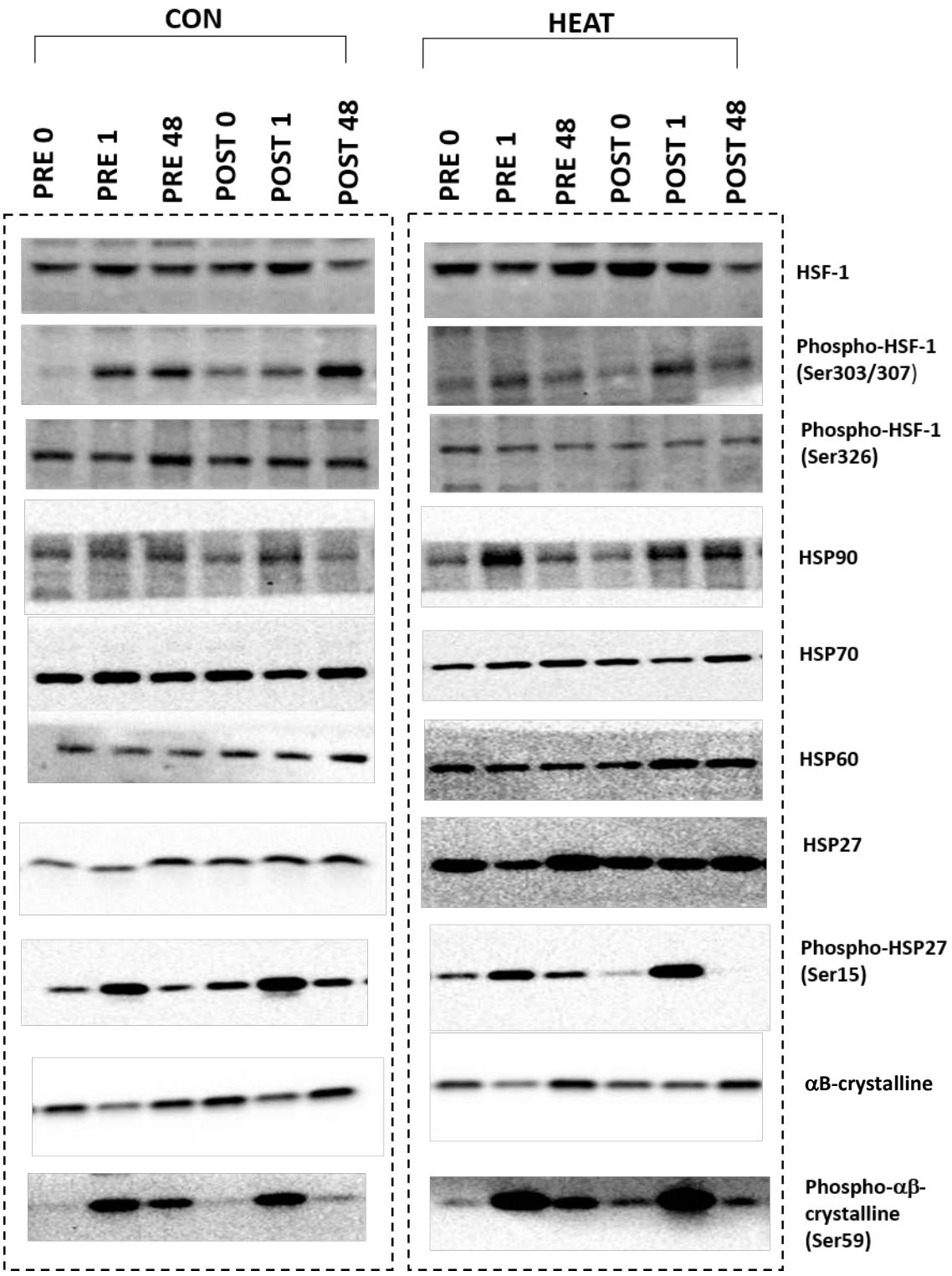


Figure 6.7. Representative western blots for all HSPs quantified. PRE; Pre intervention. Post; Post ten week RE or RE and HS intervention. Biopsies extracted acutely before, one and 48 hours after a single RE session pre and post the intervention

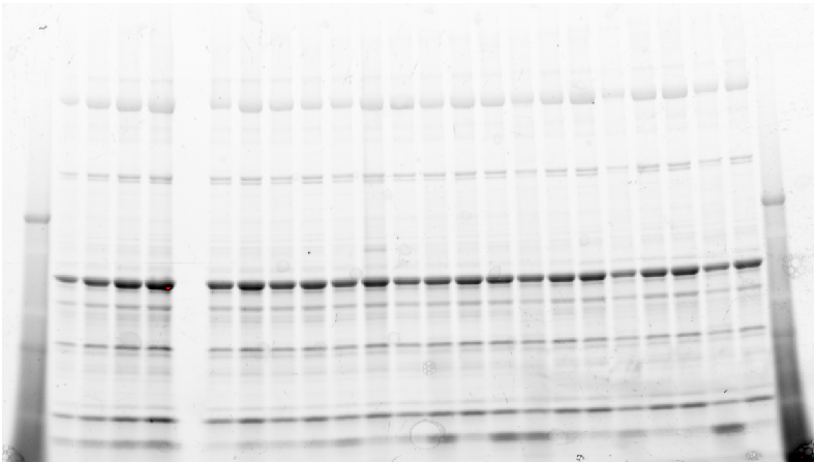


Figure 6.8. Representative stain free image

6.4 Discussion

The aim of this investigation was to examine the effect of HS on the HSP response to acute and chronic RE. As reported above, several time effects were observed with the phosphorylated levels of HSF- 1 (Ser303/307), αB-crystalline (Ser59) and HSP27 (Ser15). There were no interactions observed between the CON and HEAT group pre or post intervention, with the exception of a single interaction pre intervention in the levels of HSP90. Overall, none of the large HSP expression levels were altered as a result of acute or chronic RE. Furthermore, full body HS had no effect on the HSP response to RE, acutely or chronically. A strong phosphorylation response to RE was observed acutely one hour post, however, there was no training effect observed.

6.4.1 Heat shock factor-1

In this investigation, we report that exercising under full body HS did not impact the expression levels of HSF-1 in response to acute or chronic RE. Moreover, it does not affect the activation levels of HSF-1, as evidenced by the lack of improvements on the phospho-HSF-1 (Ser326/327) levels at the same time points. We saw difference in the HSF-1 levels in response to RE or HS acutely at one and 48 hours post stress *in the vastus lateralis*. Furthermore, the responses were not different after a ten week RE intervention. *In vivo*, Tanabe and colleagues saw that heat shocked chicken embryos (40°C) demonstrated elevated HSF-1 levels. Interestingly, they noted that HSF-1 levels did not increase any further between 40°C and 44°C (Tanabe et al., 1997). Locke and colleagues full body heat shocked rats for 15 minutes, maintaining core temperature above 42°C and examined the skeletal muscle 24 hours after stress. They saw that HSF-1 activation occurred only above a core temperature of 41°C in the rat skeletal muscle (Locke and Tanguay, 1996). Locke further saw that rats heat shocked for ten minutes to 40°C, 41°C and 42°C degrees showed activation of HSF-1 and hour following

the shock (Locke, 2000). However, they noted that these observations may not apply to a live animal (Locke and Tanguay, 1996). Additionally, they measured HSF-1 activation via heat shock element (HSE) bound HSF-1 and not via phosphorylation. There exists no evidence in skeletal muscle to discern whether HSE binding occurs after phosphorylation, as HSF-1 undergoes a large number of post translational modifications and exact mechanisms are yet to be fully understood (Anckar and Sistonen, 2011). It is worth noting that a potential reason for the lack of improvements seen in our study could be due to the fact that a different modes of quantification phosphorylation. It has been previously reported that HSF-1 is kept inactive by chaperoning HSPs (predominantly HSP90), when the cell is not under stress (Anckar and Sistonen, 2011) and activated upon stress. HSF-1 undergoes trimerisation once activated in order to achieve DNA binding capabilities. The activation or inhibition of HSF-1 trimerisation occurs via hyper-phosphorylation and is triggered by increasing concentrations of misfolded proteins in the cell under duress (Zou et al., 1998, Morimoto, 2002). Therefore, phosphorylation is a valid indicator of HSF-1 activation and inactivation. While the evidence is limited, based on our results, in the human skeletal muscle, HSF-1 is not activated at CMT ~38°C. It is possible that, at least in the skeletal muscle, the heat load is not sufficient to activate HSF-1 to induce translation of new HSPs. Locke's observations of HSF-1 activation only above 40°C in the rat skeletal muscle support this notion (Locke and Tanguay, 1996, Locke, 2000). This is further supported by the fact that we saw no upregulation in any of the HSPs that are under direct translational control of HSF-1 (discussed below). Moreover, we report for the first time that there is no cumulative effect on the activation threshold after ten weeks of three full body heat sessions a week. This indicates that the HSF-1 response is acute and does not display any chronic adaptive responses to prolonged HS.

To our knowledge, this is the first investigation of the HSF-1 response to RE or RE combined with HS in human skeletal muscle. We found that total expression levels of HSF-1 did not change in response to RE or HS pre or post intervention. Furthermore, the activated HSF-1 response, quantified phosphorylation at Ser326 was similar and, the deactivated HSF-1 (Ser303/307) levels showed a clear upward trend acutely after RE. There exists a very limited body of literature on exercise and its potential to induce or activate HSF-1. Several rat studies have showed that HSF-1 content upregulates in the cardiac muscle after chronic exercise (wheel running), aiding in adaptive cardiac hypertrophy to exercise (Sakamoto et al., 2006, Tian et al., 2020). In rat skeletal muscle, Locke showed that, after surgical overloading, HSE bound HSF-1 levels increased at days one, two and three (correspondingly HSP27 and 70 levels also increased) (Locke, 2008).

As mentioned above, there have been multiple phosphorylation sites identified on HSF-1. Among those, phospho-HSF-1 (Ser303/307) has been identified as prominent sites for inhibition (Anckar and Sistonen, 2011). In the current investigation, the acute stress of heavy RE caused an upregulation of phospho-HSF-1 (Ser303/307) 48 hours post-RE after the ten-week intervention in the CON group (Figure 6.2). Moreover, a trend of increasing phospho-HSF-1 (Ser303/307) levels were seen acutely one hour post exercise in both groups and was sustained at 48 hours (Figure 6.2),indicating an elevation of the inhibited HSF-1 levels, despite being subjected to mechanical stress. To our knowledge, there are no previous human studies which have reported the acute or chronic HSF-1 deactivation response to exercise (RE or endurance). However, we speculate that, acutely post heavy RE, the stressed skeletal muscle cell could be temporarily suppressing HSF-1. Given the heavy stress load, it is possible that the HSF-1 activation response may have occurred immediately post the exercise bout to initiate the cyto-protective HSP response in response to RE. Furthermore, at one hour post, the cell is actively suppressing the protective HSF-1 activity to allow anabolic hypertrophic response to commence which aligns with the observations discussed in chapter five. However, no other study has investigated this potentially sequential response. There is likely an overlap between the two responses where the HSP response subsides as anabolic synthesis increases. Phospho HSF-1 (Ser303/307) levels in the CON group increased significantly 48 hours post an RE session after the ten-week intervention (Figure 6.2), however the same was not observed in the HEAT group or in the CON group pre intervention. However, it is important note that there is a trend of increased levels of HSF-1 (Ser303/307) at one hour post RE in both groups pre and post intervention. This observation supports the notion that HSF-1 maybe inhibited at one hour post RE to potentially allow anabolic synthesis to begin. Overall, there appears to be no training effect on the acute HSF-1 response after ten weeks of full body RE. However, HSF-1 activity may be inhibited via phosphorylation of Ser303/307. HS applied at 40°C, which in our investigation raised CMT to ~38°C did not have an impact on HSF-1 response to RE. Thus, there appears to be a threshold temperature for HS to start impacting on the cells as a stressor in the skeletal muscle to induce the transcription of large and small HSPs.

6.4.2 HSP90

As mentioned above, in non-stressed cells, HSP90 forms a complex with HSF-1, keeping the modulator inactive, and the interaction undergoes rapid dissociation when the cell is under stress (Zou et al., 1998). In this investigation HSP90 levels were significantly higher in the

HEAT group compared to CON group at baseline pre intervention. However, no differences were observed at any other time points or between groups. The significant difference between the groups at baseline is likely anomalous given that the HEAT biopsies were extracted under identical conditions to CON biopsies. The participants in both groups were anthropometrically matched and were identically nutrient controlled overnight and had refrained from exercise prior to the biopsy and were fasted at the point of muscle extraction. Furthermore, pre intervention biopsies were extracted at 23°C in both groups. Moreover, in the non-stressed cell the basal HSP90 plays a "blocker" role and not auto induced (Zou et al., 1998). There was more individual variability observed in the HEAT group where two participants displayed markedly higher values compared to the rest pre intervention a pattern not seen post intervention. The lack of differences in HSF-1 and HSF (Ser326) at any of time points are consistent with the lack of HSF-1 activation. In contrast to the lack of effect of HS in the present study, Hafen and colleagues observed a 38 ± 13% increase of HSP90 levels (quantified via median fluorescence intensity) from baseline after six days of repeated localised HS where CMT was raised by 3.9 ± 0.4°C to a peak muscle temperature of ~40°C (Hafen et al., 2018) compared to our study's CMT of ~38°C. In addition, Hafen and colleagues also reported that HSP90 levels increased significantly after ten days of repeated HS at a comparable CMT to their previous study (Hafen et al., 2019). Similar to the observations seen with regards to HSF-1, it appears that the difference observed in the final observations are predicated upon the peak muscle temperature. In support of this, Ishan and colleagues saw an upregulation of the HSP90 mRNA levels by 64% 30 minutes after one hour of full body HS where CMT peaked at 38.8 ± 0.5°C. However, they did not observe the same results when CMT was raised to 38.1 ± 0.1°C via localised HS (Ihsan et al., 2020). These observations indicate that to induce a pronounced HSP90 response in the skeletal muscle with the application of HS, the temperature may require to be raised above 38.5-39°C. Furthermore, our findings indicate that there appears to be no chronic, cumulative HSP90 response to HS at muscle temperatures below 38.5-39°C.

In the current investigation, RE did not have an acute or chronic impact on the expression levels of HSP90. To our knowledge there are no previous studies that have investigated the HSP90 response to RE, or any other form of exercise, in human skeletal muscle. In their *in vitro* study, subjecting cultured rat myoblasts to HS (41°C), mechanical stretch and a combination of both, Goto and colleagues reported that HSP90 levels reduced in the supernatant (cytoplasmic) in all three conditions compared to the non-stressed cells. However, HSP90 levels increased significantly in the pellet (structural) by 58%, 95% and 135% respectively for the three conditions (Goto et al., 2003). These results indicate a possible translocation event where

HSP90 levels increase in response to mechanical stress accumulating alongside structural proteins in a possible chaperoning role. Moreover, the HSP90 response in the pellet was more sensitive to stretch compared to HS, indicating a more prominent response to mechanical stress. However, the combined influence of mechanical stretch and HS produced the greatest HSP90 response (Goto et al., 2003). Our results indicate that full body RE and full body RE combined with HS does not influence the acute or chronic HSP90 response in human skeletal muscle one hour or 48 hours post stress. Concurrent HS applied at 40°C does not improve upon the lack of HSP90 response to RE in the human skeletal muscle where CMT is maintained below 39°C. However, it is possible that HSP90 levels did increase in response to RE, given the muscle damage caused, but was immediately upregulated post RE and was not registered due to the timing of the biopsies in our investigation.

6.4.3 HSP72

Our results show that full body HS does not trigger a HSP72 response in the *vastus lateralis* acutely post HS. Moreover, we saw no effect of ten weeks of chronic HS on the acute HSP response to RE either. Conversely Goto and colleagues saw a 251% increase in the HSP72 levels in the pellet (structural proteins) of rat myotubes subjected to one hour of 41°C HS, but saw no improvements in the supernatant (cytoplasmic) (Goto et al., 2003). Which similar to HSP90, indicates a strong translocation response to HS, where HSP72 accumulates around structural proteins, potentially playing a protective role against HS. After a single bout of hot water immersion at 45°C, increasing CMT to 39.5 ± 0.2 °C, no HSP72 levels did not improve at HSP72 levels 48 hours (or seven days) after stress (Morton et al., 2007). Hafen and colleagues, saw no induction of HSP72 immediately after a single bout of HS but observed a 45 ± 18.8% increase in HSP 72 levels in the *vastus lateralis* after six days of repeated localised HS where CMT was raised by 3.9 ± 0.3°C to a peak CMT of ~40°C (Hafen et al., 2018). Conversely, Kim and colleagues did not observe any elevations of the HSP72 levels after eight weeks of localised HS after only raising CMT to ~38°C (Kim et al., 2020). Ihsan and colleagues saw a 362% increase in mRNA levels after whole body HS where CMT was raised to 38.8 ± 0.5°C, but not after localised HS to 38.1 ± 0.1°C (Ihsan et al., 2020). Similarly, no elevation in the transcription levels of HSP72 was observed 30 minutes after acute HS (Kuhlenhoelter et al., 2016). Similar to HSP90, HSP72 response to HS seems to be predicated upon the muscle temperature increasing above 38.5-39°C. The available data as well as our results support the notion that, in humans, the HSP72 response is predicated upon the CMT and potentially needs to surpass 38.5-39°C in order to induce acute translation for structural muscle protection as well as increased chaperone activity

We observed no changes in HSP72 levels between groups or over time acutely post a heavy RE session or post a ten week RE intervention. It has been previously shown that, HSP72 levels increased 203 ± 37% 24 hours after a high intensity eccentric bout in the *vastus lateralis* (Paulsen et al., 2007). Moreover, 48 hours after a single bout of eccentric bicep curls, Thompson and colleagues observed a HSP70/HSE increase of 1034% in the *biceps brachii* (Thompson et al., 2001). In the *vastus lateralis,* Liu and colleagues saw 181%, 405%, 456% and 363% increases of HSP72 respectively at the end of each week of a four week high intensity rowing intervention with highly trained rowers. They reported that the HSP response correlated with training intensity (Liu et al., 1999). They reinforced the intensity dependent nature of the HSP72 response to exercise in the skeletal muscle in a follow up study in a similar highly trained cohort of rowers (Liu et al., 2000). After five to eight weeks of localised RE, a significant increase in the HSP72 levels have been reported in untrained cohorts and interestingly, still elevated levels compared to baseline after eight weeks of detraining in the *triceps brachii* (Gjøvaag and Dahl, 2006). After 2 and 11 weeks of RE three days a week, cytosolic HSP70 levels increased by a combined 146 ± 51% with no difference between 2nd and 11th weeks in the *vastus lateralis* independent of the training volume (Paulsen et al., 2012). Conversely, Fyfe and colleagues saw after seven weeks of full body RE, there was no training effect on the HSP72 levels from pre intervention, nor did it improve on the acute response to RE at one or 48 hours post-exercise (Fyfe et al., 2019). In accumulation, our results agree with Fyfe's observations, where after ten weeks of progressive training, we saw no improvements in the acute HSP72 response to RE at one or 48 hours. The reason for the discrepant findings may be because it appears that acute HSP72 response to exercise is very much intensity dependent. As shown above by Liu as well as Gjøvaag colleagues, the HSP72 response in the skeletal muscle to RE is seen in a does response manner with increasing intensity (Liu et al., 2000, Liu et al., 2004, Gjøvaag and Dahl, 2006). It appears from above evidence that, RE that is more power or force driven as opposed to hypertrophy/strength driven induces a marked HSP72 response in comparison to interventions such as ours or one that was utilized by Fyfe and colleagues, where the muscle damage appears to higher, which inturn justifies the upregulation of HSP72. Another interesting observation is that us as well as Fyfe tested cohorts that were untrained, compared to the highly trained cohorts tested by Liu as well as Gjøvaag. Therefore, it appears that the HSP72 response in the skeletal muscle is not training state dependent. It would have been justified to expect higher levels of muscle damage in the untrained cohorts, thereby triggering a prominent response, even at the relatively lower intensities.

The above evidence indicates to the clear possibility of a stress threshold for total HSP72 protein content elevation in the skeletal muscle. It is to be noted that, as a prominent molecular chaperone to newly synthesised proteins and denatured proteins, the cell appears to maintain a relatively high basal level of HSP70 while unstressed, especially in the skeletal muscle (Liu et al., 2004) which maybe another contributing reason to why hypertrophy driven mechanical stress did not raise a HSP72 response acutely post RE. *In vitro,* Goto and colleagues saw a significant increase in the HSP72 the supernatant (92%) and in the pellet of rat myoblasts subjected to mechanical stretch after one hour of HS 41°C. Interestingly, HSP72 increased in the supernatant with the combined stress. But was lower in magnitude to that of induced by heat alone in the pellet (Goto et al., 2003). Locke and colleagues showed that in rats subjected to full body HS for 15 minutes with core temperature at 42°C prior to overloading via synergistic ablation, HSP72 levels improved more over control (non HS and non-ablation) at days 1-3, 5 and 7 post intervention (Frier and Locke, 2007). The lack of improvement in HSP72 levels seen in our investigation with the combined stress of RE and full body HS applied concurrently suggests that HS does not induce a HSP72 response from the skeletal muscle cell below a certain threshold. Even in combination with mechanical stretch, the cell is not put under enough stress to warrant translation of novel HSP72. A less likely reason is that the response occurs immediately post and is subsiding to at one hour where anabolic synthesis begins.

6.4.4 HSP60

To our knowledge, our investigation is the first to explore the acute total expression level response of HSP60 pre and post chronic RE or RE combined with concurrent HS in the skeletal muscle. We observed no effect of heat between the groups. Moreover acute or chronic RE or HS did not influence the HSP60 levels in *vastus lateralis.* The lack of effect of HS in our study is consistent with the findings of Morton and colleagues who saw no improvements in HSP60 levels after a single bout of hot water immersion, raising CMT to 39.2 ± 0.2°C. They reported that expression levels were not different from baseline at 48 hours or seven days. Similarly, Hafen and colleagues, raised CMT by 3.9 ± 0.39°C (~40°C) from baseline with a single bout of HS in the *vastus lateralis* and saw no changes in the HSP60 levels (Hafen et al., 2018). Moreover, six days of repeated consecutive heat exposure bouts did not alter HSP60 levels compared to baseline (Hafen et al., 2018). These are the only investigations apart from ours that have quantified the impact of HS on HSP60. Our results are comparable acutely one hour and 48 hours post HS in the lack of improvement in the HSP60 levels after single and repeated bouts of HS. Key to note the difference in peak CMT achieved was ~2°C between ours (~37°C), Mortons's (~39°C) and Hafen's (~40°C). Which implies that acute HSP60 response in the

skeletal muscle maybe independent of muscle temperature. Combined, these results imply that, that elevated muscle temperature, even at 40°C, is not adequate to trigger a HSP60 response. Interestingly, these results further suggest that even though HSP60 has been identified as a key chaperone that prevents the denaturation of pre-existing proteins and mediates the ATP refolding under high stress in the mitochondria (Martin et al., 1992), it is not elevated by the same stress levels as HSP72 or HSP90.

In high intensity endurance exercise inducing oxidative stress, Morton and colleagues saw a 139 ± 23% peak increase in the HSP60 levels from the pre-exercise values. (Morton et al., 2008, Morton et al., 2006a). The same authors have further reported that higher level of basal HSP60 is observed (*vastus lateralis*) in endurance athletes compared to a sedentary cohort (Morton et al., 2006b). While limited, these observations seem to suggest that HSP60 is more responsive to endurance exercise with high energy turnover, compared to the hypertrophy driven slow contractions of our RE. This may reflect its role as a mitochondrial chaperone given the consistent mitochondrial stress in endurance athletes. In the current study, mitochondrial stress was likely limited as seen with our mitochondrial biogenesis data (reported in Chapter Seven) where none of the markers improved chronically post RE. Thus, our observations indicate that HSP60 may not be triggered by RE which exerts less oxidative stress, regardless of the training volume and is more intensity dependent and responds to oxidative stress rather than increasing mechanical stress via overloading.

6.4.5 HSP27

In this investigation, total HSP27 levels were not different between the groups showing no impact of HS. After one hour of hot water immersion at 45°C raising CMT to a peak of 39.5 ± 0.2 °C, HSP27 levels did not improve from the contralateral non-heated control leg at 2 and 7 days after intervention (Morton et al., 2007). Similarly, Hafen and colleagues saw no improvements in the total HSP27 levels after single or repeated bouts of HS in the *vastus lateralis* after reaching a peak CMT by 3.9 ± 0.39°C (~40°C) (Hafen et al., 2018). Conversely, after four and eight weeks of chronic HS, Kim and colleagues showed significant improvements in HSPB1 (HSP27), however, CMT was not reported but suggested a potential peak CMT of ~38°C. Our results agree with the acute results of Hafen but contradict with the chronic observations of Kim. It is key to identify at this juncture that the HSP27 protein response is the inverse of that was observed for HSP72 in the same studies. This points to a potential stepwise HSP stress response where small HSPs are triggered at lower muscle temperatures (~38°C) (Kim et al., 2020) and large HSPs such as HSP72 and HSP90 are triggered at higher muscle

temperatures (>39°C) (Hafen et al., 2018) separated by a potential threshold muscle temperature of 38.5-39°C. However, we only investigated the two most prominent small HSPs among many and other less investigated small HSPs may respond differently.

In addition, we saw no effect of RE, acutely after one or 48 hours or chronically over ten weeks on the HSP27 response. HSP27 has been previously shown to upregulate acutely after RE. However, our acute results contradict with that of Thompson and colleagues where they reported 234% increase in the HSP27 levels 48 hours post an acute RE intervention in the *biceps brachii*. However, Thompson and colleagues prescribed localised RE in the non-dominant arm which may explain the different HSP response (Thompson et al., 2001). In our investigation, the limb selection was randomised. It has been shown that the degree of muscle damage or stress is greater in the non-dominant limb compared to the dominant limb after RE (Svensson et al., 2018). Therefore, generating a more prominent HSP27 response compared to a dominant limb or limb randomised examination. They further reported that after a high force bout of eccentric exercise, the HSP27 response increased significantly but the response was less prominent when the identical bout was repeated four weeks later (Thompson et al., 2002) implying a repeated bout effect. Paulsen and colleagues saw that cytosolic HSP27 levels decreased by 51 ± 13% after high intensity eccentric bout at 96 hours in the *vastus lateralis* (Paulsen et al., 2007). After an acute bout of RE, Folkesson and colleagues saw a 6.3% (0%-32%) increase in granular accumulation levels of HSP27 in type II (fast) fibres (Folkesson et al., 2008). They further reported that HSP27 accumulation was more prominent in type II fibres of long term RE practicing individuals compared to endurance exercise (Folkesson et al., 2013). After five-eight weeks of training improved HSP27 levels by 71% regardless of the training intensity. However, they noted that high intensity exercise induced much greater HSP27 reactions compared to low intensity (Gjøvaag and Dahl, 2006). In culmination, the above evidence suggests that in response to RE, HSP27 first undergoes translocation concentrating at the structural proteins (i.e., cytoskeleton). Upon further stress is synthesised anew. Moreover, HSP27 appears to accumulate in slow type fibres in response to chronic RE, potentially playing an adaptive, protective role.

The total stress load is spread across the whole body would explain the less pronounced response compared to localised, focused heat application to a selected muscle. In general contrast to the available body of work in HSP27 response to RE, we did not observe an acute improvement of the total HSP response to RE. High intensity eccentric RE applies more damage on the skeletal muscle compared to hypertrophy driven slow contraction rate (Kraemer et al., 2002) modalities of RE such as ours. While Folkesson reported that HSP27 was perhaps

intensity independent statistically, the most extreme values were seen in the highest intensity group. The lack of HSP27 response observed after ten weeks of RE is justified as Thompson and colleagues demonstrated a clear repeated bout effect after just four weeks where HSP27 response was distinctly depleted to an identical bout of RE. It is further to be noted that we did not fractionate our samples. It has been shown that HSP27 shows a tendency towards translocation after RE (Vissing et al., 2009) and a distant possibility for our lack of observations is the non-separation of cytosolic and cytoskeletal fractions.

6.4.6 αB-crystalline

Similar to HSP27, there was no impact of HS observed acutely or chronically on the total αB-crystalline levels. Morton and colleagues saw that after one hour of hot water immersion at 45°C (raising CMT by 3.6 ± 0.5°C) achieving a peak CMT of 39.5 ± 0.2°C, αB-crystalline levels did not improve from the contralateral non-heated control leg at 2 and 7 days after intervention similar to HSP27 (Morton et al., 2007). However, after four and eight weeks of chronic HS, Kim and colleagues showed significant improvements in the αB-crystalline levels, further displaying a corresponding response to HSP27 (Kim et al., 2020). To our knowledge, these are the only two studies that tested the impact of HS as a sole stressor in the skeletal muscle. It appears that the αB-crystalline response closely mimics HSP27 in responding to acute and chronic HS (Paulsen et al., 2007). Further supporting the notion that small HSP response is triggered at lower CMT as the first line of defense against stress. We propose that the HS level in the present study was not large enough to trigger the translation of αB-crystalline acutely at one hour or at 48 hours. We further argue based on our chronic results that the chronic response observed by Kim and colleagues were more predicated upon the CMT of ~38°C that they achieved rather than a cumulative effect of the repeated HS given they saw no difference between week four and week eight (Kim et al., 2020) given the fact after ten weeks of repeated bout HS, we saw no improvements in the levels of αB-crystalline at a CMT of ~36°C.

We saw no impact of RE acutely or chronically on the total expression levels of αB-crystalline. The number of studies that have investigated the impact of RE on αB-crystalline is limited. Folkesson and colleagues saw higher density staining in the type I fibres of individuals with a history of RE, however, Folkesson's study did not include an actual RE intervention therefore non-conclusive in terms of the RE response (Folkesson et al., 2013). However, Morton and colleagues saw no improvement in the αB-crystalline levels at 24, 48 ,72 hours or seven days post a non-damaging treadmill run (Morton et al., 2006a). It has also been seen that after 11

weeks of high force RE, αB-crystalline levels increased in the *vastus lateralis* but the same was not seen after two weeks of training (Paulsen et al., 2012). Conversely, Fyfe and colleagues saw no improvements in the levels of αB-crystalline after seven weeks of heavy RE intervention similar to the intervention in this study (Fyfe et al., 2019). Similar to HSP27, novel αB-crystalline induction appears to be exercise intensity dependent and responsive to the rate of muscle damage caused by high force exercise. Our results support this notion. Furthermore, we did not see a training effect on the acute αB-crystalline to RE as pre intervention levels were not different from the post intervention levels at the same time point, further strengthening the fact that the αB-crystalline induction is only triggered acutely.

We also report for the first time that concurrent full body HS applied at 40°C with RE does not alter the total expression levels of αB-crystalline. As discussed above HS and RE are capable of elevating the small HSP response acutely and chronically. However, an improved response to HS seems to be predicated upon the muscle temperature, the exercise intensity and, the level of muscle damage. Phosphorylated HSP27 and αB-crystalline.

Interestingly, we observed significant elevations in the phospho-HSP27 (Ser19) levels one hour post RE in both groups, declining to pre-exercise levels post 48 hours. However, no difference was observed between the groups. While this rapid phosphorylation supports the notion that small HSPs are major component in the first line of cellular defense against cellular stress, the lack of difference between the groups raises an interesting and previously unreported phenomena. HSP response to cellular stress may occur in three steps. Rapid phosphorylation of existing small HSPs such as HSP27 and αB-crystalline. Novel translation of small HSPs followed by translation of large HSPs at the highest stress levels.

Immediately after an acute bout of HS, Hafen and colleagues saw reduced levels of phospho-HSP27 but did not see any changes from control 24 hours after six days of repeated bout HS. In both instances CMT was raised by 3.9 ± 0.31°C. This is the only other study that has quantified phospho-HSP27 levels in humans in response to HS other than our investigation. The changes are not fully comparable as our acute measurement occurred at one hour and not immediately post. The repeated bout effect is more comparable as both investigations saw no impact of heat. However, further comparison is difficult. Given they quantified the phosphorylated HSP content via magnetic bead multiplex, it is possible that they quantified total phosphorylation rather than a specific residue as we did in our investigation. The panel kit specified by (Hafen et al., 2018) indicates for HSP27 (Ser78/82). Moreover, compared to western blots, bead multiplex may provide more specificity given it uses a pair of antibodies

compared just on in western blots. However, the multiplex requires two highly specialized reagents which might impact the development of the panel. In the current literature they are both accepted methods. We report that phsopho-HSP27 (Ser15) increased significantly one hour after an acute bout of full body RE in the skeletal muscle. The phosphorylation of HSP27 has been linked with acute cytoskeletal support, especially under eccentric contractions (Koh, 2002). However, the knowledge on human skeletal muscle is limited and poorly understood. HSP27 is phosphorylated at multiple sites such as Ser82, which has shown to be upregulated under stress (Larkins et al., 2012b, Fyfe et al., 2019). With this observation, we support the suggestion that HSP27 is rapidly phosphorylated after slow eccentric-concentric movement in response to the mechanical stress protecting the cytoskeleton (Koh, 2002) receding to pre RE levels within 48 hours.

We report also that phospho-αB-crystalline (Ser59) levels increased significantly acutely post RE and declining to pre-exercise levels at 48 hours. Similar to HSP27, phospho-αB-crystalline (Ser59) has been shown to interact with the cytoskeleton acutely post eccentric exercise and has been shown to acutely respond to cellular stress in general (Larkins et al., 2012b). Our results are in agreement with previous results by Fyfe and colleagues (Fyfe et al., 2019). Acutely post a similar bout of RE to ours they saw increased phosphorylation at Ser59 levels one hour after RE and saw no chronic training effects (Fyfe et al., 2019). Based on these observations it is apparent that acutely post a heavy RE session small HSP αB-crystalline is phosphorylated at Ser59, possibly as a response to the mechanical stress.

We also report for the first time that full body HS combined with RE did not have an additive effect on the phosphorylation levels of HSP27 or αB-crystalline acutely one hour post or 48 hours. Furthermore, over a ten-week period concurrent HS had no cumulative effect on the phospho-HSP (Ser15) or phospho-αB-crystalline (Ser59) response to RE. As discussed above in small HSP response to HS, the lack of additive phosphorylation may be attributed to the fact that full body HS applied at 40°C did not raise CMT significantly in the HEAT group (36.79 ± 0.58°C) compared to the CON (35.95 ± 0.53°C) group.

We note that the phosphorylation response of the small HSPs does not demonstrate the repeated bout attenuation effect seen in the translation of small HSPs such as HSP27 and αB-crystalline (Thompson et al., 2002). It appears that, in the skeletal muscle under mechanical stress, the rapid phosphorylation of HSP27 and αB-crystalline is not moderated by previous cumulative stress. Alternatively, and more likely, the absence of repeated bout effect could be due to the fact that our intervention was progressive RE, where the mechanical stress was higher as the

training progressed in a previously untrained cohort. Training effects of ten weeks of RE on strength were seen in our investigation (Chapter Three). Therefore, it should reduce the magnitude of small HSP phosphorylation to an identical RE session after ten weeks of training if phosphorylation were to be impacted by repeated bouts. However, our results show this not to be the case. This observation further indicates that immediately after heavy RE, the rapid phosphorylation of existing small HSPs, especially HSP27 and αB-crystalline is potentially over compensatory.

It is evident from the above discussed evidence that the small HSP response is foremost among the cellular stress response in the skeletal muscle. It is further clear that the response is intensity and stress level dependent and is positively correlated with the degree of muscle damage in the case of mechanical stress or overload. It is further apparent that there is a magnitude based separation between the large HSP and small HSP response to stress, heat or mechanical (Kim et al., 2020, Fyfe et al., 2019, Paulsen et al., 2012, Larkins et al., 2012a, Liu et al., 2000, Hafen et al., 2019b). In the case of HS, based on available evidence, a large HSP response is triggered above a threshold of 39°C and the small HSP response at temperatures 37-38°C (Hafen et al., 2019, Morton et al., 2007). As cited above in our investigation, CMT was not raised significantly in the HEAT in the *vastus lateralis.*

In fact, HS did not improve upon any of the HSP response to acute or chronic progressive RE. Given the HSP response is not only driven by heat stress and is conducive to mechanical, oxidative and other cellular stresses such as glycogen depletion (Tang et al., 2006, Liu et al., 2004, Liu and Brooks, 2011, Febbraio et al., 2002), even with the lack of change in the muscle temperature the lack of increment is surprising. Especially acutely given a high volume of RE was performed in a 40°C climate chamber. It is likely these other stressors came in to play more during a bout in full body HS compared to the thermoneutral CON group given the overall stress load on the body is likely higher with the addition of heat even though muscle temperatures between groups were not significantly different (Eskandari et al., 2020). However, the cellular economy in the translational response has been proposed to have a protein synthesis “ceiling” and the cell reaches its maximum translational potential (Bohé et al., 2001, Rennie et al., 2002). Therefore, it is possible that the extra stress created by full body HS still fell within the stress threshold created by heavy RE. Therefore, we posit that the skeletal muscle responds in an over compensatory fashion to the heavy RE and the HSP response was focused on acutely adapting to the progressive mechanical stress.

This potentially over compensatory response is further evident by the phosphorylated small HSP response seen in our study. As discussed above, phosphorylated HSP27 and αB-crystalline have been proposed to bind the F-actin cytoskeleton in small oligomers and play a protective role in preventing cytoskeleton breakage (Mounier and Arrigo, 2002). When phosphorylated HSP27 (Ser15) has been shown to achieve conformation compatibility with binding sites on F-actin (Van Troys et al., 1999). Both HSP27 and αB-crystalline has been shown to be co-locaslised with actin filaments in the skeletal muscle (Koh, 2002, Welsh and Gaestel, 1998). While the mechanism by which this happens in the skeletal muscle is yet to be elucidated, our results indicate that there is a significant phosphorylated small HSP response that may be strongly reinforcing the cytoskeleton within the skeletal muscle during and acutely post heavy RE performed to failure compensating for the combination of stresses generated within the skeletal muscle during and acutely post RE. Moreover, as shown by Larkin and colleagues in rat models, HS generates its own phosphorylated small HSP response in rats when it is the only stress acting upon the cells (Larkins et al., 2012b). HS will generate an additive stress HSP response in combination with RE when the stress caused by RE does not take the muscle to its response "ceiling". A second likely reason is that, given the lack of difference in temperature, HS just did not have an impact. However, it has been shown that even at 35°C full body HS, the serum composition is altered in humans in response to heat (Eskandari et al., 2020).

6.4.7 Conclusion and significance of findings

In conclusion, we report that the full body HS delivered concurrently at 40°C for ten weeks is not sufficient to be an additional, effective stressor to alter the HSP response to RE in the skeletal muscle acutely or chronically, likely due to minimal effects on body and muscle temperature. However, it is also likely that the stress of RE generated an overwhelming cellular response where HS was not able to induce an added stress response at the muscle temperatures we achieved. We further report that small HSPs, HSP27 and αB-crystalline are rapidly phosphorylated after a session of heavy RE. However, there is no repeated bout effect on the magnitude of the phosphorylation response after ten weeks of progressive RE.

Limitations

One of the limitations in this study was the lack of a third test condition where the stress intervention was HS alone.

A second limitation was the fact that we did not fractionate our samples. Given the shown tendency of HSPs to translocate under stress and additional layer of information may have been found in the HSP response to RE and full body HS.

Future directions

A direct lead on from this investigation is to explore the elusive threshold temperature in the skeletal muscle that would allow HS improve upon the HSP response to RE.

Secondarily, our results further confirm the rapid phosphorylation of HSP27 and αB-crystalline acutely after RE. We posited that this might be the first step in three step HSP response to skeletal muscle stress. However, this requires further investigation and has number of muscle rehabilitation and performance improvements.

A potential future direction that is not directly stem from our investigation, we support the notion of utilising HSPs as a series of bio-markers to investigate skeletal cellular stress levels, as from our observations it is clear that HSP response is stepwise that is predicated upon the intensity and quantity of the total stress. Moreover, if the stepwise response consists of post translational modification of existing proteins, novel expression of small HSP followed by Large HSPs in response to RE or HS in humans is of interest.

A different modality of concurrent HS application where the participants receive a bout of full body HS and then continuing on with concurrent HS (so as to “heat prime” the skeletal muscle cells) is also worth investigating for a potentially increased HSP response.

Chapter 7: Chronic effect of heat stress on mitochondrial adaptations and capillarisation response to long term resistance exercise

7.1 Introduction

The potential additive effects of a secondary stressor such as heat stress (HS) applied concurrently with long term resistance exercise (RE) in improving mitochondrial function as well as acute and chronic capillarisation response in the skeletal muscle has significant performance as well as health benefits. The collective muscle adaptive response to RE further extends to mitochondrial, angiogenic responses and Ca^{2+} response. These adaptations play key roles in improving the overall muscle adaptations to RE. It has been shown previously that the magnitudes of these adaptive responses may be dictated, at least in part, by training modality, time of intervention as well as intensity (Irrcher et al., 2003, Hood, 2009, Yan et al., 2011, Parry et al., 2020) HS has also been shown to impact on these adaptations acutely and chronically in the skeletal muscle (Kuhlenhoelter et al., 2016, Liu and Brooks, 2011, Hafen et al., 2018).

Mitochondria are the most critical cellular apparatus in cellular energy turnover. Physical activity increases the mitochondrial content (generation of new reticular components) as well as quality (mitochondrial biogenesis) within the skeletal muscle (Jornayvaz and Shulman, 2010, Holloszy and Coyle, 1984, Yan et al., 2011, Stotland and Gottlieb, 2015). It has been shown that mitochondrial activity, content and quality improve noticeably in response to endurance exercise (Irrcher et al., 2003, Baar, 2004). The body of work on RE is limited compared to endurance exercise (Parry et al., 2020). Some studies have demonstrated that RE is capable of improving mitochondrial activity (Balakrishnan et al., 2010, Pilegaard et al., 2003, Wilkinson et al., 2008). However, chronic adaptive mitochondrial response to heavy full body RE has not been extensively investigated. Previous studies have reported unchanged or reduced mitochondrial biogenesis levels measured PGC-1α levels (Roberts et al., 2018). Mitochondrial content, quantified via citrate synthase (CS) activity has also shown to reduce or remain unchanged. Moreover, mitochondrial respiratory capacity, measured via oxidative phosphorylation (OXPHOS) has also been seen to remain unchanged or reduce (Roberts et al., 2018, Haun et al., 2019).

Similar to exercise, localised HS has been shown to improve mitochondrial content as well as maintain mitochondrial quality (function) in human skeletal muscle (Hafen et al., 2019, Hafen

et al., 2018). However, these improvements maybe predicated upon muscle temperature as others saw no changes (Kim et al., 2020). However, whether applying full body HS, concurrently with full body progressive RE improves upon any mitochondrial adaptations to RE is yet to be investigated. (Hafen et al., 2018, Groennebaek and Vissing, 2017).

Angiogenesis refers to the process where capillary growth, density and the adaptive remodelling of the blood vessels changes within the skeletal muscle in response to stress (Bloor, 2005).This process increases the efficiency of the muscle to perform exercise via increased circulation (Gavin, 2009) therefore, unsurprisingly exercise stimulates angiogenesis (Gavin, 2009). Factors other than mechanical stress also have the potential to trigger angiogenesis and include oxidative stress, Ca^{2+} and cell produced nitric oxide (NO) (Wackerhage, 2014, Gavin, 2009). These adaptations trigger multiple signalling pathways including CaMK/AMPK which involves the key molecular marker PGC-1α as well. However, the particular pathway via which the response occurs is yet to be determined (Gavin, 2009). Similar to mitochondrial biogenesis, the angiogenic response to endurance exercise is well documented (Bloor, 2005, Gavin, 2009). While limited in scope, some studies have shown clearly that RE increases angiogenic growth factor expression such as VEGF acutely. However, factors such as ANGPT1 remained unchanged (Gavin et al., 2007). Furthermore, endothelial nitric oxide synthase (eNOS) which is involved in regulating blood follow via the production of NO has been seen to increase with acute RE (Gavin, 2009) Multiple chronic capillarisation index markers such as capillary density, capillary contacts, capillary/fibre ratio in human skeletal muscle (McCall et al., 1996, Nederveen et al., 2016). Moreover, HS has been shown to increase transcription the angiogenic markers VEGF and ANGPT1 acutely (Kuhlenhoelter et al., 2016) and improve the phenotypic capillarisation response chronically (Kim et al., 2020, Hesketh et al., 2019, Hyldahl et al., 2021) in the skeletal muscle but the evidence sparse. Whether concurrent full body HS is able to improve upon the angiogenic response to chronic progressive RE in the skeletal muscle has not been examined before.

There is a large body of evidence regarding mitochondrial and angiogenic adaptations to endurance exercise. The evidence is markedly limited for RE, but there is enough evidence to suggest that RE does have the ability to trigger these key adaptations acutely and chronically. The body of work on these adaptive responses to HS in the skeletal muscle is further sparse (Hyldahl and Peake, 2020). But similar to RE, there is merit in what's been reported to indicate that HS maybe a useful stressor in driving mitochondrial biogenesis and angiogenesis in skeletal muscle.

A plausible additive effect of a secondary stressor such as HS applied concurrently with long term RE could benefit muscle molecular and functional adaptations to the exercise training improving performance as well as aiding in muscle rehabilitation. Therefore, in this investigation, we hypothesised that full body HS applied concurrently with ten weeks of progressive heavy RE may increase mitochondrial biogenesis and angiogenic response to RE.

7.2 Methods

7.2.1 Biopsy sequence and western blots

Mitochondrial biogenesis

Muscle biopsies were obtained at a depth of 3 -3.5cm below the epidermis from the *vastus lateralis* pre-intervention at rest (Pre 0), one hour (Pre 1) and 48 hours (Pre 48) post a resistance training session that was identical to the first training session of the intervention 72-96h prior to the commencement of the intervention. Three further biopsies were extracted post-intervention at rest (Post 0), one hour (Post 1) and 48 hours (Post 48) after an identical RE session to the first session of the intervention, which was performed ~72-96h after the last training session of the intervention. Muscle samples were analysed for expression levels of PGC-1α at all six time points. Mitochondrial complexes I-V were quantified at Pre 0 and Post 0. Methods were as outlined in Chapter Four.

Angiogenesis

Muscle samples were analysed for expression levels of VEGF, ANGPT1 and eNOS at all six time points. Methods were as outlined in Chapter Four.

7.2.2 Immunohistochemical analysis

Angiogenesis

Immunofluorescence (CD 31) was used to quantify angiogenesis. Capillary density, capillary contacts per fibre and capillary fibre periphery exchange (CFPE) were quantified at Pre 0 and Post 0. The methods were as outlined in Chapter Four.

7.2.3 Citrate synthase assay

Citrate synthase activity was quantified at Pre 0 and Post 0. Methods were as outlined in Chapter Four.

7.3 Results

In reporting results, all significant time, group and time x group effects are reported. Where time effects are present, the difference between the times are reported for CON and HEAT groups separately.

7.3.1 Mitochondrial biogenesis and citrate synthase activity

We observed no significant time, group or time x group effects in the total expression levels of PGC-1α. There were no time, group or time x group effects observed in the total expression levels of mitochondrial complexes I, II, III, V. No time or group effects were observed in the expression levels of complex IV, however, a significant time x group effect was observed (p = 0.032) (Figure 7.1). CS activity showed no change from Pre 0 to Post 0 in either group (Figure 7.2).

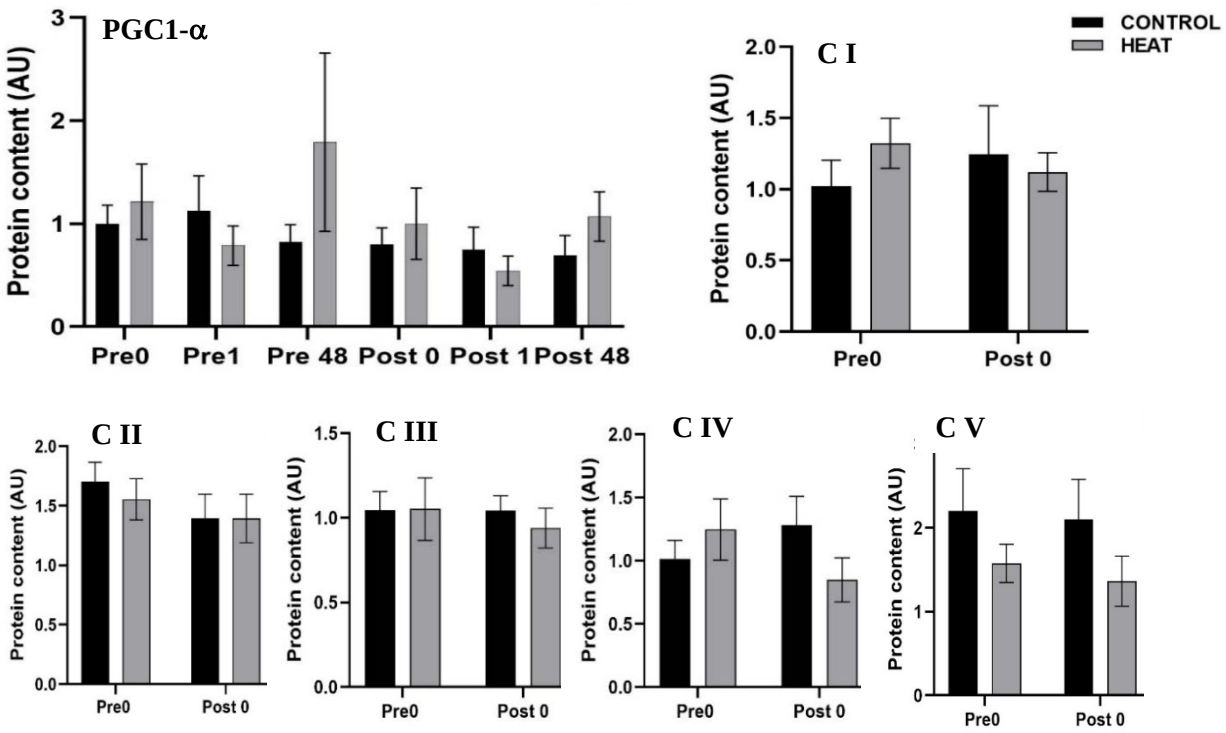


*Figure 7.1. Expression levels of total PGC 1-α and mitochondrial complexes I-V. * p <0.05*

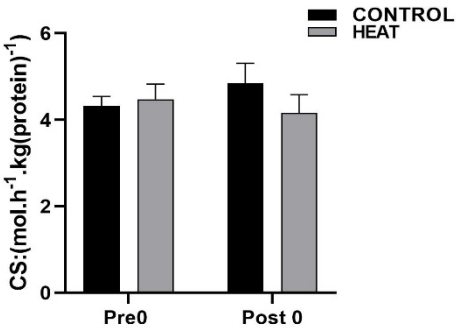


Figure 7.2. CS activity pre and post intervention

7.3.2 Angiogenesis

We observed no significant time, group or time x group effects in the total expression levels of VEGF or ANGPT1. A significant time effect was observed for eNOS in the HEAT group ($p = 0.006$). eNOS levels decreased by 62% Post 0 to Post 1 ($p = 0.047$) (Figure 7.3). No changes were observed in the CON over time.

There were no group or time x group effects observed in any of the measurements of capillarisation. Significant time effects were observed for capillary to fibre ratio ($p = 0.0002$), capillary density ($p = 0.001$), capillary contacts ($p = 0.014$) and CFPE ($p = 0.028$). In the CON group capillary to fibre ratio increased by 27% ($p = 0.007$) and capillary density by 28% Pre 0 to Post 0 ($p = 0.024$). In the HEAT group capillary to fibre ratio increased by 32% ($p = 0.016$) and capillary density by 34% ($p = 0.047$) (Figure 7.6). However, in post-hoc analysis capillary contacts and CFPE effect was not significant. However, both measurements increased from Pre 0 to Post 0. Capillary contacts in CON group increase by 17% (Pre 0 = 2.794 ± 0.136, Post 0 = 3.392 ± 0.267, $p = 0.061$) and in HEAT group by 6% (Pre 0 =2.846 ± 0.227, Post 0 = 3.040 ± 0.217). CFPE in CON group increased by 13% (Pre 0 = 6.975 ± 0.390, Post 0 = 7.939 ± 0.696) and in HEAT group by 30% (Pre 0 = 6.065 ± 0.447, Post 0 = 7.905 ± 0.737, $p = 0.054$) (Figure 7.7).

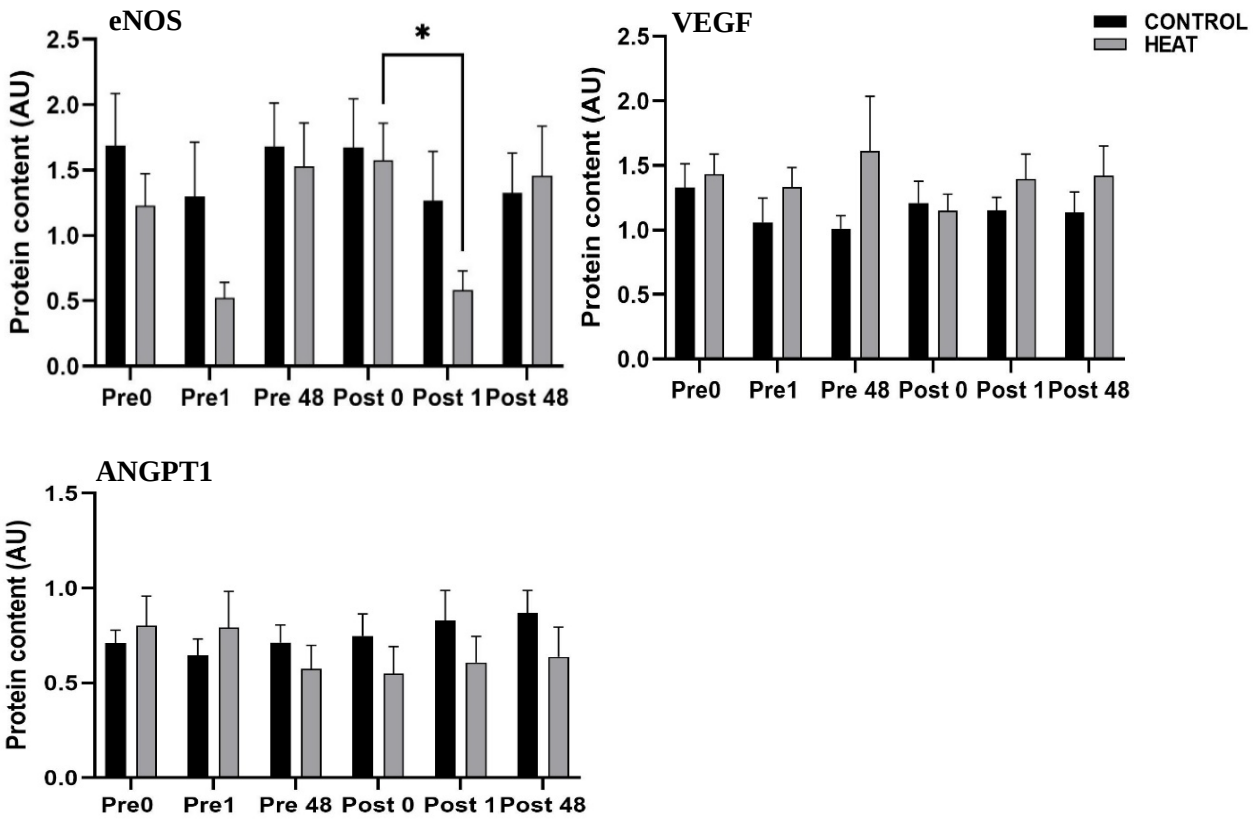


Figure 7.3 Expression levels of total eNOS, VEGF and ANGPT1 pre and post intervention. * p <0.05

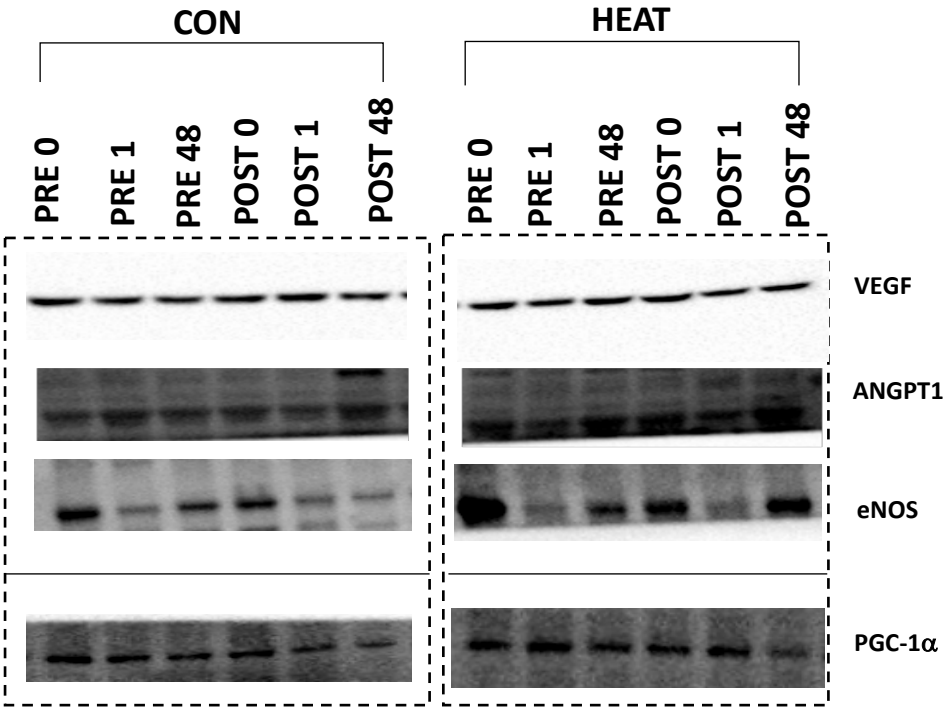


Figure 7.4. Representative blots for angiogenic makers for capillarisation and PGC 1-α for mitochondrial biogenesis

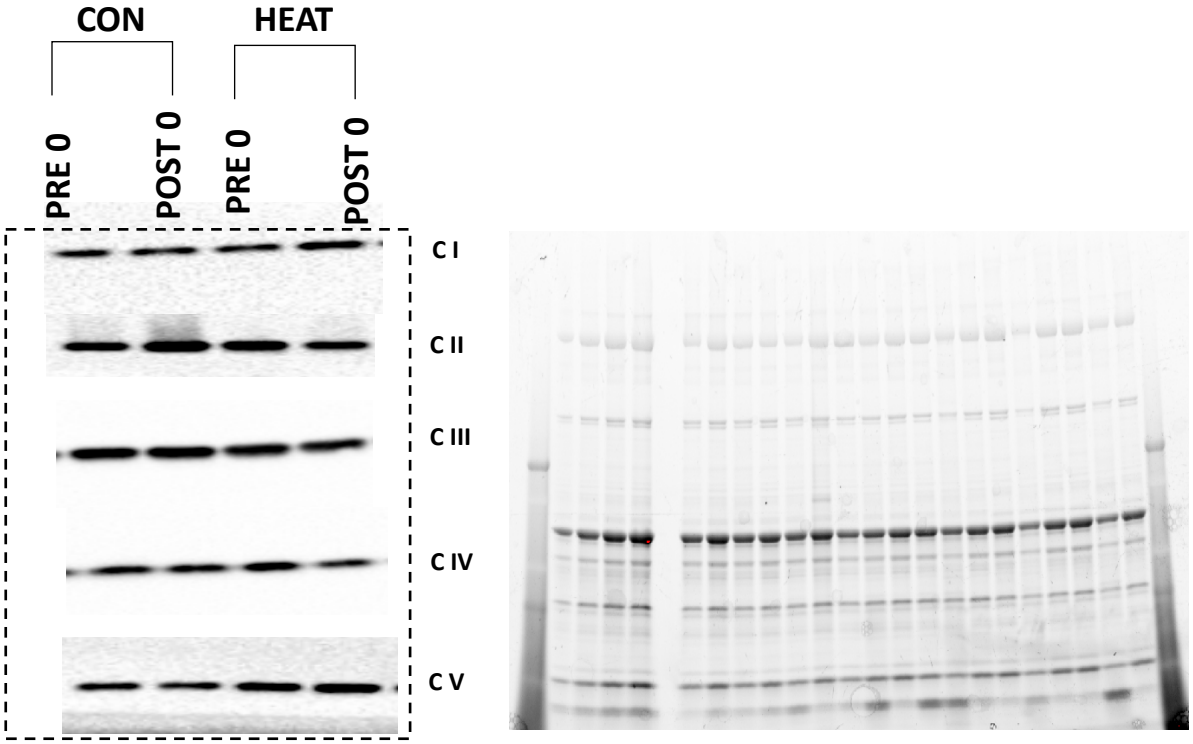
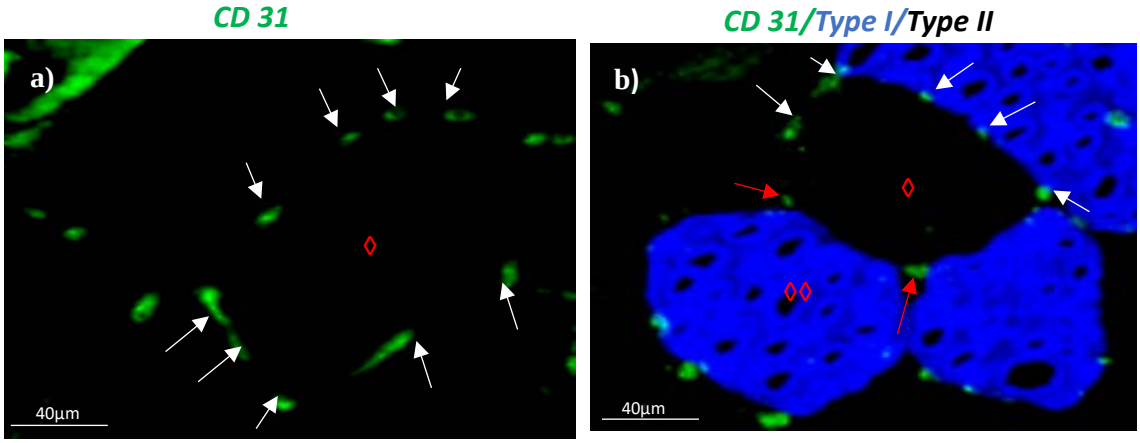


Figure 7.5. Representative blots for mitochondrial complexes I to V pre and post intervention and the representative stain free image for all western blots shown



◊: *Capillarisation indices per fibre*

Capillary contacts (CC) (In contact with a given fibre) : a) ◊ = 9

Fibre sharing factor (SF) : b) ◊:◊◊ = 2 (← = shared capillaries)

Capillary to fibre ratio (Cap/Fi): b) ◊:◊◊ = 7 x ½ + 5 x ½ = 6

CFPE index = $\frac{Cap}{Fibre}/Fibre\ perimeter\ (\mu m)$

Capillary Density = $\frac{Cap}{Fibre}/Fibre\ area$ *x* 10^6 (mm^{-2})

CC, SF, Cap/Fi and CFPE have been extensively outlined and defined in chapter 4 methods

Figure 7.6. Immunohistochemical analysis of capillarisation a) capillaries per fibre b) Fibre specific capillarisation

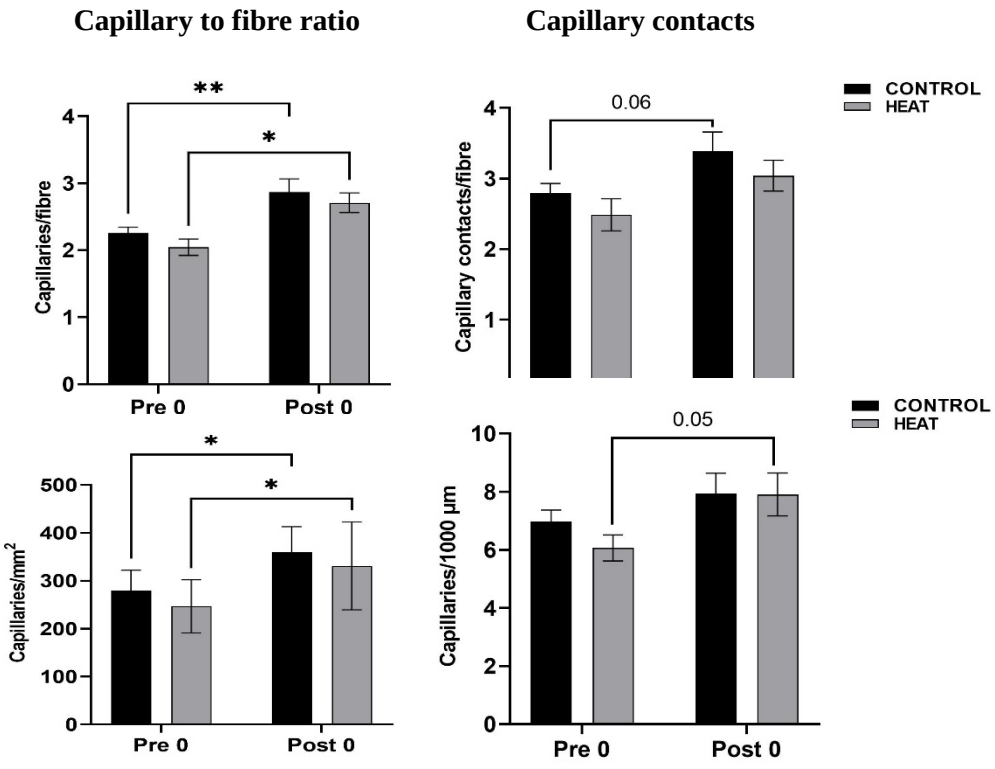


*Figure 7.7. Quantification of angiogenesis pre and post ten week intervention. * p < 0.05, ** p < 0.01*

7.4 Discussion

The aim of this investigation was to examine whether full body HS applied concurrently with ten weeks of progressive heavy RE may improve upon chronic mitochondrial biogenesis and angiogenic response to RE. Moreover, we further wanted to examine the effect of HS on acute mitochondrial biogenesis as well as angiogenic response as to full body RE.

We report that full body, progressive RE did not improve mitochondrial biogenesis and activity acutely or chronically. Moreover, full body concurrent HS did not impact mitochondrial biogenesis and activity in skeletal muscle. Total protein expression levels of key angiogenic marker VEGF, ANGPT1 and eNOS did not change acutely or chronically post RE. HS did not have an impact on the levels of VEGF or ANGPT1. However, we report for the first time that eNOS levels decreased rapidly at one hour post RE when combined with full body HS. Phenotypic capillarisation response was not different between the HEAT and CON groups. Capillaries per fibre and capillary density increased in both groups. CFPE trended up in HEAT and capillary contacts trended up in the CON group (Figure 7.7).

7.4.1 Mitochondrial biogenesis

In this investigation we found no changes mitochondrial biogenesis measured via the total protein expression levels of PGC -1α, acutely or chronically with RE, and there was no positive nor negative effect of HS. Mitochondrial respiratory capacity measured via the total expression levels OXPHOS complexes did not change in response to chronic RE or HS. Furthermore, mitochondrial content, assessed via CS activity did not change after ten weeks of progressive RE nor was it impacted by concurrent application of full body HS. It has been reported previously that overall mitochondrial protein content (measured via proteomics) increased in the skeletal muscle after 12 weeks of full body RE, however, in younger cohorts the translation response was significantly lower compared to older cohorts (Robinson et al., 2017). This observation is to be expected given, older adults show lower basal levels of mitochondrial proteins (Rooyackers et al., 1996) and could potentially be a reason for lack of change seen in our investigation given the age range of the cohort. However, most of the studies investigating the chronic effects of RE on mitochondrial content have utilised CS activity as the biomarker (Groennebaek and Vissing, 2017).

Following 12 weeks of full body RE, Porter and colleagues saw no changes CS activity or OXPHOS levels (Porter et al., 2015). Partially contradicting this report, Roberts and collgues saw CS activity decrease after 12 weeks of RE, while PCG-1α and OXPHOS levels were

unchanged in anabolic high as well as low responders (Robinson et al., 2017). Similarly, after six week of high volume RE lead to a decrease in CS activity (Haun et al., 2019). After eight weeks (two sessions a week) of full body RE (leg and bench press only), in normoxia and hypoxia (14.4% oxygen for total of 40 minutes before and after session), no changes in total PGC-1α were seen in either group, however, CS activity decreased by 9% ($p < 0.05$) in both groups in the *vastus lateralis* (Kon et al., 2014). In combination, the previous reports are equivocal on CS activity response to chronic RE, but a significant majority indicates a reduction while some show no change (Parry et al., 2020). The concept of mitochondrial dilution has been proposed as a very likely reason as to decrease or no change in mitochondrial content in response to chronic RE (Parry et al., 2020). Mitochondrial dilution suggests that the rate of increase in fibre hypertrophic response exceeds the rate of mitochondrial biogenesis, thereby reducing or maintaining the overall mitochondrial content normalised to fibre volume (Parry et al., 2020). Our results discussed in Chapter 5 showed muscle fibre CSA improved significantly in the CON group while HEAT group trended up. Therefore, our observation of unchanged CS levels following ten weeks of full body RE are in agreement with this notion. This observation is further supported by the fact we saw no change in the PGC-1α levels pre and post intervention. If there had been no mitochondrial biogenesis, with the increasing CSA, PGC-1α levels would have decreased when normalised against total protein. Moreover, total respiratory capacity remained unchanged, as seen by the unchanged OXPHOS levels pre and post intervention, which further supports the increased levels of mitochondrial biogenesis. Our results support the observations by Robinson and colleagues (Robinson et al., 2017) who reported similar results.

To our knowledge, we are the first to investigate the acute effects of full body RE on mitochondrial biogenesis. We saw no changes in PGC-1α levels at one and 48 hours post an RE session. In a previous study, Wilkinson and colleagues have reported the elevation of mitochondrial protein synthesis four hours post a single bout of isokinetic contractions. However, they measured mitochondrial activity via fractional synthesis rate compared to expression levels of PGC- 1α in our investigation (Wilkinson et al., 2008). Given PGC-1α is mitochondrial gene regulator and fractional synthesis rate measures translated content, an increase in PGC-1α levels would have meant increased biogenesis following RE. In support, Donges and colleagues saw no improvements in the mRNA levels of PGC- 1α one hour after a single bout of leg extensions and saw no subsequent improvements in fractional synthesis rate (Donges et al., 2012). Interestingly, Coffey as well as Burd and colleagues observed that acute mitochondrial protein synthesis was exercise intensity dependent and that RE modalities resembling endurance

exercise (high volume) tend to elevate mitochondrial activity compared to heavy hypertrophy oriented RE (Burd et al., 2012, Coffey et al., 2006). Given the hypertrophy oriented nature of our full body RE protocol, where the muscle is not subjected to extensive oxidative stress (Burd et al., 2010), it appears that the mechanical stress is not a sufficient stressor to instigate acute mitochondrial biogenesis compared to oxidative stress caused by endurance exercise or low high volume RE, that mimics endurance exercise. In support, Leek and colleagues saw a significant improvement in CS activity that one hour post RE that fell to baseline at 72 hours (Leek et al., 2001) supporting previous observation by Tonkonogi and colleagues where CS activity was seen to elevate after acute bout of prolonged exercise (Tonkonogi et al., 1997) perhaps due to high ATP turnover at fatigue (Gorostiaga et al., 2012). While we can report that PGC-1α levels are unchanged pre and post RE, we cannot confirm whether full body RE increased aerobic mitochondrial potential acutely post full body RE.

There was no clear effect of HS or HS combined with RE seen on mitochondrial biogenesis, content or respiratory capacity. In the first study to demonstrate the ability of HS driven mitochondrial biogenesis in humans, after six repeated bouts of HS using shortwave diathermy over six days (two hours per day) to the *vastus lateralis* increasing CMT by 3.9 ± 0.31°C to a peak of ~40°C, Hafen and colleagues observed improved levels of C I and C V along with PGC-1α levels, but no changes in CII, III,IV or CS activity (Hafen et al., 2018). In a similar study investigating the effect of repeated HS on muscle atrophy, Hafen reported significant increases in total expression levels of PGC -1α after ten days of repeated heat exposure (two hours a day), and significant increases (maintained without decreasing compared to immobilised control) in all OXPHOS complexes. The CMT raised reported was 4.2 ± 0.29 °C at 3.5cm, to a peak of ~40°C (Hafen et al., 2019). However, Kim and colleagues observed no changes in the OXPHOS and CS activity after eight weeks of localised HS at an estimated peak CMT of ~38°C (increase of ~3 °C) (Kim et al., 2020) in the *vastus lateralis.* Ihsan and colleagues saw no improvements in the mRNA levels of PGC-1α after a bout of full body HS where peak CMT reached 39.1 ± 0.3°C (Ihsan et al., 2020). In our investigation, we observed a peak CMT of 36.79 ± 1.55 °C in the HEAT group (with RE) and 35.94 ± 1.51 °C in the CON group in the *vastus lateralis* inducing a CMT raise of ~ 2°C in both groups. It appears when comparing the muscle temperatures at the same depth in the same muscle, mitochondrial biogenesis might be predicated upon the muscle temperature achieved, therefore the total cumulative heat load applied. Within the scope of available evidence, it could be argued that CMT needs to be raised by ~4° (~40°C) to illicit a mitochondrial response acutely and chronically. Furthermore, Hafen's observations indicate that repeated, continuous HS maybe the more effective stress methodology compared to non-

continuous HS such as ours (Hafen et al., 2019, Hafen et al., 2018). It is worth noting however, HSP 60 levels, discussed in chapter six as a potential marker of mitochondrial stress levels, did not increase acutely or chronically post HS combined with RE in our investigation. Similarly, total HSP 60 levels did not alter in Hafen's investigation after repeated HS (Hafen et al., 2018). This suggests a high threshold temperature for mitochondrial stress and subsequent mitochondrial biogenesis in the human skeletal muscle. Moreover, the overall presence of HS when performing hypertrophy driven, high volume RE is not sufficient to instigate a biogenesis response. Additionally, the variability in the heating modalities between the limited number of studies available is worth noting with regards to the difference in results seen as well. The focussed deep muscle heating offered by diathermy, targeting a smaller area compared to the more dispersed application of full body HS.

7.4.2 Angiogenesis

Angiogenic response (or the adaptive growth remodelling of the blood vessels) is triggered via external stressors such as exercise. Similar to mitochondrial stress, angiogenesis shows a more pronounced response to oxidative stress (chronic endurance exercise) compared to mechanical stress in the human skeletal muscle. Furthermore, the response has been found to be intensity and duration dependent. Moreover, patterns and rate of muscle blood flow has also been associated with improved angiogenesis (Bloor, 2005, Hudlicka et al., 1992, Laughlin and Armstrong, 1982).

VEGF

We did not observe improvements in the total expression levels of VEGF at one and 48 hours acutely post RE in CON or HEAT groups. Ten weeks of full body RE did not improve upon the acute VEGF response to RE. The limited available evidence suggests that acute and chronic RE are capable of inducing a positive angiogenic marker response. Trenerry and colleagues saw that VEGF transcription (mRNA) levels increased significantly ($p < 0.05$) at four hours post a single acute bout of leg extension but did not see an improvement at two hours while trending up (Trenerry et al., 2007). Similarly, Gavin and colleagues observed that after an acute bout of RE (knee extensions) levels of muscle VEGF protein expression increased significantly by 15% ($p = 0.019$) at two and four hours along with plasma VEGF levels ($p = 0.017$) at the same time points (Gavin et al., 2007). Their observations were similar that of acute aerobic exercise at similar time points (Gavin et al., 2007). Gustaffson and colleagues saw increased translation levels of VEGF 24 hours following seven kick training sessions (over ten days) (Gustafsson et al., 2002). Moreover, Yeo and colleagues observed increased levels of VEGF in plasma ($p <$

0.05) post a chronic RE intervention of eight weeks (Yeo et al., 2012). Furthermore, they observed that moderate intensity RE may be more effective in driving the angiogenic response compared to high intensity exercise (Yeo et al., 2012). Kon and colleagues also reported significant elevation of VEGF-B levels from pre intervention after 8 weeks of moderate intensity full body RE (Kon et al., 2014). These results contradict our findings on acute and chronic levels.

It has been suggested that PGC-1α may play a regulatory role in VEGF expression during exercise (Arany et al., 2008). In this investigation we did not observe changes in the levels of PGC 1-α as was reported above, which may support our observation of unchanged VEGF levels over the intervention. However, Kon and colleagues saw no improvements in PGC-1α, but still saw VEGF-B levels increase after eight weeks of RE (Kon et al., 2014). Induction of VEGF expression is a coordinated process that appears to be regulated by multiple signalling cascades. Interestingly, Takahashi and colleagues saw a strong relationship between Akt and VEGF in cultured human skeletal muscle cells (C2C12 myoblasts) during hypertrophic growth (Takahashi et al., 2002) and a temporal, consequential VEGF transcription, showing that Akt may be essential for VEGF secretion during muscle growth (Takahashi et al., 2002). As reported in chapter five, we saw phospho-Akt (Ser473) levels increasing at one hour post RE in both groups. This observation possibly indicates towards the fact that VEGF transcription is possibly initiated at one hour after a bout of RE but not translation which on the current evidence appears to occur between two to hours post RE (Gavin et al., 2007).

Overall, the contradiction in observations between Trenerry, Gavin (Trenerry et al., 2007, Gavin et al., 2007) and ours could be due to the fact that, they were acute, high volume RE session of knee extensions that focused on the quadriceps from which the muscle was extracted compared to ours where lower body exercise was only single component and perhaps drove a more universal response across the body but did not increase locally at the quadriceps. A more likely and previously posited reason is that the high frequency of contractions seen in moderate intensity RE similar to above cited studies is more likely to initiate VEGF induction compared to lower frequency of contractions used in our intervention. We conclude that this contradiction is due to the difference in exercise intensity. As seen from above investigations, VEGF response is more conducive to high volume exercise compared low volume exercise. Hypoxia is one of the most potent inductors of VEGF (Bloor, 2005). High volume RE likely creates comparably more potent hypoxic stimuli to low volume RE, generating a more prominent VEGF response.

Whereas RE with contraction low frequency seen in hypertrophy driven interventions such as ours where upstream regulators such as Akt and PGC-1α are down regulated or unchanged

acutely post RE. A third and possibly the most likely explanation could be the fact that in the skeletal muscle, VEGF transcription response begins within the 2-4 hours post exercise, however, the translation maybe further delayed (Trenerry et al., 2007). Therefore, at one hour post exercise we have may have missed the acute translation response.

This notion of delayed translation of VEGF is supported by the observations of Kuhlenhoelter and colleagues where they reported significant increments of VEGF mRNA levels at 30 minutes and 90 minutes post lower body HS intervention. However, they did not observe similarly elevated levels at two hours post (they did not measure total protein expression levels).While the stressors are different between the two studies, Gavin and Kuhlenhoelter both used localised, lower body stressors where translation was increased ~two hours and transcription diminished approaching the ~ two hour mark (Kuhlenhoelter et al., 2016). Furthermore, Ihsan and colleagues reported that after full body HS at 50°C (50% RH, core temperature maintained at 39°C) for one hour, VEGF mRNA levels increased significantly at 30 minutes (Ihsan et al., 2020). Crucially, they did not observe similar improvements after one hour of localised HS where CMT was raised to a peak of 38.1 ± 0.6 ° C. Interestingly, their localised heating method was highly comparable to Kuhlenhoelter's who observed a significant elevation in VEGF mRNA levels at the same time points. Albeit, with a 90 minute intervention compared to Ihsan's one hour (Ihsan et al., 2020, Kuhlenhoelter et al., 2016). Additionally, chronic localised HS applied over eight weeks (five days a week, 90 minutes a session) did not improve total protein expression levels of VEGF at 48 hours post termination of the intervention at a perceived CMT of ~38°C (Kim et al., 2020), similar to our observations in both CON and HEAT groups. 24 hours after ten days of localised HS to the immobilised *vastus lateralis,* total VEGF protein content did not decrease significantly compared to immobilisation only group where a significant reduction was seen indicating VEGF synthesis (Hyldahl et al., 2021). The heat application method utilised in this study has previously has been reported to raise CMT to ~40°C (Hafen et al., 2019). Collectively, it appears that, while an acute VEGF response is observed following acute RE and HS, translation only occurs post ~ two hours. Therefore, it is possible that at one hour post, the total expression levels do not change and at 48 hours post returns to baseline levels. Even with the combined stress of RE and HS, it is possible that translational response of VEGF is delayed while transcription is more immediate. However, no investigation previously has quantified total protein VEGF expression levels within one hour of acute RE or HS. Furthermore, to our knowledge this is the first investigation to report that long term HS does not have a cumulative effect on acute VEGF response to RE. A limitation of our study was the lack quantification of transcription of VEGF, which would have allowed us to draw more relatable comparisons with the previous studies. Finally, VEGF response may be

muscle temperature dependent as Hyldahl and colleagues saw potential regulation of total protein content at ~40°C compared to lack of improvement in ours at ~37°C.

ANGPT1

We did not observe changes in the total expression protein levels of ANGPT1 acutely one or 48 hours post RE. There was no chronic effect observed after ten weeks of full body RE. Similarly, Gavin and colleagues did not observe an elevation in ANGPT1 levels at two or four hours post an acute bout of RE (Gavin et al., 2007). Moreover, Yeo and colleagues reported elevated levels of ANGPT1 post eight weeks of knee extensions indicating a chronic effect similar to VEGF in plasma (Yeo et al., 2012). While angiopoietins are key capillarisation markers, they cannot induce angiogenesis and are more involved in vascular remodelling and maturation post angiogenesis (Bloor, 2005). Therefore, the lack of induction acutely post RE is not surprising. However, we expected an increase in ANGPT1 levels chronically post RE given the improvements in capillarisation seen post training in a previously untrained cohort (discussed below). A likely reason is that at the moment of extraction of the post intervention biopsy, 72-96 hours following the last session of the RE intervention, ANGPT1 levels decreased to pre intervention levels having temporarily increased during the intervention .

We saw no acute effects of HS on ANGPT1 protein levels, nor did we observe a chronic effect of HS after ten weeks of heat application. Kuhlenhoelter and colleagues did not observe elevated levels of ANGPT1 mRNA levels at 30 minutes or two hours post, acute localised HS to the *vastus lateralis* (thigh heating) or to the lower body (Kuhlenhoelter et al., 2016). But, reported a significant ($p < 0.05$) fold change difference mRNA in the heat treated group compared to control in thigh heating. Interestingly, they did not see the same results when lower body heating was applied (thighs and calves) (Kuhlenhoelter et al., 2016). However, since there was no time effect seen with heat treatment, the difference between the groups does not provide evidence on the acute effect of HS (Kuhlenhoelter et al., 2016). Using the same heating method (localised water perfusion), Kim and colleagues did not see improvements in total protein expression levels of ANGPT1 after four or eight weeks of localised HS intervention (Kim et al., 2020).

Our chronic results agree with the observations by Kim and colleagues. We also report for the first time that there appears to be no effect of chronic RE or RE combined with HS, on ANGPT 1 protein expression. While the work on ANGPT1 response to RE or HS is limited, it does appear that its role as a capillary stability promoting factor as opposed to an angiogenic growth factor (Gavin, 2009), is more a chronic response than acute (Bloor, 2005). Given that Kim as well as Kuhlenhoeltor and colleagues used a heating method that has shown to raise CMT ~38°C

compared to our 36.79 ± 1.55, we suggest that ANGPT1 response in human skeletal muscle is not triggered chronically below ≤ 38°C. Moreover, it does not appear to be a sufficient additive stressor to supplement RE at these muscle temperatures.

eNOS

The facilitation of blood flow regulation and vasodilation is mediated by eNOS by managing the release of nitric oxide from the muscle vascular endothelium, and potentially regulates VEGF and mitochondrial respiration (Gavin et al., 2000). However, the available evidence on eNOS response to RE or HS on human skeletal muscle is limited.

In this investigation we observed that total protein eNOS levels did not alter from baseline at one hour and 48 hours pre and post the ten week RE intervention in the CON group. However, in the HEAT group eNOS levels decreased significantly at one hour after an acute RE session post intervention and trended down one hour after an acute RE session pre intervention (Figure 7.3). To our knowledge, we are the first to report a potential acute impact of HS on the total eNOS levels in the skeletal muscle acutely post RE. The available evidence on HS induced changes in eNOS levels is limited. In their eight week heat intervention, Kim and colleagues reported a significant elevation in the total eNOS levels from baseline at four weeks (mid intervention) and eight week (post intervention). They suggested that this may have aided in averting a decline in capillary indices over time due to inactivity. However, we believe that this observation maybe due to vasodilation caused by the HS where body attempts to dissipate the energy (Nielsen et al., 1990) from the *vastus lateralis*. It has been previously reported that passive heat therapy improves NO dependent subcutaneous microvascular dilation (Brunt et al., 2016). The blood flow increase caused by consistent localised HS over eight weeks and may not be related to the maintenance of capillary incidence. This raises the question as to why eNOS levels decreased under HS when combined concurrently with full body RE in our investigation. As this observation contradicts our hypothesis that the combination of HS and RE, would trigger an acute increased vasodilation response increasing muscle blood flow.

Kellogg and colleagues demonstrated clearly that NO plays a dominant role in sustaining vasodilation under heat stress (skin temperatures comparable to ours) (Kellogg Jr et al., 1999). They further established that at milder heat applications, the inhibition of NO synthase (NOS) did not completely attenuate the vasodilation indicating a secondary regulator (Kellogg Jr et al., 1999). This notion was further established by Minson and colleagues (Minson et al., 2001). In a very similar heat intervention to ours Hesketh and colleagues established that passive full body HS applied at 40°C (40% RH) for six weeks in a young sedentary cohort improved eNOS content

8% ($p < 0.001$) at 48 hours post intervention. They further reported that phospho-eNOS (Ser1177) levels increased by 4% form pre intervention ($p = 0.05$) in the *vastus lateralis* (Hesketh et al., 2019). They further established that the results seen after passive HS were comparable to a six weeks of moderate intensity continuous exercise training (Hesketh et al., 2019). This report combined with the reports of Kim and colleagues where they reported no changes in the phospho-eNOS (Ser1177) levels post eight weeks of intervention and our observations shows a highly interesting facet of the skeletal muscles eNOS response to HS. The acute reduction of eNOS levels at one hour post RE in the HEAT group may indicate rapid degradation of protein content. A phenomenon not seen in the CON group. We are the first to report this observation. Given RE and HS both induce vasodilation, we expected a lager increase in the total eNOS levels when the stressors were combined. The paradoxical occurrence of sympathetic vasoconstriction (inhibited blood flow) with increasing exercise intensity in the skeletal muscle has previously been suggested (Buckwalter and Clifford, 2001).Moreover, it has been seen that vasoconstriction leads to oxidative stress thereby causing reduced eNOS activity (Förstermann et al., 2017, Rodriguez-Porcel et al., 2017, Sena et al., 2018). Based on this evidence, we speculate whether, HS combined with RE caused an acute bout of vasoconstriction, thereby causing hypoxic conditions within the skeletal muscle. Which lead to the acute degradation of eNOS.

Chronically, Hesketh, in an almost identical chronic heat intervention to ours, and Kim in their chronic localised heat intervention saw improvements in the eNOS content (Hesketh et al., 2019, Kim et al., 2020). Whereas we did not see chronic effect of HS when applied concurrently with RE. Kim and colleagues estimated a peak CMT of ~38°C (did not directly measure). Hesketh and colleagues did not measure CMT either. But, given their intervention parameters were almost identical to ours at 40°C (30% RH) we make the assumption that it would be similar to ours albeit slightly lower given there was no exercise component. Therefore, it would have been justified to expect an additive chronic effect in our study on the eNOS content as was our hypothesis. One possible explanation for the lack of a chronic effect in our study could be due to the concurrent application of RE. As it was seen with the with the small HSP response in Chapter six, the skeletal muscle cell is concurrently responding to the mechanical stress of RE and HS, but the response to RE is prioritised. The stress input by HS does not generate an adequate amount of extra stress to warrant improved eNOS content chronically.

7.4.3 Phenotypic capillarisation response

We investigated the chronic phenotypical capillarisation response to RE and RE combined with HS by quantifying capillary density, capillary to fibre ratio, capillary contacts and CFPE pre and

post intervention (Figure 7.7). Capillary density and capillary to fibre ratio increased significantly in both groups. While not statistically significant ($p < 0.05$), capillary contacts ($p = 0.061$) and CFPE ($p=0.054$) trended considerably high post intervention in the HEAT group. However, it was evident that full body HS applied at 40°C (30%RH) did not have an effect on chronic RE driven capillarisation. Kon and colleagues saw two cohorts performing eight weeks of RE under normoxic and hypoxic conditions improve capillary to fibre ratio. Significantly so in the hypoxic group ($p <0.05$) and a strongly trend up in normoxic group ($p = 0.06$) (Kon et al., 2014). The training volume was much lower in their study compared to ours. In response to 16 weeks of high volume full body RE (four times a week), Nederveen and colleagues reported significant improvements in capillary to fibre ratio, CFPE and fibre specific (type II) elevations in capillary contacts and capillary to fibre ratio in the *vastus lateralis* (Nederveen et al., 2017). We quantified overall capillarisation (non-fibre specific), however, their observations are highly comparable to ours. Therefore, we further confirm that chronic RE improves skeletal muscle capillarisation.

Hyldhal and colleagues saw no improvements in capillary density, CPFE or capillary to fibre ratio after ten days of localised HS to the *vastus lateralis* (Hyldahl et al., 2021) however, the lack improvements in this study is due to the shorter time frame of the intervention. Conversely, after six weeks of repeated bout full body HS, Hesketh and colleagues observed a 21% increase in capillary density, 15% increase in CFPE, 9% increase in capillary contacts and 12% in capillary to fibre ratio ($p <0.05$) in the *vastus lateralis* (Hesketh et al., 2019). After eight weeks of localised HS, Kim and colleagues observed a significant treatment effect in number of capillary contacts, capillary to fibre ratio and CFPE in type II fibres but did not see an overall improvement in the same parameters for type I fibres (Kim et al., 2020). However, both reports disagree with ours given the fact that we did not observe an additive effect of HS on the improvements in response to chronic RE. The observations by Hesketh and colleagues are of great interest as there HS parameters are highly comparable to ours. Crucially, the improvements observed by HS alone by Hesketh are lower compared to RE alone in our investigation. This is justified as the mechanical stress load of progressive ten week RE program in combination with HS. However, Hesketh reported that the improvements by HS were comparable to moderate intensity continuous exercise training. Therefore, the lack of additive effect by HS in our intervention is surprising, especially given the similarities between the CMT at the same depth in the same muscle. However, similar to the observations with eNOS content, it is possible that when HS is the sole stressor it consistently triggered vasodilation and improved cutaneous and subcutaneous blood flow over six weeks (Hesketh et al., 2019), triggering the adaptive capillarisation.

Whereas, we propose that in our investigation the skeletal muscle's capillarisation adaptations to chronic, heavy progressive RE already compensates for the vasodilation and blood flow improvements caused by the full body HS thereby leaving no room for an additive improvement by HS.

7.4.4 Ca^{2+} response

Ca^{2+} was previously introduced and discussed for it is potential role in anabolic synthesis in Chapter 5. It has been reported that stress induces Ca^{2+} transience (Hudson et al., 2016, Hilton et al., 2015). Earlier it had been suggested that eNOS activation maybe Ca^{2+} dependent (Fleming, 2010) (Hudson et al., 2016). However, evidence since has shown that stress induced eNOS activation may be independent from Ca^{2+} influx (Fleming, 2010). However, there is adequate evidence to suggest that Ca^{2+} movement is temperature sensitive. For the first time to our knowledge, we quantified a potential Ca^{2+} response to RE or HS in the human skeletal muscle, by investigating the levels of TRPV1, a polymodal ion channel that has been found to be sensitive to HS and exercise (all investigations in mice) (Hudson et al., 2016). We saw no changes in the total protein expression levels at any of the time points. Furthermore, we did not observe any differences between CON and HEAT groups acutely or chronically. Given the CMT did not increase above ~38°C in either group, it is plausible that the stress magnitude was not large enough to warrant novel synthesis of TRPV1 channels. Additionally, the mechanical stress of heavy, full body RE did not impact the TRPV1 channel translational response either.

In summary, we hypothesised that given RE and HS shown the capability to drive mitochondrial adaptions as well as acute and chronic angiogenic responses within the skeletal muscle as standalone stressors, combining concurrent application of full body HS at 40°C with progressive hypertrophy driven RE may provide an enhanced response compared to either stressor alone.

Mitochondrial biogenesis, content or respiratory capacity was unchanged after ten weeks of heavy, progressive RE. Given the myofibre hypertrophy in response to RE, the unchanged levels of mitochondrial content post intervention implies biogenesis. Full body HS applied concurrently at 40°C did not have an additive effect on mitochondrial biogenesis response to RE. It appears that full body HS does not supply an adequate amount of supplementary stress to instigate a differential response. Furthermore, our results in combination with previous (Kim et al., 2020, Hesketh et al., 2019) findings strongly imply that chronic HS raising CMT by only ~2-3°C to peak of ~38°C is not capable of triggering mitochondrial adaptations. As evidenced by Hafen and colleagues' (Hafen et al., 2019b, Hafen et al., 2018), raising CMT by ~4°C to a peak of

~40°C might be more appropriate. Finally, even though CON group CSA improved markedly more than HEAT group, mitochondrial measurements were not different between the groups.

Acute angiogenic marker response of VEGF and ANGPT1 were not different between the HEAT and CON groups. It is possible that we may have missed the elevating of VEGF given the timing of our biopsy at one hour post as it has been shown that VEGF transcription is elevated rapidly post stress but translation is latent. A more likely reason is that acute and chronic VEGF response is exercise intensity dependent. Low intensity, high contractile frequency exercise seem to trigger acute and chronic angiogenic responses (Yeo et al., 2012, Gavin et al., 2007). Full body HS did not raise CMT from CON significantly, therefore had no impact on the acute or chronic VEGF response. ANGPT1, involved in vascular remodelling (Bloor, 2005) rather than angiogenesis is justified in showing no acute improvements. However, the lack of chronic improvement was surprising given the capillarisation response observed.

One of our key findings in this study was that eNOS levels decreased significantly one hour post RE with the addition of full body HS. We speculate that this phenomena might be a result of acute oxidative stress caused by a bout of vasoconstriction caused by the combination of RE and HS in the *vastus lateralis*, leading to degradation of eNOS. This was not observed in the control group. We further report that capillarisation response to chronic RE (ten weeks) does not improve with concurrent full body HS applied at 40°C. Chronic full body HS as a single stressor is capable of instigating capillarisation (Hesketh et al., 2019), however, when combined with heavy progressive RE does not have an additive effect on the capillarisation response. When combined with HS, capillarisation driven by mechanical stress appears to take priority. A core temperature of 38.18 ± 0.27 °C and peak CMT of 36.79 ± 1.55 °C is not sufficient supplement the capillarisation response to RE in the human skeletal muscle.

7.4.5 Conclusion and significance of findings

In conclusion, full body HS applied at 40°C (30% RH) concurrently with RE is not sufficient to alter mitochondrial biogenesis or elevate the angiogenic makers VEGF and ANGPT 1 acutely. Chronic HS did impact on mitochondrial biogenesis (PGC-1α) content (CS activity) or respiratory capacity (OXPHOS) responses to RE. However, despite a marked difference in fibre CSA (Chapter Five), mitochondrial biogenesis, content or signalling was not different between the groups. Concurrent full body HS causes the acute decrease of eNOS one hour post RE. This finding might carry implications against resistance training in hot environments. Furthermore, while full body HS applied at 40 °C does not improve the chronic capillarisation response to RE.

Limitations

In this investigation, we did not quantify Phospho-eNOS (Ser1177) levels along with total eNOS levels which would have provided further insight in to the acute eNOS response to RE and HS. Moreover, a mid intervention biopsy at five weeks in both CON and HEAT groups would have provided a more comprehensive picture of the capillarisation response to RE and HS. Especially markers such as ANGPT1 (and ANGPT2) which are involved in chronic capillarisation adaptions. Additionally, transcription quantification at the same time points investigated for translation would have provided a further layer of data in order to more completely understand the molecular response, especially for VEGF. Finally, measuring fractional mitochondrial synthesis rate alongside the biomarkers would have provided a more complete picture in demonstrating mitochondrial content and biogenesis. Moreover, additional acute biopsies later than one hour post would have been beneficial to establish a more comprehensive time course as we may have missed upregulation at one hour and at 48 hours.

Future directions

The exploration of a threshold muscle (or core body) that will have an additive effect on the angiogenic and mitochondrial adaptations to heavy RE is of great interest. Moreover, mechanics of the acute degradation of eNOS under the combinative stress of full body HS and RE warrants further investigation.

Chapter 8: General Discussion

8.1 Thesis hypothesis and aims

In this investigation we examined the acute and chronic effects of full body HS on performance and muscle molecular adaptations to long-term RE. It is well documented that RE can improve muscle hypertrophy as well as performance. While limited in comparison to RE, there is evidence to show that HS is also capable of improving muscle contractility (Racinais et al., 2017) and instigating muscle protein synthesis and thereby, hypertrophy (Goto et al., 2011). Furthermore, a limited number of studies have shown that when combined with RE, HS can improve the muscle molecular responses to RE (Kakigi et al., 2011) as well as upon performance measures, especially in low intensity exercise. However, the effect of chronic concurrent full body HS on performance and muscle molecular adaptations to high intensity RE has not been previously investigated.

Therefore, the primary aim of this thesis was to investigate the effects of chronic, concurrent HS on performance adaptations to RE. Secondarily, how the cellular signal transduction pathways regulating skeletal muscle adaptations to RE are altered with long term concurrent heat stress were also investigated.

We hypothesised that chronic full body HS applied concurrently to progressive RE may improve upon performance adaptions to RE as well as the phenotypic and molecular muscle adaptations to RE in the skeletal muscle.

In testing these hypotheses, we firstly sought to establish a reliable muscle heating protocol, given the variable efficacy and inconsistencies with available previously used methods in achieving stable deep muscle temperatures.

8.2 Summary of key findings by experimental chapter

8.2.1 Chapter 2

In chapter two, we explored the possibility of establishing a reliable and practical localised muscle heating protocol that was applicable during exercise. We trialled three heating methods (Exo2® recreational heat wrap, SEFAR® heating mesh and GameReady® Med4Elite™ water perfusion device) and assessed their ability to improve muscle temperature above 38.5-39°C, which we hypothesised was the threshold for improved performance and molecular adaptations to HS in the skeletal muscle. However, all three methods proved unsuitable for practical application. The Exo2® recreational heat wrap and SEFAR® heating mesh methods were

deemed unsuitable due to high discomfort ratings reported by the users during application. Finally, GameReady® Med4Elite™ water perfusion device method failed to raise core muscle temperature above 39°C. Moreover, it failed to meet our test requirements in preserving an adequate range of motion during concurrent RE. In conclusion, we were not able to establish a safe, reliable heating protocol that allowed for the increasing of the muscle temperatures we required whilst preserving the range of motion required for concurrent application with RE.

8.2.2 Chapter 3

In this study we investigated the effect of chronic concurrent, full body HS on performance adaptations to chronic full body RE. Ten weeks of RE improved the absolute lower body strength as well as relative upper body strength of the participants. However, no improvements were seen in speed, agility or peak force measures. Moreover, full body HS applied at 40°C concurrently to long term progressive RE over ten weeks did not have an additive effect on the performance measures investigated.

8.2.3 Chapter 5

In this investigation we explored the acute anabolic synthesis signalling and chronic skeletal muscle hypertrophy response to ten weeks of full body RE. Moreover, we examined whether chronic full body HS was capable of improving upon the anabolic synthesis signalling and hypertrophic response to RE. Key kinases in the mTOR pathway were activated acutely post RE both pre and post intervention. However, there was no acute effect of HS observed on the anabolic synthesis signalling response to RE. Moreover, there was no training or cumulative effect of HS on the anabolic synthesis signalling responses after ten weeks. Statistically meaningful improvements were seen in the CSA of fibre types I and II in CON group but not in the HEAT group. Moreover, CSA improvements in the CON group were markedly higher in comparison to the HEAT group in response to full body RE. Satellite cell content improved in both groups, however, was not different between the groups. Furthermore, myonuclear density improved in the CON group only. Ten weeks of progressive RE improved appendicular LMM in both groups while upper body LMM improved without reaching significance in both groups. Lower body and total LMM reached statistical significance in the CON group but not in the HEAT group. While there were no group interactions observed, it appears that full body HS may attenuate myofibre hypertrophy.

8.2.4 Chapter 6

We further investigated the effect of full body HS on the regulatory activation/deactivation of HSF-1 as well as the response of selected small and large HSPs to RE acutely and chronically.

RE rapidly phosphorylated small HSPs (HSP27 and αB-crystalline) acutely post RE. However, HS had no additive effects on the magnitude of phosphorylation pre or post intervention. In addition, large HSP content was not influenced by HS. Thus, it appears that the full body heat load delivered concurrently at 40°C was not sufficient to improve the large or small HSP response in skeletal muscle to RE acutely or chronically.

8.2.5 Chapter 7

In this chapter, the impact of full body HS on the angiogenic as well as mitochondrial biogenesis response to RE was examined. The mitochondrial biogenesis marker PGC-1α did not respond to acute or chronic RE combined with full body HS. Moreover, HS did not improve upon the mitochondrial activity or content after ten weeks of RE. Key angiogenic markers did not change in response to acute or chronic RE. Interestingly, HS caused eNOS levels to decrease acutely following the post-training RE session only. As capillary contacts as well as capillary density increased in both groups without a difference between groups, it appears that full body HS did not improve upon the phenotypic angiogenic response to RE.

To the best of our knowledge, this is the first investigation to combine concurrent full body HS with progressive, chronic full body RE. We saw no adverse effects on the participants from the combined stress of 40°C HS and RE. A strength of this study was the rigorous training under strict supervision and the high training compliance. Ten weeks of progressive resistance training improved absolute lower body strength as well as relative upper body strength. Overall, acute full body HS did not have an impact on the performance adaptions to RE. The skeletal muscle molecular and phenotypic response to RE was not enhanced upon by HS. Moreover, HS appears to have a negative effect on fibre hypertrophy when applied concurrently with RE. However, requires further investigating with a larger sample size to delineate the validity of this observation.

8.3 General discussion

8.3.1 Heat, resistance exercise and performance

The ability of chronic, structured RE to improve muscle hypertrophy and performance aspects such as strength is well established (Kraemer et al., 2002). A limited number of studies have shown that HS has the ability to improve performance aspects in the skeletal muscle, especially acutely (Binkhorst et al., 1977, Bergh and Ekblom, 1979, Asmussen et al., 1976, Casadio et al., 2017) and some chronically (Goto et al., 2011, Racinais et al., 2017). However, some chronic HS interventions saw no improvements (Stadnyk et al., 2017, Labidi et al., 2020). The limited

number of studies that have investigated localised HS have predominantly done so with the HS applied to the *vastus lateralis*. However, it has also been shown that passive full body HS (acclimation) improves skeletal muscle contractility and aerobic exercise performance (Racinais et al., 2017, Lorenzo et al., 2010). Based on the limited evidence, it appeared at the inception of our hypothesis that chronic HS driven performance improvements are predicated on CMT reaching > 38.5°C (Goto et al., 2011) and was not improved in the cases when below 38.5°C (Stadnyk et al., 2017). We further identified that there was a high degree of variability in the muscle temperatures achieved by the heating methods used in different investigations (Draper et al., 1998, Hawkes et al., 2013, Draper and Ricard, 1995, Ichinoseki-Sekine et al., 2008). Moreover, several methods were not permissive to the concurrent application of RE and HS due to their inability to preserve the ROM of the exercise performed. Therefore, in chapter two we attempted to develop a reliable localised heating protocol/device that was capable of improving CMT over 38.5-39°C reliably and consistently. However, we were not able to establish a method that fulfilled the criteria of improving muscle temperature above 38.5-39°C while simultaneously preserving ROM and ensuring user well-being during exercise. Therefore, in Chapter Three, we chose to combine full body HS (i.e., heat chamber), which has been shown to improve muscle contractility (Racinais et al., 2017), with full body RE, in a chronic concurrent intervention to investigate performance enhancements by HS.

In applying full body HS in chapter three, the issue of preserving ROM was eliminated and allowed for the practising of full body, high load RE. We hypothesised that with the previously shown improvements in muscle contractility with passive full body HS, chronic concurrent HS and further improve upon the performance gains of ten weeks of, progressive RE. As previous reports have shown that passive full body HS applied at 40°C (30%RH) improved aerobic exercise performance (Lorenzo et al., 2010), we chose to apply the same parameters for concurrent RE as the participants would perform RE for long periods of time. We sought to ensure that the HS did not impede the ability of the participants to perform the exercise, while applying a larger combined stress load. The RPE values for each session were not different between the groups throughout the intervention, indicating that full body HS did not negatively impact on the participants' physical capacity to perform the exercise. This is further supported by the fact that training compliance was not different between the groups. It would have been justified to expect a higher average RPE in the HEAT group given the sensory displeasure of performing heavy RE in a hot environment. However, we note the limitation in the reporting of RPE values by a previously untrained, non-athlete cohort as they had no referential training previously to compare their level of exertion to exercising in HS.

To our knowledge, we reported for first time in chapter three that concurrent full body HS does not improve upon the performance gains by RE. In this intervention, absolute lower body strength as well as relative upper body strength improved in both groups. In this previously untrained cohort, these improvements are expected as untrained cohorts respond in a linear manner to progressive RE compared to previously trained cohorts (Narici et al., 1996, Ahtiainen et al., 2003). Therefore, by recruiting a previously untrained, recreationally active cohort we looked to maximise the impact of RE as well as potential effects of HS.

Surprisingly, we did not see a significant increase in CMT or the core temperature in the HEAT group compared to the CON group in chapter three. Consequently, CMT did not improve over the hypothesised threshold of 38.5-39°C. Performance levels in explosive movements such as sprint speed as well as lower and upper body peak force did not improve in either group regardless of the temperature. This could be due to the slow eccentric-concentric contractions of the hypertrophy/strength driven training modality. It has been shown that to improve explosive power and force chronically, higher velocity movement training is more suitable (Kraemer and Ratamess, 2004) in terms of training specificity (Hakkinen et al., 1998). The chosen RE intervention in this study combined single and multiple–joint eccentric-concentric exercises at 60-70% 1RM loading (3 sets x 8-12 repetitions) with periodisation. The prescribed loading scheme was based on literary recommendations for novice trainers for hypertrophy and strength improvements while potentially improving explosive adaptations as well (Kraemer and Ratamess, 2004). Therefore, it was justified in its application to drive hypertrophy, strength as well as force and speed adaptions. We hypothesised that potential additive effects of HS maybe more markedly evident in the case of force and speed as the contribution from the RE was not optimised towards these particular performance aspects. As discussed above, improved contractility appears to be linked to improved muscle temperatures reaching an excess of 38.5-39°C in the human skeletal muscle (Binkhorst et al., 1977, Goto et al., 2011, Racinais et al., 2017). Given that our investigation in chapter three did not elevate muscle temperature significantly, it appears that the contractile properties were not substantially altered to have an additive effect over the improvements by RE in strength. Moreover, HS was not adequate to supplement RE to meaningfully improve explosive performance.

8.3.2 Molecular and phenotypic muscle responses to heat and resistance exercise

Anabolic synthesis

Acute RE upregulates anabolic synthesis in the skeletal muscle. Continuous upregulation of protein synthesis via RE leads to a net positive accumulation of proteins within the skeletal

muscle, chronically manifesting as hypertrophy (Folland and Williams, 2007, Brook et al., 2015). Moreover, it has been previously established that HS as a standalone stressor is capable of improving acute anabolic synthesis in vitro (Goto et al., 2003), in rodent skeletal muscle (Goto et al., 2004, Kojima et al., 2007) as well as human skeletal muscle (Kakigi et al., 2011, Ihsan et al., 2020). Furthermore, chronically localised HS showed the ability to improve muscle size (Goto et al., 2011) Therefore, in chapter five, we investigated whether full body HS applied concurrently can improve upon the acute anabolic synthesis response to RE. Thereby either expediting or improving upon the chronic hypertrophic response to RE. We observed that key kinases involved in the mTOR anabolic synthesis pathway upregulated acutely (one hour) post a bout of RE pre and post the ten week intervention. However, concurrent HS did not improve upon this response. Conversely, in the only other concurrent HS study investigating the anabolic synthesis response in humans, Kakigi and colleagues showed an improved anabolic response after localised HS (Kakigi et al., 2011).

Our conflicting results with those of Kakigi and colleagues may be due to two reasons with the major being the CMT difference. In Kakigi's investigation, they reached a CMT > 40°C via microwave diathermy compared to ~38°C in our investigation with full body HS. We hypothesised that full body HS combined with full body RE will improve upon the acute and chronic response. However, it appears that the overall temperature elevation in the muscle in the HEAT was not an adequate stressor to supplement the typical anabolic synthesis response to RE. Regardless of the RE intensity, it appears that for HS to be effective it requires to exceed 38.5-39°C to improve upon the anabolic synthesis response in the human skeletal muscle. Moreover, as it has been previously seen in rodents (Yoshihara et al., 2013) that the anabolic synthesis response to HS maybe independent of other stressors (such as RE) and may only be activated above a certain temperature threshold. This notion is partially supported by our observations in chapter five of the temperature sensitive polymodal ion channel, TRPV1 levels that has been shown to upregulate at temperatures >38.5-39°C in the skeletal muscle (Hudson et al., 2016). In our investigation, TRPV1 levels were unchanged acutely after RE in the heat group.

A second speculative reason for the divergent findings between our investigation and Kakigi's is that, there may be an anabolic synthesis response ceiling or a threshold, where the skeletal muscle is responding to one stressor overwhelmingly in our case, the mechanical stretch of full body RE. This idea is partially supported by our observations in chapter six where the rapid phosphorylation of small HSP was not different between the groups despite the added stress of full body HS.

Chronic hypertrophy

In chapter five we saw total LMM improve significantly in the CON group. The HEAT group improved but fell short of statistical significance at $p = 0.06$. While appendicular LMM improved in both groups, upper body LMM did not improve in either group while lower body LMM only showed improvements in the CON group. In an untrained cohort, the overall chronic LMM improvements seen are justified (Mitchell et al., 2013). Moreover, the overall LMM improvements coincided with the acute activation and upregulation of the key kinases in the anabolic synthesis pathway. However, no training effect on the mTOR response was seen post intervention. This alludes to the fact that the acute response to RE is not influenced by ten weeks of RE training in previously untrained cohorts.

The fibre specific hypertrophy response was intriguing in this investigation. The fibre CSA of both Type I and II both improved significantly in the CON group by 27% and 33% respectively however, the HEAT group did not improve significantly. While there was no statistically meaningful group interaction, the HEAT group improved only by 13% and 5% respectively. Moreover, the myonuclear density, which has been posited to increase as a key hypertrophic response to RE by fusing with the existing fibres via mitosis (Bruusgaard et al., 2010), only improved in CON group. Taken together this evidence alludes to a potential chronic fibre hypertrophy attenuation effect in the presence of full body HS. This observation has not been seen previously with the application of HS. However, a similar trend was observed after seven weeks of RE combined with cold water immersion. The CSA improvement in type II was greater in response to RE only compared to the combined stress (Fyfe et al., 2019).

Therefore, it appears that there is a detrimental effect on the myofibre hypertrophy when full body RE is combined with a secondary stressor. With HS the impact appears to be on both fibre types. Moreover, it is possible that the lack of statistically meaningful improvement in the lower body LMM in the HEAT group was due to this attenuated fibre CSA. This effect is independent of muscle temperature and appears to be the effect of the presence of a secondary stressor as there was no significant difference in CMT between the two groups. It is difficult to establish the reason for this effect as the anabolic synthesis was not influenced by HS. However, a likely explanation could be increased rates of protein breakdown (Tipton et al., 2018) in the HEAT group compared to the CON group. Interestingly, a recent examination of full body HS alone has shown improvements in fibre CSA in identical heating conditions after six weeks (Hesketh et al., 2019). Therefore, we suggest that even without an improvement in CMT, the additional chronic presence of HS does attenuate the myofibre hypertrophy in combination with RE. This

effect is further independent of acute anabolic synthesis response to RE. Interestingly, with RE, the synthesis response has been shown to last in excess of five hours (Terzis et al., 2008). We speculate whether in the presence of chronic HS, the anabolic synthesis response to RE is down-regulated earlier than ~5 hours due to the skeletal muscle cell being chronically "overstressed". Over ten weeks, such a scenario could lead to a reduced overall protein synthesis rate resulting in a reduced hypertrophy response. Moreover, based on our observations, we speculate whether this was due to attenuation of myonuclear proliferation consequently, reducing the hypertrophy by mitosis. As in rat skeletal muscle, myonuclear proliferation improved with just HS and promoted muscle regeneration (Oishi et al., 2009). This notion of "overstress" is supported by previous observations that low intensity exercises followed by localised HS (Goto et al., 2007) improved hypertrophy. Interestingly, lower body strength, as seen in chapter three, improved in both groups and was not different between groups. Therefore, strength was not impeded. However, in highly trained athletes, chronic strength improvements have been seen even in the absence of hypertrophy (Hakkinen et al., 1988). We report that, even with potentially attenuated fibre hypertrophy due to chronic full body HS, strength improvements may still be observed in untrained cohorts, possibly due to adaptive neuromuscular function gained via ten weeks of progressive RE (Hakkinen, 1994, Häkkinen and Keskinen, 1989). This notion is further supported by the fact that strength is task dependent (Ratamess et al., 2009) and the large neural learning component in performing complex strength tasks (Rutherford and Jones, 1986) where perhaps the contribution of hypertrophy is secondary. However, The lack of blunting of strength despite the discrepancy in hypertrophy between the groups, further brings to focus the recent questions about hypertrophy and maximal strength not being strongly correlated (Buckner et al., 2016), however, this aspect RE is highly debated with some studies showing a clear correlation (Hornsby et al., 2018). Lastly, activated SC content improved in both groups comparably to other RE investigations lasting ≥ ten weeks (Nederveen et al., 2017, Bellamy et al., 2014) with no group difference.

8.3.3 Heat stress, resistance exercise and angiogenesis in the skeletal muscle

In chapter seven we investigated whether HS can improve upon the angiogenic response to RE in the skeletal muscle. All chronic angiogenic adaptations improved in both groups with capillary density and capillary contacts reaching statistical significance which is comparable to previous chronic RE interventions (Nederveen et al., 2016). Moreover, identical full body chronic HS application alone improved, albeit at lesser degree to RE, capillarisation over six weeks in the skeletal muscle (Hesketh et al., 2019). Therefore, it appears that in the skeletal muscle, the overall chronic capillarisation may potentially increase and plateau in response to one overwhelming

stress. In an untrained cohort, it appears that the skeletal muscle overcompensates for the larger stress by improving blood flow, leaving no further room for improvements with a secondary stressor.

Moreover, in chapter five we saw activated Akt levels increase acutely in response to RE, but not further elevated by the combination of HS. It has been shown that hypertrophy associated VEGF synthesis is regulated by the activation of Akt. Intramuscular activation of Akt improved capillary vessel formation in the rabbit skeletal muscle (Takahashi et al., 2002). However, in chapter seven we saw no improvements in acute VEGF or ANGPT1 levels pre or post intervention at the same point. However, it is highly likely that VEGF upregulation occurs latently after one hour and we potentially missed the improved response with our choice of time points. However, since VEGF transcription has been seen to upregulate with increasing muscle temperature (Kuhlenhoelter et al., 2016), it would have been justified to perhaps expect an additive effect on the response to RE. This could be due to the lack of improvement in the muscle temperature in our intervention or the fact that maximal VEGF transcription response had already occurred in response to RE in the skeletal muscle.

Heat, resistance exercise and vasodilation in skeletal muscle

We further observed in Chapter Seven that eNOS levels decreased acute post RE in the HEAT group but not in the CON group. eNOS, the endothelial NO synthase, has also been shown to be under the control of activated Akt in cells (Fontana et al., 2002, Fleming, 2010). Moreover, HSP90 (examined Chapter Six) may serve as molecular scaffold for the activation of eNOS via phosphorylation by Akt (Fontana et al., 2002). In addition, a key mTOR kinase AMPK has also been shown to regulate eNOS via phosphorylation, again with the scaffolding support of HSP90 in endothelial cells (Schulz et al., 2005). However, one major limitation here was that we did not quantify phosphorylated eNOS (Ser1177), which is most common target site for activation of eNOS by Akt and AMPK (Fleming, 2010). It has been previously suggested increasing exercise intensity could lead to sympathetic vasoconstriction (Buckwalter and Clifford, 2001). We posit whether the presence of full body HS at 40°C, even without a significant difference in CMT from CON, caused a relative increase in exercise intensity, leading to a bout of acute vasoconstriction. It has been shown before that vasoconstriction leads to oxidative stress, which in-turn causes reduced eNOS activity (Förstermann et al., 2017, Sena et al., 2018).

8.3.4 Mitochondrial biogenesis, respiratory capacity and content

We saw no effect of full body HS on mitochondrial biogenesis, respiratory capacity or mitochondrial content acutely or chronically. Mitochondrial content and respiratory capacity

measured via CS activity and OXPHOS content were unchanged from pre intervention in response to ten weeks of heavy RE. This observation is consistent with previous chronic RE interventions (Haun et al., 2019, Roberts et al., 2018) where mitochondrial biogenesis occurred in response to chronic RE as CS levels were unchanged. The observation of unchanged mitochondrial content is possibly seen due to the fact the rate of biogenesis was matched to the rate of hypertrophy, therefore when normalised against the total protein content, post intervention it does not signify the improvement in mitochondrial content and respiratory capacity. This supports the idea of mitochondrial dilution in response to chronic RE. Interestingly, as we reported in Chapter 5, CON group fibre CSA improved markedly over the HEAT group, but mitochondrial marker levels were not different between the groups. In this investigation, both CON and HEAT groups undertook identical load matched protocols. If the mitochondrial biogenesis response to RE was only dependent on the load stimulus, it would have been justified to expect higher mitochondrial content and respiratory capacity in the HEAT group, given the CSA improvements were less leading to reduced mitochondrial dilution. It therefore, raises the question whether mitochondrial biogenesis to RE is limited by the attenuation of fibre hypertrophy. Alternatively, chronic application of full body HS concurrently with full body RE maybe attenuating mitochondrial biogenesis as it does muscle fibre CSA in the human skeletal muscle. As localised HS has previously been seen to induce and maintain mitochondrial activity (Hafen et al., 2018, Hafen et al., 2019), it is possible that the combinatory application of HS (even without a difference in CMT) is detrimental to mitochondrial biogenesis, content and respiratory activity.

8.3.5 Heat, resistance exercise and the HSP response in the skeletal muscle

In chapter six we investigated the large and small HSP response as well as the regulatory control of HSF-1 to concurrent full body HS and RE. We saw no difference in the HSP response between the groups. Given the additional stress of full body HS at 40°C, it would have been justified to expect a meaningful difference between the groups. However, as with the anabolic synthesis response, this was not the case, and we suspect for similar reasons. The first reason being the lack of difference in muscle temperature between the groups. In rodents, a temperature gradient has been seen to elevate large and small HSPs at ~40°C and 38-40°C respectively (Larkins et al., 2012a, Locke, 2008, Oishi et al., 2002). For the first time to our knowledge, we posit that the HSP response to cellular stress in human skeletal muscle is stepwise. In the order of phosphorylation of basal small HSPs, translation of small HSPs followed by the translation of large HSPs. In Chapter Six we saw a rapid phosphorylation of small HSPs (HSP27 & αB-crystalline) acutely post RE. Here, the lack of difference between the groups could be due to a

secondary reason, where the rapid phosphorylation response to RE reached the response threshold. Therefore, HS not was not able to further potentiate an additional response. Phosphorylated small HSPs have been shown to stabilise the cytoskeleton in the skeletal muscle cell under mechanical stress to mitigate acute muscle damage (Mounier and Arrigo, 2002). Therefore, it is likely that the high level of acute muscle damage caused by full body RE (represented by the *vastus lateralis*), required a threshold phosphorylation response of small HSPs.

8.4 Overall Limitations and considerations

In this thesis, we investigated the potential benefits of concurrent full body HS on the chronic performance adaptions to full body RE. Moreover, the acute and chronic effect of HS on muscle molecular and phenotypic adaptions in the human skeletal muscle was also examined.

The limitations of testing these questions have been discussed throughout the thesis, specific to each experimental chapter. Here, a number of overall limitations and considerations are presented.

8.4.1 Sample size

The thesis investigation was powered adequately to detect some within and between group changes. However, the observed lack of statistical significance achieved in some variables while showing a tendency towards of the study could be due to the fact the sample sizes were not adequate enough under stringent post-hoc analysis such as Bonferroni. However, we chose that over less stringent tests such as Tukey or Newman-Keuls to increase the robustness of the analysis and decrease false discovery. In some parameters statistical significance was only observed in pooled mixed effects analysis, failing to reach significance when compared group wise (i.e. CFPE and capillary to fibre ratio).

8.4.2 Study design

The overall robustness of the thesis would have benefited from the inclusion of a HS only control group. However, this would have meant the recruitment of a third group. Recruitment was challenging due to the invasive nature of the study including multiple biopsies and the overall 12 week commitment requirement (with familiarisations and post intervention biopsies). Moreover, a heat only group would have had to rest in a heated chamber (40°C) for ~60-90 min for 30 sessions and the duration would have had to be adjusted to time match the longer training sessions towards the end of the RE intervention for consistency. The cost-benefit to the potential

participants was not justified as biopsies would have been required as well without receiving the health benefit of exercise.

Compliance to study requirements

Given the untrained, recreationally active nature of the cohort, it is possible that there were deviations from the study requirements over the course of 12 week period of participation. For instance, participants were asked to refrain from any physical activity on the days before performance testing. However, it is possible that this request was not strictly adhered to in some instances, potentially impacting the outcomes.

8.5 Practical significance of thesis

HS is being widely used as a rehabilitation method in the skeletal muscle by athletes as well as a potential method of attenuating muscle atrophy in older demographics. The advantages of HS has been examined in different modalities for acute performance improvements (predominantly endurance). However, the use of full body concurrent HS for chronic RE for potential improvements in overall athletic performance had not been investigated before. We report that using HS as a secondary stimulus during high intensity RE does not additively improve performance aspects. Moreover, post RE rapid degradation of eNOS seen in the HEAT group may be due to oxidative stress caused by a rapid bout of vasoconstriction. Furthermore, application of HS concurrently with full body RE may chronically attenuate the myofibre hypertrophy of type I and type II fibres in the human skeletal muscle. We may have also established an upper threshold for point of diminishing returns for the combination of HS and RE. However, given there was no strength loss HEAT group, even with attenuated myofibre hypertrophy, could beneficial for athletes restricted by categories who are looking to improve strength without gaining muscle mass. However, these observations require further validation with larger samples sizes. Finally, rapid phosphorylation of small HSPs appears to be a frontier stress response to mechanical stress. Finally, this thesis study indicates towards a “ceiling” in anabolic synthesis as well as cytoprotective response in the skeletal muscle cell in response to RE which has also been previously suggested in response to ingestion of amino acids (Rennie et al., 2002, Bohé et al., 2001).

8.6 Future directions

The potential future directions stemming from this thesis are elaborated on a chapter specific basis. Given the degree of variability in muscle temperatures, heating modalities and the exercise interventions in the literature, we attempted to establish a reliable heating protocol that would

allow for more control over the muscle temperature. This would have allowed for the dose-response testing of the elusive potential threshold temperature in the skeletal muscle for potential additive responses of HS. Therefore, the examination of this threshold temperature is one of the key future directions. Furthermore, combining localised HS with low intensity localised RE has previously shown additive benefits over just exercise. Therefore, combining full body HS with full body RE, but with lower intensity may have potential performance, health and rehabilitation benefits. Moreover, the apparent negative impact of full body HS on muscle fibre hypertrophy when combined with high intensity requires further exploration. Lastly, the reasons for the degradation of eNOS acutely post RE when combined with full body HS is also of great interest.

8.7 Thesis conclusion

Overall, ten weeks of chronic RE improved lower body strength and relative upper body strength, however it had no effect on power, agility or speed. LMM, fibre CSA, SC content and myonuclear density improved in response to RE. Moreover, capillary to fibre ratio and capillary density showed improvements as well. In the anabolic synthesis pathway, key kinases were upregulated acutely post RE. Furthermore, small HSPs were rapidly phosphorylated acutely post RE, but no chronic training effect was seen with the RE intervention. Angiogenic signalling markers did not respond to RE. Mitochondrial biogenesis, respiratory capacity as well as content was unchanged post intervention in both groups. Given the mitochondrial protein content as well as CS activity is normalised against overall total protein content, the lack of change post intervention can be attributed to the phenomena of mitochondrial dilution against RE. Moreover, HS did not impact upon acute mitochondrial biogenesis response to RE. Full body HS applied at 40°C applied concurrently to long term progressive RE over ten weeks did not have an additive effect on the performance measures. There was no effect of HS on the HSP response to RE acutely or chronically. Moreover, no impact of HS was observed on the chronic angiogenic response to RE. However, chronic mitochondrial biogenesis maybe attenuated with the concurrent application of full body RE and HS.

Finally, concurrent full body HS at 40°C failed to raise core muscle temperature in the *vastus lateralis* significantly from the control non-heat stressed group. Therefore, the lack of effect of HS on performance aspects could be due to the fact that CMT was unchanged.

However, even without a significant difference in CMT, the application of full body HS concurrently with heavy full body RE appears to attenuate myonuclear proliferation, myofibre hypertrophy in type I and II, as well as overall LMM hypertrophy. These observations require further investigation with larger sample sizes. Moreover, the lack of difference in mitochondrial

content, respiratory and biogenesis makers between groups despite the marked difference in myofibre CSA between groups suggest that mitochondrial adaptations to RE might be limited by fibre hypertrophy.

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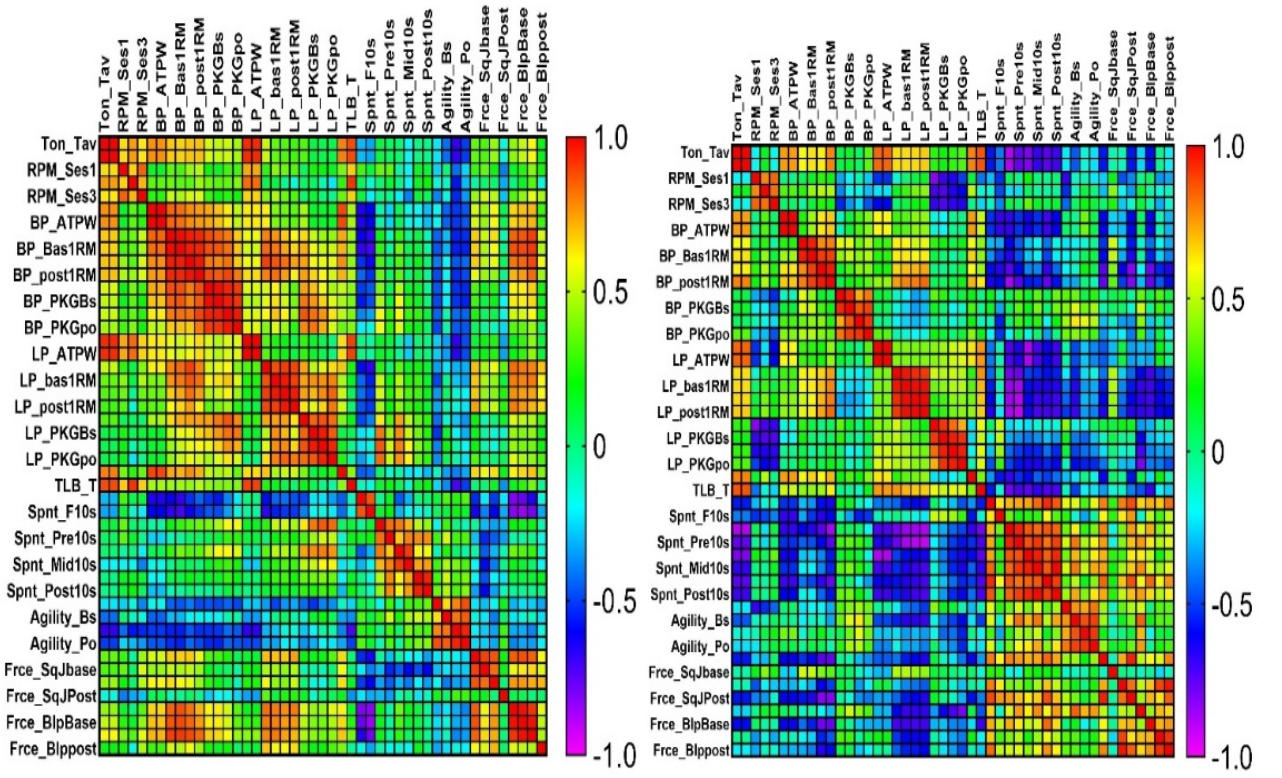
Supplementary material



Ton_Tav=Total average tonnage

RPM_Ses1= Average RPM session1

RPM_Ses3= Average RPM session1

BP_ATPW= Bench press average tonnage per week

BP_Bas1RM= Bench press baseline 1RM

BP_post1RM = Bench press post intervention 1RM

BP_PKGBs= Bench press per KG of LMM baseline

BP_PKGpo= Bench press per KG of LMM post intervention

LP_ATPW= Leg press average tonnage per week

LP_bas1RM= Leg press baseline 1RM

LP_post1RM= Leg press post intervention 1RM

LP_PKGBs= Leg press KG of LMM baseline

LP_PKGpo= Leg press KG of LMM post intervention

TLP_T= Total leg press tonnage

Spnt_F10m = Sprint familiarization (10m)

Spnt_Pre10m= Sprint pre intervention (10m)

Spnt_Mid10s= Sprint mid intervention (10m)

Spnt_Post10s = Sprint post intervention (10m)

Agility_F = Agility familiarization

Agility_ B = Agility pre intervention

Agility_Po = Agility post intervention

Frce_SqJbase = Force square jump pre intervention

Frce_SqJPost = Force square jump post intervention

Frce_BlpBase = Force ballistic push up pre intervention

Frce_Blppost = Force ballistic push up post intervention

Figure 0.1. Pearson's correlation matrices for performance measurements in CON and HEAT group

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