The Effect of Environmental Temperature on Substrate Oxidation and Extracellular Heat Shock Proteins in Response to Heart Rate Matched Moderate-Intensity Cycling in Endurance Trained Males

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Abstract

Endurance exercise combined with environmental heat stress alters substrate metabolism and upregulates extracellular heat shock protein 70 (HSP70) concentrations. Previous research has studied these stimulatory effects of heat stress at matched absolute, external work rates. In the real-world, however, a reduction in absolute work rate typically occurs when training and/or competing in hot environments compared to temperate conditions. The aim of the present investigation was to determine how heat stress impacts substrate oxidation rates and plasma HSP70 expression during endurance exercise regulated by relative physiological work rates, or heart rates (HR). Ten endurance-trained, male cyclists performed two experimental trials in an acute, randomised, counterbalanced cross-over design. Each trial involved a 90-minute bout of cycling exercise at a specified HR (95% of the HR associated with VT_1) in either 18 (TEMP) or 33˚C (HEAT), with ~60% relative humidity. Mean power output (17 ± 11%, *P* = 0.0005) and whole-body energy expenditure (14 ± 8%, *P* = 0.0003) were significantly lower in HEAT. Sweat rate was significantly greater in HEAT (87 ± 63%, *P* = 0.0003). Whole-body carbohydrate oxidation rates were significantly lower in HEAT (19 ± 11%, *P* = 0.002), while fat oxidation rates were not different between-trials. Plasma HSP70 expression was not increased with exercise in either environment. The heat stress-induced reduction in carbohydrate oxidation was associated with the observed reduction in power output (r = 0.64, 95% CI, 0.01, 0.91, *P* = 0.05) and augmented sweat rates (r = 0.85, 95% CI, 0.49, 0.96, *P* = 0.002), while the lack of plasma HSP70 accumulation during endurance exercise may be explained by insufficient exercise duration, inadequate thermal stress, and/or the cycling-exercise modality employed in this present study. These data contribute to our understanding of how moderate environmental heat stress is likely to influence substrate oxidation and plasma HSP70 expression in an ecologically-valid model of endurance exercise.

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Attestation of Authorship

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.”

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Ethics Approval

All experimental procedures of this thesis were approved by the Auckland University of Technology Ethics Committee on 2nd June 2021 (AUTEC reference number 21/121, Appendix 1).

Chapter 1: Introduction

1.1 Background

During endurance exercise, carbohydrates and fatty acids are the primary substrates oxidised to support the adenosine triphosphate (ATP) turnover required for repeated skeletal muscle contraction (Hawley & Leckey, 2015; O'Brien et al., 1993; Watt et al., 2002). In humans, while fat energy storage is effectively unlimited in the context of exercise (Gonzalez et al., 2016; Maunder et al., 2018), endogenous carbohydrate sources, primarily stored as muscle glycogen, can be depleted to near-zero concentrations after exercise of sufficient length and intensity (Ahlborg et al., 1967; Bergström et al., 1967; Bergström & Hultman, 1967; Gonzalez et al., 2016; Hermansen et al., 1967). Indeed, early studies indicated that muscle glycogen depletion associates with fatigue during endurance exercise (Bergström & Hultman, 1967; Hermansen et al., 1967).

Exposure to environmental heat stress stimulates various physiological responses during endurance exercise, including increases in internal temperatures (Febbraio et al., 1994a; Febbraio et al., 1994b; Fernández-Elías et al., 2015), dehydration (Febbraio et al., 1996a; Febbraio et al., 1998; Wilson et al., 1977), and increases in circulating adrenaline concentrations (González-Alonso et al., 1999; Hargreaves et al., 1996b; Powers et al., 1985). These appear to exert metabolic alterations, including a shift toward carbohydrate utilisation and away from fat metabolism (Febbraio et al., 1994a; Febbraio et al., 1994b; Hargreaves et al., 1996a; Jentjens et al., 2002). Furthermore, endurance exercise performed in hot environments also stimulates the expression of heat shock protein 70 (HSP70), which is a protective mechanism to prime and/or enhance immunologic responses and improve recovery (Fleshner et al., 2003; Fleshner & Johnson, 2005).

Importantly, these stimulatory effects of exposure to environmental heat stress on carbohydrate metabolism (Febbraio et al., 1994a; Jentjens et al., 2002; Maunder et al., 2020; Young et al., 1985) and HSP70 expression (Gibson et al., 2014; Walsh et al., 2001; Whitham et al., 2006; Yamada et al., 2007) during endurance exercise have been observed at matched absolute work rates (i.e., power outputs or speeds). In the real-world, however, a reduction in the absolute work rate typically occurs when exercise is performed under heat stress in comparison to equivalent exercise performed in temperate conditions (Boynton et al., 2019; Lorenzo et al., 2010; Maunder et al., 2021a; Maunder et al., 2021b). Therefore, it would be useful for athletes and practitioners exposed to environmental heat stress during training sessions to understand if these stimulatory effects remain present when endurance exercise is performed at matched relative work rates, or heart rates.

Therefore, the purpose of the present thesis was to investigate the following research questions:

1. What is the effect of moderate environmental heat stress on substrate oxidation rates during heart rate-matched moderate-intensity cycling?
2. What is the effect of moderate environmental heat stress on plasma HSP70 expression in response to heart rate-matched moderate-intensity cycling?

I hypothesised that the lower absolute work rate achieved when exercising under moderate environmental heat stress would reduce both energy expenditure and whole-body carbohydrate oxidation rates, but that heat exposure would still increase expression of extracellular HSP70 due to greater increases in core temperature during prolonged cycling exercise.

1.2 Structure and organisation of the thesis

This thesis is set out into five chapters, beginning with this general introduction Chapter 1 (Table 1). Chapter 2 is a literature review; here I provide a narrative overview of the importance of substrate metabolism and HSP70 expression during endurance exercise, and how these are affected by environmental heat stress. In Chapter 3, I describe the methods of the acute, randomised, counterbalanced cross-over study design adopted in this thesis, and also include experimental procedures and how the collected data were analysed. Subsequently, I present the key findings of this thesis in Chapter 4. In Chapter 5, I aimed to elaborate on the findings of the present thesis, with comparison to existing research, and therefore to answer my research questions. Finally, references and appendices are listed at the end of this thesis.

Table 1. Purposes of each chapter in this thesis

Chapter	Title	Purposes
1	Introduction	General introduction to the contents of this thesis
2	Literature review	Narrative literature review demonstrating the importance of substrate metabolism and HSP70 expression to endurance exercise performance, and discussing how these are affected by environmental heat stress
3	Methods	Description of the methods, experimental procedures, and data analyses used in this thesis
4	Results	Demonstration of the key findings of this experimental study
5	Discussion	Discussion on the observed findings of this thesis, with comparison to existing research, and therefore answer the research question *"What is the effect of moderate environmental heat stress on substrate oxidation rates and plasma HSP70 expression in response to heart rate-matched moderate-intensity cycling?"*

Chapter 2: Literature review

2.1 Introduction to substrate metabolism during endurance exercise

Carbohydrates and fatty acids are the primary substrates oxidised to support ATP turnover during endurance exercise (Hawley & Leckey, 2015; O'Brien et al., 1993; Watt et al., 2002), while the oxidation of amino acids contributes only ~5% to total energy expenditure (Tarnopolsky, 2004). In humans, endogenous carbohydrate is stored as glycogen, primarily in skeletal muscles (~80%) and the liver (~10-15%), with minor amounts stored in other tissues including the heart and brain (Jensen et al., 2011). It is estimated that ~500 g of glycogen is stored in skeletal muscles and ~100 g is in the liver (Jensen et al., 2011). Given that 1 g of carbohydrate provides ~4.07 kcal of energy during moderate- to high-intensity exercise at 50-75% $\dot{V}O_{2max}$ (Jeukendrup & Wallis, 2005), these endogenous carbohydrate stores are finite (<3,000 kcal), and can be depleted to near-zero concentrations after exercise of sufficient length and intensity (Ahlborg et al., 1967; Bergström et al., 1967; Bergström & Hultman, 1967; Gonzalez et al., 2016; Hermansen et al., 1967). In contrast, human fat storage is effectively unlimited in the context of endurance exercise (Maunder et al., 2018). Endogenous fat is stored as triacylglycerol, primarily located in subcutaneous and visceral adipose tissue cells (Frayn et al., 2003), and also situated in muscle fibres, known as intramuscular triglyceride (IMTG). Indeed, very lean individuals of 70 kg with 10% body fat have ~7 kg of endogenous fat stores (Maunder et al., 2018), which can provide ~68,250 kcal of energy as 1 g of fat provides ~9.75 kcal of exercise energy (Jeukendrup & Wallis, 2005). Theoretically, ~7 kg of fat is sufficient to provide energy for ~80 hours at cycling at 200 W, assuming gross efficiency of 20%, or ~14.3 $kcal \cdot min^{-1}$ of energy expenditure.

Skeletal muscle glycogen and liver-derived blood glucose are the primary forms of carbohydrate utilised as metabolic fuels during exercise, and the relative contributions made by these two carbohydrate sources to total energy metabolism vary with the exercise intensity and duration (Coggan, 1991). Intramuscular glycogen provides the quantitively most important carbohydrate fuel source during prolonged, moderate- to high-intensity exercise (Bergström et al., 1967; van Loon et al., 2001), and with sufficient length and intensity, skeletal muscle glycogen concentrations can decrease to ~2-7% of pre-exercise contents (Ahlborg et al., 1967; Bergström et al., 1967; Bergström & Hultman, 1967; Hermansen et al., 1967; Hultman & Bergström, 1967). Furthermore, various studies have used these data to support the hypothesis that a reduction in muscle glycogen availability may limit endurance capacity (Bergström et al., 1967; Hargreaves et al., 1995; Hermansen et al., 1967). Indeed, it has been well documented that the ability of muscle to continue contracting is seriously compromised when skeletal muscle glycogen is reduced to low concentrations despite an abundance of other metabolic sources (Bergström et al., 1967), which limits endurance capacity (Bergström et al., 1967; Hargreaves et al., 1995; Hermansen et al., 1967). Similarly, hepatic glycogen concentration is also substantially reduced following prolonged exercise (Casey et al., 2000; Gonzalez et al., 2015; Jeukendrup et al., 1999; Petersen et al., 2004). Mechanistically, liver glucose output is increased to support the rise in muscle glucose uptake and maintain blood glucose concentration; hypoglycaemia may occur when liver glycogenolysis and gluconeogenesis is not able to synthesise sufficient glucose to match glucose uptake (Ahlborg et al., 1994; Wasserman, 2009). There is some evidence that hepatic glycogen may similarly limit endurance capacity (Casey et al., 2000), suggesting that the maintenance of hepatic glycogen concentration is likely to have implications for endurance performance (Gonzalez et al., 2016).

Fat oxidation also makes a significant contribution to total energy expenditure during exercise, with plasma free fatty acids and IMTG serving as the primary fat sources (Hargreaves & Spriet, 2020; Martin et al., 1993; Romijn et al., 1993; van Loon et al.,

2001). During moderate-intensity exercise (~60-65% $\dot{V}O_{2max}$), the peak absolute rate of fat oxidation occurs, providing ~50% of total energy expenditure (Hargreaves & Spriet, 2020), although this varies substantially between-individuals (Maunder et al., 2018). Existing literature indicates that the absolute and relative contribution made by fat oxidation to energy turnover decreases with a greater reliance on carbohydrate oxidation when exercise intensities increase above moderate intensity, while the contribution of fat oxidation to total energy expenditure becomes more predominant as exercise duration increases, which is likely due to the depletion of glycogen stores intensity (Jeukendrup et al., 1998; Romijn et al., 1993; Watt et al., 2002).

Amino acid oxidation during endurance exercise contributes only between <1 and 8% of total ATP provision (Tarnopolsky, 2004), although this contribution might be influenced by factors such as carbohydrate availability (Lemon & Mullin, 1980; Riddell et al., 2003; Tarnopolsky et al., 1995). Lemon and Mullin (1980) observed a greater amount of sweat urea nitrogen excretion during 1-h cycling exercise at ~61% $\dot{V}O_{2max}$ under carbohydrate-depleted condition in comparison to a carbohydrate-loaded condition. These data indicate that the initial muscle glycogen concentration might play an important role in protein catabolism during exercise. More recently, Howarth et al. (2010) reported that exercise-induced muscle glycogen depletion combined with a low-carbohydrate diet prior to 2-h knee extensor exercise at ~75% $\dot{V}O_{2peak}$ resulted in a greater increase in net skeletal muscle protein breakdown during exercise, compared with high-carbohydrate consumption. Evidence suggests that these findings were possibly due to greater protein degradation when carbohydrate availability was low, and a decrease in protein synthesis late in exercise (Howarth et al., 2010). Indeed, it has been reported that net protein oxidation was not significantly different between rest and during 6-h exhaustive exercise with carbohydrate consumption, suggesting that prolonged exhaustive moderate-intensity exercise did not result in an increase in protein degradation and/or a decrease in protein synthesis (Koopman et al., 2004). Therefore, in many studies of substrate metabolism during exercise, protein oxidation is assumed to be negligible.

2.2 Why is endogenous carbohydrate depletion important?

2.2.1 Implications of muscle glycogen depletion for fatigue

The role of muscle glycogen depletion in fatigue associated with endurance exercise has been studied extensively (Hermansen et al., 1967; Nielsen et al., 2011; Ørtenblad et al., 2013). Simplistically, depletion of muscle glycogen concentration may lead to fatigue when carbohydrate availability is insufficient to support the energetic demands of the exercise intensity (Bergström & Hultman, 1967; Hermansen et al., 1967; Ørtenblad et al., 2013). This is supported by findings from several studies reporting depletion of muscle glycogen content to very low concentrations (~2 to 25% of pre-exercise values) at exhaustion during moderate-intensity exercise (Ahlborg et al., 1967; Bergström et al., 1967; Bergström & Hultman, 1967; Hermansen et al., 1967; Hultman & Bergström, 1967), and by studies reporting increased exercise capacity following interventions that successfully increased pre-exercise muscle glycogen content (Ahlborg et al., 1967; Angus et al., 2000; Bergström et al., 1967; Carter et al., 2003; Hermansen et al., 1967; Stellingwerff & Cox, 2014).

However, exhaustion during open-ended moderate-intensity exercise capacity trials occurs with lowered, but not zero, muscle glycogen content. Accordingly, the specific mechanism linking muscle glycogen depletion to fatigue is likely more nuanced than simply ‘running out’ of glycogen. A recently posited mechanism to reconcile these findings is known as the localisation hypothesis (Ørtenblad & Nielsen, 2015; Ørtenblad et al., 2013). Glycogen stores within muscle can be identified in intramyofibrillar, intermyofibrillar, and subsarcolemmal subcellular compartments. There is evidence that the rate of glycogen utilisation during prolonged endurance exercise in different subcellular compartments is distinct (Nielsen et al., 2009). Specifically, greater depletion of intramyofibrillar compared to intermyofibrillar and subsarcolemmal glycogen has been observed during prolonged exercise (Marchand et al., 2007; Nielsen et al., 2011). These data suggest that intramyofibrillar glycogen is preferentially utilised in prolonged

exercise, and therefore greater depletion of this subcellular store may induce fatigue even when the glycogen content of the whole muscle fibre is still considerable (Marchand et al., 2007). Although the functional roles of the different subcellular glycogen stores in human skeletal muscle remains speculative, existing evidence reveals a relationship between the content of intramyofibrillar glycogen and fatigue resistance capacity in rodent models as measured by repeated tetanic contractions elicited by action potential depolarisations (Nielsen et al., 2009), and between intramyofibrillar glycogen content and tetanic Ca^{2+} handling (Nielsen et al., 2014; Ørtenblad et al., 2011). Intramyofibrillar glycogen may have a specific role in excitation-contraction (E-C) via the apparent dependence of Na^{+}-K^{+}-ATPase activity on glycolytic ATP synthesis (Jensen et al., 2020).

Indeed, a recent study in rats appeared to show the Na^{+}-K^{+}-ATPases are dependent on glycogen from the intramyofibrillar store only, whereas myosin ATPases are supplied by the oxidation of intra- and intermyofibrillar, and sarcoplasmic reticulum Ca^{2+} ATPases utilise intermyofibrillar glycogen (Nielsen et al., 2022). Therefore, it is plausible that depleted intramyofibrillar glycogen availability, prior to the attainment of very low global muscle glycogen concentrations, may have a role in fatigue during prolonged exercise.

2.2.2 Implications of muscle glycogen depletion in signalling related to training adaptation

Several studies have suggested that performing endurance exercise with low glycogen availability might stimulate exercise-induced signalling responses associated with enhanced oxidative capacity (Cox et al., 2010; Hulston et al., 2010; Morton et al., 1985; Yeo et al., 2008), and potentially improve endurance performance (Cochran et al., 2015; Hansen et al., 2005). Hansen et al. (2005) reported that a ten-week intervention of endurance knee-extensor exercise with low glycogen availability significantly increased resting muscle glycogen content in humans (from ~400 to ~700 $mmol \cdot kg^{-1}$, from before

to after 10 weeks of training, $P < 0.05$), indicating an increase in muscle glycogen stores. More recently, Hulston et al. (2010) found that muscle-derived triacylglycerol oxidation significantly increased after a 3-week training intervention of endurance exercise with low muscle glycogen content (from 16 ± 1 to 23 ± 1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, $P < 0.05$), but this was not observed from participants who trained with high muscle glycogen content. Furthermore, these authors observed that endurance exercise training with low muscle glycogen was sufficient to increase mean power output during a 60-min cycling time trial (from 278 ± 11 to 307 ± 10 W, from pre- to post-training, $P < 0.05$), although the improvement in performance was similar in the two intervention groups (10.5% vs. 10.2%, in low vs. high glycogen content groups). These findings, therefore, indicate that endurance exercise training with low muscle glycogen might improve the capacity for fatty acid oxidation to a greater degree than training with normal glycogen levels (Hulston et al., 2010).

Mechanistically, exercise-induced expression and activation of peroxisome proliferator-activated γ-receptor co-activator 1α (PGC-1α), which is responsible for the activation of mitochondrial transcription factors including nuclear respiratory factors (NRF-1 and -2), and the mitochondrial transcription factor A (Tfam) (Jäger et al., 2007), has been suggested to influence adaptative responses to endurance exercise training (Knuiman et al., 2015). Mathai et al. (2008) demonstrated a relationship between muscle glycogen stores the changes in PGC-1α protein abundance. Indeed, increases in PGC-1α activity and mitochondrial volume enhance oxidative capacity via the increase in fatty acid β-oxidation and mitigating glycogenolysis (Margolis & Pasiakos, 2013), that allows skeletal muscle glycogen to be spared, and therefore plausibly results in the delay of muscular fatigue and enhanced exercise performance. Therefore, existing literature supports that carbohydrate metabolism may have a role both acute fatigue and stimulation of training adaptations to endurance exercise.

2.3 Environmental heat stress and substrate metabolism during endurance exercise

Environmental heat stress is a potent stimulus for whole-body metabolic alterations during acute prolonged exercise (Edwards et al., 1972; Febbraio et al., 1994b). From a metabolic standpoint, increased rates of carbohydrate metabolism (Kozłowski et al., 1985; Rowell, 1974; Sawka et al., 1985; Young et al., 1985), and decreased fat utilisation (Fink et al., 1975; González-Alonso et al., 1999), have been observed when performing prolonged exercise in hot compared to temperate conditions. Specifically, greater muscle glycogenolysis (Febbraio et al., 1994a; Febbraio et al., 1994b), whole-body carbohydrate oxidation rates (Jentjens et al., 2002), hepatic glucose output (Hargreaves et al., 1996a), and muscle and blood lactate accumulation (Febbraio et al., 1994a; Jentjens et al., 2002) have been reported when prolonged exercise is performed in a hot environment in comparison to temperate conditions, along with decreased fatty acid utilisation (Gagnon et al., 2020). Furthermore, evidence has shown these effects may be exacerbated when endurance exercise is performed with carbohydrate feeding, as exposure to heat stress decreases exogenous glucose oxidation, with an increase in muscle glycogen utilisation in comparison to the same exercise procedures in a temperate environment (Jentjens et al., 2002). Several mechanisms have emerged as contributors to these metabolic effects of exposure to heat stress during prolonged exercise, including increases in internal temperatures (Febbraio et al., 1994a; Febbraio et al., 1994b; Fernández-Elías et al., 2015), dehydration (Febbraio et al., 1996a; Febbraio et al., 1998; Wilson et al., 1977), and increases in circulating adrenaline concentrations (González-Alonso et al., 1999; Hargreaves et al., 1996b; Powers et al., 1985).

2.3.1 Increases in internal temperatures

Exercise performed under heat stress induces a larger increase in core temperature than when the same exercise is performed in temperate conditions, due to the reduced thermal gradient for heat dissipation (Febbraio et al., 1994a; Febbraio et al., 1996b; Hargreaves et al., 1996a). The larger rise in core temperature associated with prolonged exercise performed under heat stress likely contributes to the associated metabolic effects. Febbraio et al. (1996b) observed a greater increase in rectal temperature during 40-min cycling exercise at 65% $\dot{V}O_{2max}$ in temperate environment (20˚C) compared with the same exercise performed in cool environment (3˚C). In this investigation, muscle and blood lactate and blood glucose concentrations were similar between 20 and 3˚C conditions, while plasma catecholamines and net muscle glycogen utilisation were higher at 20˚C environment. These findings suggest that blunting the rise in core temperature during exercise resulted in a reduction in muscle glycogenolysis in exercising humans (Febbraio et al., 1996b), and therefore that the greater core temperature increase during exercise performed under environmental heat stress may contribute to the observed effects on carbohydrate metabolism.

Furthermore, intramuscular temperature increases in proportion to the workload during exercise (Saltin & Hermansen, 1966), and the increase in muscle temperature is more pronounced in magnitude when exercising under environmental heat stress (Febbraio et al., 1994a; Febbraio et al., 1994b; González-Alonso et al., 1999; Hargreaves et al., 1996a). Several studies suggest elevated muscle temperature stimulates muscle glycogenolysis during exercise (Edwards et al., 1972; Febbraio et al., 1996a; Starkie et al., 1999). For example, Febbraio et al. (1996a) reported that 60-min passive, single-legged heating, which increased the *vastus lateralis* temperature by 2.2˚C from pre-heating temperature and continued to increase significantly during exercise, resulted in greater skeletal muscle glycogen metabolism during 2-min cycling exercise at 115% $\dot{V}O_{2max}$ in comparison to the same exercise protocol without pre-heating treatment. Importantly, core temperature was not different between the trials, suggesting that the

metabolic effects were attributable to the heating of muscle itself (Febbraio et al., 1996a). In a subsequent study, Starkie et al. (1999) used water-perfused cuffs to heat one limb, and cool the other, prior to 20-min cycling exercise at 70% $\dot{V}O_{2peak}$. Muscle glycogen utilisation was increased in the heated limb (208 ± 23 vs. 118 ± 22 $mmol \cdot kg^{-1}$), again suggesting that carbohydrate utilisation during exercise responds to muscle temperature (Starkie et al., 1999).

2.3.2 Dehydration

Exercise-induced dehydration occurs when fluid intake does not match fluid loss, primarily via sweating. Sweat rates are elevated during exercise performed in hot environments, as sweating is the primary means of heat dissipation during exercise (Wendt et al., 2007). Existing literature indicates that a progressive dehydration during exercise contributes to increased core temperature (Costill et al., 1970; Hamilton et al., 1991; Montain & Coyle, 1992), reduced stroke volume (Hamilton et al., 1991; Saltin & Stenberg, 1964), and increased heart rate (Hamilton et al., 1991; Montain & Coyle, 1992; Saltin & Stenberg, 1964). Exercise-induced dehydration *per se* stimulates muscle glycogenolysis during endurance exercise in both temperate (~20-23˚C) (Hargreaves et al., 1996b; Logan-Sprenger et al., 2013; Logan-Sprenger et al., 2015; Logan-Sprenger et al., 2012) and hot (~33-35˚C) (Fernández-Elías et al., 2015; González-Alonso et al., 1999) environments, in comparison to the same exercising trials with the maintenance of euhydration by fluid ingestion. Mechanistically, González-Alonso et al. (1999) reported that a cycling exercise to exhaustion at ~60% $\dot{V}O_{2max}$ in a hot environment (35 °C, 40-50 % rH) combined with progressive dehydration resulted in greater reductions in contracting leg blood flow and cardiac output were observed, and a greater difference in femoral arterio-venous O_2 as compared with the similar exercise duration and intensity with the maintenance of euhydration. These authors suggested that even if contracting limb blood flow during prolonged exercise under heat-stress conditions is decreased, the

difference in arterio-venous O_2 is increased accordingly, so that local O_2 availability is not compromised (González-Alonso et al., 1999; González-Alonso et al., 1998). Furthermore, González-Alonso et al. (1999) also showed that a decrease in muscle blood flow with dehydration did not affect either the deliveries of glucose and free fatty acids (FFA), or lactate removal during exercise, while dehydration during exercise under environmental heat stress resulted in elevated carbohydrate oxidation and lactate production. Therefore, it has been suggested that the hyperthermic, rather than circulatory, effects of dehydration are more likely to be the stimulation of carbohydrate metabolism when exercise is performed with dehydration (González-Alonso et al., 1998).

2.3.3 Increases in circulating adrenaline concentrations

Plasma adrenaline and/or noradrenaline concentrations increase to a greater extent during endurance exercise under environmental heat stress compared to equivalent exercise performed in temperate conditions (Dolny & Lemon, 1988; Febbraio et al., 1994a; Hargreaves et al., 1996a; Niess et al., 2003; Parkin et al., 1999), and this may contribute to the increase in intramuscular glycogen utilisation during endurance exercise in the hot environments (Febbraio et al., 1994a; Febbraio et al., 1996b; Hargreaves et al., 1996b). Febbraio et al. (1998) demonstrated that an adrenaline infusion prior to and during 40-min cycling exercise at ~70% $\dot{V}O_{2peak}$ in temperate conditions (20-22˚C) resulted in a greater decrease in muscle glycogen concentration during exercise, and thus total muscle glycogen utilisation was greater (303 ± 30 vs. 224 ± 37 mmol·kg^{-1}, $P < 0.01$). These data therefore suggest a role for circulating adrenaline concentrations in the stimulation of carbohydrate metabolism. Combining this with previous findings from the same laboratory reporting that net muscle glycogen utilisation and circulating adrenaline concentration were greater during 40-min exercise at 70% $\dot{V}O_{2max}$ in 40 vs. 20°C conditions (Febbraio et al., 1994a; Febbraio et al., 1994b), the literature supports that the elevated adrenaline concentrations during exercise

performed under heat stress contribute to the stimulatory effects on carbohydrate metabolism.

2.4 Summary

In summary, carbohydrate availability during endurance exercise has implications in both acute fatigue and training adaptations relevant to endurance performance. Endurance exercise performed under environmental heat stress appears to exert metabolic alterations, including a shift toward carbohydrate and away from fat metabolism (Febbraio et al., 1994a; Febbraio et al., 1994b; Hargreaves et al., 1996a; Jentjens et al., 2002). However, these effects have only been studied at matched absolute work rates, i.e., the same running speeds (Marino et al., 2001), or at power outputs (Febbraio et al., 1994a; Jentjens et al., 2002; Maunder et al., 2020; Young et al., 1985). In the real-world, however, a reduction in the absolute work rate is typically observed when exercise is performed under heat stress observed compared to the same exercise performed in temperate conditions (Boynton et al., 2019; Lorenzo et al., 2010; Maunder et al., 2021a; Maunder et al., 2021b). As available evidence indicates the importance of exercise intensity on substrate utilisation during endurance exercise (Boynton et al., 2019; Lorenzo et al., 2010; Maunder et al., 2021a; Maunder et al., 2021b), it is possible that carbohydrate metabolism may be decreased with the reduction in absolute work rates when endurance exercise is performed in environmental heat stress. Therefore, it might be useful for practitioners if there is more relevant investigation into the stimulatory effect of exposure to heat stress during endurance exercise using matched relative physiological work rates, or heart rates on substrate metabolism.

2.5 Introduction to heat shock protein expression during endurance exercise

Cells respond to various stressors by synthesising a family of highly conserved polypeptides, which are known as stress proteins (Ashburner & Bonner, 1979; Salo et al., 1991). Heat shock proteins (HSPs), which were initially found to be upregulated with elevated temperature, have been the most widely investigated of the stress proteins (Liu & Steinacker, 2001). For example, classic work reported that temperature alterations induced structural modifications in the salivary gland chromosomes of *Drosophila melanogaster* (Ritossa, 1962). While transcription of most genes was suppressed, a 70,000-dalton heat shock protein (HSP70) increased with heat-shock treatment (Ashburner & Bonner, 1979; Ingolia et al., 1982). Subsequent to these initial data, the synthesis of HSPs in response to increasing temperature has been demonstrated in tissues of various organisms, such as yeast (Ingolia et al., 1982), rats (Lim et al., 1984), and humans (Mues et al., 1986).

The HSPs are categorised based on their molecular weight. These include small HSPs, HSP20, HSP60, HSP70, HSP90, and HSP100 (Krueger et al., 2019; Morton et al., 2009b). In the present thesis, my focus is on the HSP70 family, which is considered as the most temperature sensitive and highly conserved of the HSPs (Kregel, 2002; Morton et al., 2009b). Four main isoforms of the HSP70 family are observed in mammalian cells, the most prominent of which include an abundant, constitutive isoform, HSP73, and a highly stress-inducible isoform, HSP72, both of which have been extensively studied (Welch & Suhan, 1986). Two other HSP70 isoforms are not heat inducible including glucose-regulated protein 75 (GRP75) and GRP78 (Locke, 1997). Although both HSP72 and HSP73 isoforms share common protein sequence alignments, they are synthesised in different circumstances (Kregel, 2002). Under unstressed conditions, HSP73 is constitutively expressed in the cytoplasm to chaperone misfolded and denatured proteins, which possibly facilitates renaturation (Welch & Suhan, 1986). On the other

hand, HSP72 quantities are not substantial under unstressed conditions, but HSP72 can be rapidly synthesised in the cytoplasm to respond to stressors (Lewis & Pelham, 1985), including exercise and temperature (Fehrenbach et al., 2005; Gibson et al., 2014; Skidmore et al., 1995; Whitham et al., 2007).

2.6 Roles of the HSP70 family

Exposure of cells to stressors, including hyperthermia, anoxia, ultraviolet irradiation, and toxic drugs, can result in damage to cellular structures. Although the direct link is not clear, there might be a relationship between these structural damages and cell death (Mosser et al., 1997). Cell death often occurs through a self-destruction pathway, which is known as apoptosis (Gabai & Sherman, 2002; Hale et al., 1996; Vaux & Strasser, 1996). The apoptotic response is important for maintenance of the appropriate quantity of cells in all multicellular organisms (Mosser et al., 1997), as it eliminates cells impaired by either physiological or environmental stressors, and therefore enhances organism survival (Beere et al., 2000). Several studies have indicated that deregulated apoptosis is an underlying basis of numerous human pathologies, ranging from viral infection (Rinkenberger & Korsmeyer, 1997) to cancers (Evan & Vousden, 2001).

Under hyperthermic conditions (43-45˚C), protein denaturation and aggregation have been observed in the nuclear matrix of mammalian cells, which is considered as the most thermally labile component (Gabai & Sherman, 2002; Roti Roti et al., 1998). Following exposure to heat shock, protein adsorbs to the nuclear matrix, resulting in an increase in the protein associated with isolated nuclei or nuclear matrices. Indeed, it has been documented that changes in protein contents were observed after exposure to heat shock of 40˚C for 20 min, or following duration as short as 5 min at 47˚C (Roti Roti et al., 1998). Evidence indicates that protein denaturation plays an important role in cell killing

through the activation of a programmed cascade of events leading to cell demise (Calderwood & Hahn, 1983; Gabai & Sherman, 2002).

Heat shock can modulate apoptosis via various signalling pathways including death-signalling pathways and survival pathways (Gabai & Sherman, 2002). Briefly, the major element of the heat-activated apoptotic cascade is efflux from mitochondria of cytochrome *c*, which activates a cascade of caspases, including caspase-9 and caspase-3, and subsequently leads to the execution of apoptosis (Gabai & Sherman, 2002). One of the two important death-signalling pathways relates the activation of stress kinase c-Jun NH_2 terminal kinase (JNK). This pathway involves stress-induced efflux of cytochrome *c* and possibly via cleavage of Bid protein, a proapoptotic BH3-only member of the Bcl2 group (Tournier et al., 2000), or phosphorylation of anti-apoptotic proteins (Kharbanda et al., 2000; Srivastava et al., 1999; Yamamoto et al., 1999). Another heat-induced apoptotic pathway via JNK activation involves the induction of Fas ligand with subsequent engagement of the caspase-8 and caspase-3 cascades (Sreedhar et al., 2000). Furthermore, heat shock can also trigger survival pathways through the activation of Akt and extracellular signal-regulated kinase (ERK) (Ma et al., 2001; Woessmann et al., 1999). In general, the Akt activation was observed to inhibit JNK, that possibly occurs through the induction of JNK-inhibitory protein-1, while ERK can interfere with Fas-induced activation of caspase-8 (Tran et al., 2001), and cytochrome *c*-induced activation of caspase-3 (Erhardt et al., 1999).

Cells respond to heat-induced apoptosis through the expression of HSPs, which is a protective mechanism for interfering with the cell death programme (Gabai & Sherman, 2002). It has been suggested that HSP70 is synthesised as a conserved response to environmental stress (Deshaies et al., 1988). With strong cytoprotective effects, HSP70 acts as a molecular chaperone that accompanies misfolded and denatured proteins to maintain cellular homeostasis (Whitham & Fortes, 2008), and subsequently contributes to the development of thermotolerance (Li et al., 1995; Liu et al., 1992). Evidence indicates that the expression of HSP70 family alone was sufficient to reduce protein

aggregation in the nucleus and accelerate refolding of luciferase after heat shock (Nollen et al., 1999; Stege et al., 1994).

A single bout of exercise *per se* imposes stress on the thermoregulatory system which elicits a multitude of physiological responses (i.e., increases in core and/or local muscle temperatures, and stimulation of sweat loss) (Whitham & Fortes, 2008). As a result, training programmes involving repetitive exercise episodes over a sufficient period result in heat shock responses, including activation of heat shock transcription factors, changes in HSP expression, and upregulation of mRNA transcription (Horowitz, 2016; Quindry et al., 2007; Staib et al., 2007). Given the roles described above that HSPs have in managing cellular stress, the cumulative effect of these heat shock responses might be to support adaptations to exercise training and subsequent performance (Hawley et al., 2018). Expression of HSP70 is highly responsive to prolonged exercise training (Liu et al., 1999; Sandström et al., 2008), and this may potentially reflect the intramuscular hyperthermia, reactive oxygen species (ROS) accumulation, and metabolic stress associated with exercise (Liu et al., 1999). Furthermore, as molecular chaperones, HSPs may be specifically implicated in supporting the mitochondrial biogenesis sought with endurance training by chaperoning newly-synthesised, nuclear-encoded mitochondrial proteins from the nucleus to the mitochondria (Henstridge et al., 2014; Skidmore et al., 1995; Young et al., 2003). Therefore, stimulation of intramuscular HSP70 expression may be a fundamental adaptative response to manage the cellular stress generated by exercise.

2.7 Factors influencing the expression of HSP70 in skeletal muscle

Exercise-induced HSP70 expression has been observed in various organs and tissues of both rodents and humans, including blood (Fehrenbach et al., 2005; Gibson et al.,

2014), liver (Salo et al., 1991), brain (Walter et al., 1998), heart (Ruell et al., 2004; Salo et al., 1991), and skeletal muscle (Febbraio & Koukoulas, 2000; Skidmore et al., 1995). In humans, the induction of HSP70 expression in skeletal muscle following both a single bout of endurance exercise (Puntschart et al., 1996) and repeated training (Liu et al., 1999; Sandström et al., 2008) has been documented. As mentioned above, this upregulated expression of HSP70 may be in response to potential exercise-induced stressors including elevated internal temperature (Skidmore et al., 1995), reduced pH (Liu et al., 1999), increased Ca^{2+} concentration (Hawley et al., 2006), production of reactive oxygen species (ROS) (Salo et al., 1991; Zuo et al., 2000), increased metabolic rates (Liu et al., 2000), and changes in substrate availability (Febbraio & Koukoulas, 2000; Febbraio et al., 2002b). Although the precise mechanisms for how each of these exercise-associated physiological stressors results in augmented muscle HSP70 expression is not clear, the influence of common exercise characteristics, such as increases in temperatures, exercise intensity, and carbohydrate availability, have been more extensively studied.

2.7.1 Elevated temperature

Thermal stress has been considered as a primary stimulus for activating the synthesis of HSPs, especially HSP70 which is considered as the most thermally sensitive HSP family (Locke & Noble, 1995; Mizzen & Welch, 1988; Welch & Suhan, 1986). Supporting evidence for the role of environmental temperature in upregulating HSP70 expression is provided from various experimental approaches in rodent skeletal muscle. For example, Salo et al. (1991) observed that a 2-h hindlimb incubation at 42˚C increased HSP70 concentrations in contracting muscles of exercising rats by over two-fold when compared with the contralateral hindlimb incubated at 37˚C for 2 h, suggesting that a ~3-5˚C increase in incubation temperature is sufficient for upregulating HSP70 expression. Oishi et al (2002) directly heated rat hindlimb muscles and then maintained at 42˚C for 60 min,

by increasing the air temperature in a stainless steel can inserted into the soleus and plantaris muscles. They found that both rectal and non-heated hindlimb muscle temperatures increased slightly following 60-min internal, passive muscle heating (by 1.1 and 1.3˚C, respectively), but augmented HSP72 expression was only observed from the soleus and plantaris muscles in heated hindlimb, not the contralateral hindlimb (the medial gastrocnemius muscle). Indeed, the expression levels of HSP72 in the soleus and plantaris muscles of contralateral non-heat-stressed hindlimb was not different from age-matched sedentary rats (Oishi et al., 2002).

Based on the known robust induction of skeletal muscle HSP70 in response to exercise-induced hyperthermia, it was postulated that elevations in core and contracting muscle temperatures might be an initiating factor in HSP70 activation during prolonged exercise (Henstridge et al., 2016). Kim et al. (2004) observed a significant increase in rectal temperature in exercising rodents after treadmill running to exhaustion in a temperate (23˚C) and a hot (41˚C) environments, but no main effect was found in a cool environment (12˚C). Furthermore, significant increases in HSP70 accumulation in soleus muscle were observed at exhaustion both temperate and hot conditions, but not in the cool environment. Therefore, based on the unchanged rectal temperature in the cool environment, it was suggested that exercise-associated skeletal muscle HSP70 levels might depend on increased core temperature in rodent models (Kim et al., 2004). Skidmore et al. (1995) reported that the combination of environmental heat stress and exercise-induced hyperthermia (a 3˚C increase in core temperature) resulted in greater expression of post-exercise HSP70 in the gastrocnemius and soleus muscles of rats compared with resting rats. However, it should be noted that exercise-associated expression of skeletal muscle HSP70 occurs in a specific manner as the increased HSP70 contents were only observed in the locomotor muscles of the hindlimb, but not in the extensor digitorum longus muscle, which is unlikely to be activated to a significant extent during treadmill running. However, elevations in HSP70 accumulation were also observed in rodent skeletal muscles following exercise with a thermal clamp (the

maintenance of core body temperature at 38˚C) in a cool environment (14˚C), suggesting that stimulators other than heat stress might also contribute to the expression of HSP70 in contracting muscles during prolonged exercise (Skidmore et al., 1995).

Although evidence for the augmented HSP70 contents in response to elevated environmental temperatures is well documented in animals, comparable findings in humans are relatively sparse. Morton et al. (2006) reported that a bout of 45-min treadmill running at a speed corresponding to the lactate threshold in a temperate environment (~18˚C) significantly increased both rectal and the *vastus lateralis* muscle temperatures, and observed a significant increase in the accumulation of HSP70 in skeletal muscle. In a subsequent study from the same laboratory, Morton et al. (2007) demonstrated that although passive heating through 1-h of immersion in hot water (45˚C) increased both rectal (1.5 ± 0.8˚C) and the *vastus lateralis* muscle (3.6 ± 0.5˚C) temperatures to values comparable to those seen during exercise performed under environmental stress, no main effect of the passive heating treatment was observed for HSP70 expression. These data indicate that without an exercise stimulus, increases in both core and local muscle temperatures were insufficient to mediate the exercise-induced production of HSP70 in skeletal muscle, indicating the importance of contractile activity for stimulating HSP70 expression in skeletal muscle (Morton et al., 2007). More recently, Cuthbert et al. (2019) observed that mean core temperature during endurance exercise was not statistically different between environmental temperatures at 7, 20, and 33˚C despite mean skin temperature that was significantly higher in a hot environment (33˚C) than temperate (20˚C) and cool (7˚C) environments. They found that a 60-min cycling exercise at 60% $\dot{V}O_{2peak}$ in all three environments increased HSP70 mRNA expression in the *vastus lateralis* muscle by over two-fold at 3-h post-exercise compared with pre-exercise concentration, but no significant difference in expression was observed between temperatures (P = 0.103). These findings could be, at least partly, explained by the similar thermoregulatory response between environments. Collectively, these findings demonstrate that thermal stress is not the sole signal responsible for the augmentation

of HSP70 expression, thus suggesting additional signals arising during exercise could possibly play important roles in the stimulation of HSP70 expression in skeletal muscle.

2.7.2 Exercise intensity

Exercise intensity, via effects on factors such as muscular ischemia, glycogen depletion, and reactive oxygen species, may influence the HSP70 response (Liu et al., 1999). To date, several studies have investigated the effect of a single exercise bout at various intensities on HSP70 expression in animal skeletal muscle. Milne and Noble (2002) observed HSP70 expression in rat soleus and *vastus lateralis* muscles following treadmill running at various intensities (15 to 33 $m \cdot min^{-1}$), and reported HSP70 expression in the soleus significantly increased at a slower running speed (15 $m \cdot min^{-1}$), but subsequently expression decreased as exercise intensity progressed. The soleus muscle is rich in slow oxidative fibres (Armstrong & Phelps, 1984), and is extensively recruited during normal locomotion and standing posture (Roy et al., 1991). These may indicate an unloading function of the slower contracting soleus muscle when surrounding, faster oxidative and/or glycolytic fibres are recruited at higher running speeds (Milne & Noble, 2002). In accordance, they reported HSP70 expression in the *vastus lateralis* was only stimulated at running speeds of 24 $m \cdot min^{-1}$ and above. In contrast to the soleus, the major composition of red and white portions of the *vastus lateralis* muscle is fast oxidative glycolytic and fast glycolytic, respectively (Armstrong & Phelps, 1984). These differences in muscle fibre type composition and oxidative capacity may explain why exercise-associated increased expression of HSP70 in the *vastus lateralis* occurred at higher running speeds (Laughlin & Armstrong, 1982). Therefore, exercise-induced increases in HSP70 expression in skeletal muscles appeared to exhibit an intensity-dependent threshold, relating to their known recruitment patterns (Milne & Noble, 2002).

Although the response of intracellular HSP70 expression to a single bout of exercise has been less frequently studied in humans, available evidence demonstrates relatively consistent findings between animal and human models. Puntschart et al. (1996) reported that a single bout of 30-min treadmill exercise at the individual anaerobic threshold was sufficient to increase HSP70 mRNA expression in *vastus lateralis*. Therefore, it suggests that HSP70 expression in skeletal muscle is responsive to exercise intensity, rather than duration, as exercise-associated HSP70 upregulation in human skeletal muscle was observed even though exercise was only 30 minutes in duration (Puntschart et al., 1996). More recently, Magalhães et al. (2010) also observed an increase in intracellular HSP72 expression at the end of a 90-min treadmill exercise at 50% $\dot{V}O_{2peak}$ in unacclimated individuals. Collectively, these supporting evidence from both animal and human studies suggest that a single exercise bout of sufficient intensity can elicit a rise in HSP70 expression in contracting skeletal muscle.

Stimulation of HSP70 expression may mediate exercise training-induced remodelling of skeletal muscle (Hawley et al., 2018; Horowitz, 2016; Liu et al., 1999). Brinkmann et al. (2019) observed that three months of endurance exercise training increased the resting *vastus lateralis* HSP70 content of individuals with non-insulin-dependent type 2 diabetes mellitus. Liu et al. (1999) measured resting HSP70 expression in the *vastus lateralis* muscle of highly trained rowers during four weeks of rowing exercise training, which consisted of four one-week training phases with different training loads. The HSP70 expression fluctuated throughout the intervention, and increased to reach a maximum of five times the pre-training concentration. Indeed, this study reported that the greatest increase in HSP70 expression, which was 123% from the previous training week, was observed at the end of the second phase, which contained the maximum exercise load. Furthermore, a reduction in HSP70 expression was detected at the end of the fourth training phase when the rowers trained with reduced load. These findings suggest that the HSP70 response to endurance exercise training might relate to the total exercise load. Conversely, Morton et al. (2009a) observed that a six-week endurance exercise

training intervention using either interval or continuous protocol did not statistically affect the resting *vastus lateralis* HSP70 content. These authors suggest that the lack of HSP70 adaptation to endurance training exercise was possibly due to insufficient training intensity of each training (Morton et al., 2009a). Based on these findings, the stimulated HSP70 expression in resting muscle following either a single bout of exercise or a period of exercise training seems to be related to exercise intensity and the amount of training load, rather than exercise duration.

2.7.3 Carbohydrate availability

Carbohydrate availability might be a factor related to the exercise-induced production of HSP70 in human skeletal muscle (Febbraio & Koukoulas, 2000; Febbraio et al., 2002b). Febbraio and Koukoulas (2000) observed that moderate-intensity cycling until exhaustion significantly increased the *vastus lateralis* muscle temperature, decreased muscle glycogen, and elicited a significant increase in HSP72 mRNA expression. Indeed, the increase in HSP72 gene expression was observed coinciding with the time-point when muscle glycogen was reduced to low levels (Febbraio & Koukoulas, 2000). In order to isolate the effects of muscle glycogen availability on the HSP72 response to exercise, Febbraio et al. (2002b) subsequently used single-legged exercise to deplete muscle glycogen prior to a 150-min bout of two-legged knee extensor exercise. In response to the two-legged exercise, HSP72 mRNA expression increased only in the *vastus lateralis* of the leg that had previously performed glycogen-depleting exercise. Recently, Dalgaard et al. (2022) observed that 4-h glycogen-depleting cycling exercise at ~75% of the maximum heart rate (HR_{max}) significantly reduced muscle glycogen content in the *vastus lateralis*, corresponding to 34% of the pre-exercise content, and statistically increased *vastus lateralis* HSP70 expression by 147 ± 99%. In this study, a significant

correlation between glycogen content and HSP70 expression within the same skeletal muscle fibres at the termination of exercise (r^2 = 0.23, P < 0.0001). However, it should be noted that causality of this correlation could not be determined, and therefore, it was not possible to indicate whether the augmented HSP70 expression was due to muscle glycogen availability *per se* or other stimulators induced by endurance exercise (Dalgaard et al., 2022). Although these data demonstrate a potential role of carbohydrate availability as a contributing factor to the exercise-induced expression of HSP70, the mechanism underpinning these responses has not yet been well defined.

2.8 Expression of extracellular HSP70

Release of HSP72 into the circulation has been observed after exposure to a variety of stressors, in animal and human models. During stress, the elevation of extracellular HSP72 expression may act a 'danger signal' to prime and/or enhance immunologic responses and improve recovery (Fleshner et al., 2003; Fleshner & Johnson, 2005). Fleshner et al. (2004) reported that a psychological model of stress, exposure of rats to cats without physical contact for 2-h, was sufficient to increase circulating extracellular HSP72. Mechanistically, rats that had been adrenalectomised prior to predatory-stress exposure showed no intracellular HSP72 response and an attenuated response of extracellular HSP72 expression in comparison to sham-operated animals. Therefore, these data suggest that the release of HSP72 into the circulation is possibly dependent on adrenal hormones (Fleshner et al., 2004). Compellingly, Johnson et al. (2005) demonstrated that pharmacological blockade of α_1-adrenoceptors, but not β-adrenoceptors, resulted in a complete elimination of the plasma HSP72 response to tail shock stress in rats. Hence, these data confirmed that stress-induced release of HSP72 into the circulation is promoted by α_1-adrenergic binding, and therefore elevated plasma catecholamines (Johnson et al., 2005). The link between catecholamines and

extracellular HSP72 release was subsequently further examined by Whitham et al. (2006). These authors stimulated sympathetic activity with caffeine supplementation to observe responses of extracellular HSP72 expression during endurance exercise in humans, based on available data suggesting that plasma adrenaline concentrations are characteristically greater in exercising humans supplemented with caffeine than a placebo (Graham & Spriet, 1995). Exercising with caffeine supplementation resulted in a greater increase in extracellular HSP72 expression during a 90-min cycling bout at ~70% $\dot{V}O_{2max}$ than exercise with a placebo. Indeed, this greater upregulation of extracellular HSP72 expression was associated with a greater increase in adrenaline concentration in response to prolonged-cycling exercise with caffeine supplementation, suggesting that catecholamines may be an important mediator of the exercise-induced increase in extracellular HSP72 expression in humans (Whitham et al., 2006).

Catecholamines have previously been observed to influence the upregulation of extracellular HSP72 (Chin et al., 1996; Heneka et al., 2003). Although the mechanism by which catecholamines mediate plasma HSP72 expression remains unclear, it is feasible that the upregulated HSP72 in the circulation through α_1-adrenoceptor pathway is influenced by the induction of intracellular HSP72 (Johnson et al., 2005; Whitham et al., 2006), especially from tissues with high resting levels of intracellular HSP72 that could theoretically be released under stress (Maloyan et al., 1999; Musch et al., 2001). One potential mechanism for HSP72 release into the circulation during times of stress relates to exosomes, small membrane vesicles secreted by various cell types containing costimulatory and antigen-presenting molecules, including HSP70 (Lancaster & Febbraio, 2005). Because the releasing mechanism of exosomes occurs in a calcium-dependent fashion on stimulation of the cell (Savina et al., 2003), and with the activation of α_1-adrenoceptors increasing intracellular Ca^{2+} concentration (Schwietert et al., 1992), it is possible that HSP72 release from tissue to the circulation occurs via this mechanism during stressful situations (Johnson et al., 2005).

Although HSP70 expression in the peripheral circulation of healthy humans was first detected in the late 1990s (Pockley et al., 1998), the tissue from which the elevated circulating HSP72 originates is still unclear. As exercise has been reported to induce HSP72 in the contracting skeletal muscle of humans (Febbraio & Koukoulas, 2000; Puntschart et al., 1996), it has been hypothesised that the release of HSP72 into the circulation might come from skeletal muscle tissue. Walsh et al. (2001) reported that serum HSP72 expression significantly increased during endurance exercise (0.13 ± 0.10 vs. 0.87 ± 0.24 and 1.02 ± 0.41 $ng \cdot mL^{-1}$, at rest vs. 30 and 60 minute of treadmill running exercise, respectively, $P < 0.05$), but a significant effect was not observed for HSP72 mRNA expression or protein content in the *vastus lateralis* muscle at the end of 60-min treadmill running exercise. Interestingly, the skeletal muscle HSP72 mRNA expression was found to increase significantly at 2-h post-exercise despite a decrease in serum HSP72 expression at the timepoint (Walsh et al., 2001). Therefore, these data may suggest that contracting skeletal muscle may not be the secretory organ responsible for the increased extracellular HSP72 expression with exercise, as the increased extracellular HSP72 expression with exercise occurred prior to elevated skeletal muscle HSP72 mRNA expression.

Since various proteins are synthesised by the liver (De Feo & Lucidi, 2002), it is possible that extracellular HSPs are also derived from the hepatosplanchnic tissue to the circulation in response to exercise-associated stressors, and arterial–venous balance experiments have been conducted to examine this hypothesis. Febbraio et al. (2002b) utilised catheters to draw blood samples from one femoral artery and two femoral veins on three occasions; prior to, during, and recovery from 150-min of two-legged knee extensor exercise, and measured arterial–venous differences in serum HSP72 expression. However, HSP72 was not detected in either arterial or venous serum, despite a two-fold increase in HSP72 expression in the *vastus lateralis*. Hence, it appears HSP72 in muscle is unlikely to be released from the contracting limb based on the measurement of arterial–venous differences (Febbraio et al., 2002b). Similarly, a

subsequent study from the same laboratory did not observe a femoral arterial–venous difference in serum HSP72 expression at any timepoint during 120-min semi-recumbent cycling exercise at ~60% $\dot{V}O_{2max}$, while a significant increase in arterial serum HSP72 expression was observed at the end of exercise (Febbraio et al., 2002a). Interestingly, a hepatosplanchnic arterial–venous difference in HSP72, that was not observed at rest, gradually increased and was significant at 60 and 120 min of exercise. This suggests that hepatosplanchnic tissue may be responsible for releasing HSP72 into the circulation during exercise (Febbraio et al., 2002a). Although these results indicate that extracellular HSP72 expression following exercise is more possibly to be derived from hepatosplanchnic tissue, rather than skeletal muscle (Febbraio et al., 2002a; Febbraio et al., 2002b), these data should be interpreted cautiously as HSP72 was measured in serum rather than plasma. Experiments have shown that extracellular HSP72 measurements are sensitive to blood handling procedures, and previous data on serum HSP72 expression have tended to be reported at the lowest point of detectable range of assay (Febbraio et al., 2002a; Lancaster et al., 2004; Walsh et al., 2001). Whitham and Fortes (2006) employed different blood handling procedures to analyse post-exercise blood samples using an enzyme-linked immunosorbent assay, and reported that serum HSP72 concentration was significantly lower than extracellular HSP72 derived from plasma using ethylenediaminetetraacetic acid (EDTA). A plausible explanation for these findings relates to the intracellular functions of HSP72 in chaperoning aggregated proteins. It is possible that extracellular HSP72 binds to proteins involved in the clotting process, and therefore results in a reduction in measured serum HSP72 concentrations (Ladenson et al., 1974).

2.9 Effects of endurance exercise in hot environments on HSP70 responses

Exercise in a hot environment increases physiological strain in the whole organism (Galloway & Maughan, 1997). To date, a number of laboratories have studied the acute effect of endurance exercise combined with thermal stress in humans, and reported changes in extracellular HSP70 expression in response to various experimental exercise protocols (Magalhães et al., 2010; Marshall et al., 2006; Périard et al., 2012; Whitham et al., 2007). Marshall et al. (2006) conducted a 2-h cycling exercise trial at 42.5% $\dot{V}O_{2peak}$ in a hot environment (38˚C, 60% rH) for two consecutive days, and reported that serum HSP72 expression significantly increased at the end of exercise compared with the resting concentration, with a similar increase was observed on both days. Similarly, Magalhães et al. (2010) also observed an increase in plasma HSP72 expression (rest vs. post-exercise) in unacclimated individuals, responding to a 90-min treadmill exercise at 50% $\dot{V}O_{2peak}$ under environmental heat stress conditions (40˚C, 45% rH). More recently, Périard et al. (2012) investigated the influence of different exercise intensities on extracellular HSP72 upregulation using an exhaustive cycling-exercise protocol at moderate- and high-intensity, 60 and 75% $\dot{V}O_{2max}$, respectively, in a hot environment (40˚C, 50% rH). They observed a significant increase in plasma extracellular HSP72 expression from pre-exercise to exhaustion for both moderate- and high-intensity exercise trials. However, this exercise-heat stress induced upregulation was not statistically different between trials despite a significant difference in core temperature at exhaustion (39.7 ± 0.4 and 39.0 ± 0.5˚C, in moderate- and high-intensity exercise trials, respectively). In this study, a regression analysis demonstrated that the increase in plasma HSP72 concentration during moderate-intensity exercise was significantly predicted by two variables; core temperature and $\dot{V}O_{2max}$ (adjusted R^2 = 0.33). In the high-intensity exercise trial, the rate of increase in core temperature was a significant predictor variable of plasma extracellular HSP72 expression (adjusted R^2 = 0.219, P = 0.039). Therefore, these observations suggest that the upregulated circulating HSP72 in

response to endurance exercise combined with thermal stress may relate to both core temperature attained and the rate of increase in core temperature (Périard et al., 2012).

Combining endurance exercise with environmental stress to induce hyperthermia has been reported as stimuli for further increasing extracellular HSP70 expression from exercise alone (Gibson et al., 2014; Whitham et al., 2007). Whitham et al. (2007) employed deep-water immersion techniques for investigating the combined and independent effects of exercise and thermal stress on extracellular HSP72 upregulation. Data demonstrate that 2-h running exercise at ~60% $\dot{V}O_{2max}$, was sufficient to increase plasma HSP72 concentrations. Interestingly, 2-h deep-water passive heating in ~38.5˚C, with an increase in core temperature (~2.3˚C), also resulted in increased plasma HSP72 expression. However, exercise and whole-body hyperthermia combined resulted in the greatest rise in plasma HSP72 expression (~6 vs. ~2.5 $ng \cdot ml^{-1}$, in exercise combined with whole-body hyperthermia vs. passive heating trials, respectively), even though core temperature was not significantly different at any timepoint between exercise-heat stress and passive heating trials (Whitham et al., 2007). More recently, Gibson et al. (2014) investigated the expression of plasma HSP72 in response to 90-min cycling exercise at 50% $\dot{V}O_{2peak}$ in temperate, hot, and intensively hot environments (at 20, 30, and 40˚C (with the relative humidity of 63, 51, and 37%, respectively). Interestingly, a significant increase in extracellular HSP72 expression was only observed following 90-min cycling exercise in 40˚C, but not in 20 or 30˚C environments. Available evidence suggests a strong relationship between core temperature and plasma HSP72 upregulation (Ruell et al., 2006; Sandström et al., 2009), and it is possible that the magnitude and duration of core temperature above 38.5˚C are the most potent stimuli for extracellular HSP72 upregulation when combined with exercise stress (Périard et al., 2012). Therefore, in this study of Gibson et al. (2014), regression analysis was performed and indicated that the rate of rectal temperature increase ($˚C \cdot h^{-1}$) and absolute change in rectal temperature (˚C) were predictors of the increase in plasma HSP72 concentration. As significant differences in both the rate of increase and absolute change in rectal temperature were

only observed in 40 vs. 30 and 20˚C conditions, but not in 30 vs. 20˚C conditions, this could explain why no significant effect of exercise combined with moderate heat stress (30˚C) was observed for extracellular HSP72 responses (Gibson et al., 2014).

As previously mentioned (Section 2.4.3), upregulated HSP70 expression is not only an acute response following a single bout of endurance exercise (Kim et al., 2004; Morton et al., 2006; Skidmore et al., 1995), but also an adaptation to that occurs in response to a period of exercise training episodes (Liu et al., 1999; Sandström et al., 2008). A series of exercise training sessions under hot conditions with the purpose of sustaining increased core temperature, which is usually generated by performing physical exercise during repeated days in the high-temperature environment, is known as heat and exercise acclimation (HA) (Moseley, 1997). Evidence indicates that HA could potentially elicit a protective thermotolerance effect to subsequent exercise session in the heat, and therefore be advantageous for performance in high environmental temperatures (Henstridge et al., 2016). However, adaptive responses of extracellular HSP70 expression to HA have been less frequently investigated in humans. Sandström et al. (2008) reported that a 15-d prolonged cycling training intervention performed in a hot environment (31.9 ± 0.9˚C) was sufficient to lower HRs at rest and during exercise, reduce in resting and exercising core temperatures, and increase power output in an ultramarathon runner when compared between day 15 and day 1 of the HA intervention, and therefore, reported successful adaptations to heat. With these physiological markers, the authors also daily measured pre- and post-exercise serum HSP70 expression, and found that the resting serum HSP70 concentration had increased over the HA intervention period. On the contrary, Yamada et al. (2007) employed a 10-day HA protocol of 100-min treadmill exercise at 56% $\dot{V}O_{2max}$ under hot conditions (42.5˚C, 27.9% rH). Although cardiovascular and thermoregulatory adaptations were reported from the observations of decreased peak HR and core temperature during HA sessions after 10 days, the HA intervention was insufficient to stimulate the adaptation of serum HSP72 expression at rest. Consistent with this literature, Magalhães et al. (2010)

reported unchanged plasma HSP72 expression from pre- to post-HA intervention of 11-d treadmill exercise in a hot-dry environment (40˚C, 45% rH), consisting of elevating core temperature by 1°C in 30 min and then maintaining it elevated for another 30 min (1% grade with 2.20 ± 0.11 and 1.69 ± 0.11 $m \cdot s^{-1}$, respectively) despite a significant increase in intracellular. In unacclimated individuals, increased plasma HSP72 expression in response to exercise combined with heat stress, which the exercise characteristics and environmental conditions were described above, might be sensed by the organism as a stress capable of inducing an immunological response. Following HA intervention, the participants of this study had become heat acclimated, and so the same exercise bout might be insufficient to stimulate cellular stress which is capable for eliciting an immunological response (Magalhães et al., 2010).

2.10 Summary

In summary, existing literature demonstrates that exercise characteristics, such as increases in core and/or local temperatures, exercise intensity, and carbohydrate availability, influence an increase in intracellular HSP70 expression, which is an acute response to maintain cellular homeostasis during exercise, and is also fundamental in facilitating the cellular remodelling processes that is inherent to training adaptation (Morton et al., 2009b). Increased extracellular HSP70 expression might be important for enhancing immunologic responses and improving recovery under stress circumstances (Fleshner et al., 2003; Fleshner & Johnson, 2005). HSP70 release from tissue to the circulation is found to increase with various physiological factors, including increased core temperature (Périard et al., 2012) and elevated plasma catecholamines (Johnson et al., 2005).

However, as for carbohydrate metabolism, it should be noted that the acute effects of exercise performed under heat stress for HSP70 expression have been studied at

matched absolute work-rates, with the percentage of $\dot{V}O_{2peak}$ as the exercise-intensity determinant (Gibson et al., 2014; Walsh et al., 2001; Whitham et al., 2006; Yamada et al., 2007). In the real-world, endurance athletes often train and compete in hot environments (Boukelia et al., 2018; Lucía et al., 2000), and they are likely to experience a reduction in absolute work rates compared to when training in temperate conditions (Boynton et al., 2019; Lorenzo et al., 2010; Maunder et al., 2021a; Maunder et al., 2021b). Given that research supports the importance of exercise intensity in the stimulation of HSP70 expression in response to exercise (Gibson et al., 2014; Morton et al., 2009a), and as previously mentioned in the substrate oxidation section, it is possible that the stimulatory effect of exposure to environmental heat stress during exercise on extracellular HSP70 expression may be at least partially blunted by the likely reduction in absolute work rates. Therefore, a more relevant comparison for practitioners considering the likely metabolic effects of performing a training session under environmental heat stress may be using matched relative physiological stress, or heart rates.

2.11 Thesis aim and hypotheses

Therefore, the aim of the present investigation was to determine how heat stress impacts on substrate oxidation rates and plasma HSP70 expression during endurance exercise regulated by relative physiological work rates, or heart rates (HR) in order to answer the following research questions:

1. What is the effect of moderate environmental heat stress on substrate oxidation rates during heart rate-matched moderate-intensity cycling?
2. What is the effect of moderate environmental heat stress on plasma HSP70 expression in response to heart rate-matched moderate-intensity cycling?

I hypothesised that endurance trained athletes would achieve a lower absolute work rate when exercising under moderate environmental heat stress (33˚C) in comparison to equivalent exercise in temperate conditions (18˚C), that would therefore reduce both energy expenditure and whole-body carbohydrate oxidation rates. However, endurance exercise combined with heat exposure would still increase plasma HSP70 expression due to greater increases in core temperature during prolonged cycling exercise.

Chapter 3: Methods

3.1 Participants

Endurance-trained male cyclists and/or triathletes participated in this study (Table 2). Participants were consistently engaging in endurance training (≥3 years), and habitually completing a self-reported 6 ± 2 $h \cdot wk^{-1}$ of cycling training. Participants were free from viral infection (>1 month), lower-limb injury (>3 months), and had not suffered with any cardiovascular disease, or previously experienced exertional heat stress illness. These eligibility criteria were established in line with published recommendations (Casa et al., 2015; Conley et al., 2014) in order to maximise the safety of athletes in the present investigation. Participants were unacclimated to exercise-heat stress, which was defined as not recently having undertaken specific heat acclimatisation training (≥6 months). Heat acclimatisation is known to stimulate physiological adaptations, including reduced exercise heart rate (Brazaitis & Albertas, 2010; Yamada et al., 2007), improved thermoregulatory controls (Bonner et al., 1976; Lorenzo et al., 2010), and metabolic alterations (Febbraio et al., 1994a), that may to influence the primary outcomes of the present study. Therefore, it was necessary to exclude those with recent acclimation to ensure a homogenous group of participants and responses were studied. Participants also met the eligibility criteria of a peak oxygen uptake ($\dot{V}O_{2peak}$) of >50 $mL \cdot kg^{-1} \cdot min^{-1}$, which was determined in the first laboratory visit.

Female athletes were excluded from this study on the basis of observed effects of the ovarian hormones, oestrogen and progesterone, on relative fat and carbohydrate metabolism during prolonged exercise (D'Eon et al., 2002). Furthermore, the resting core temperature of female athletes might fluctuate when observed at the different phases of menstrual cycle (Marshall, 1963). In order to control for these effects, experimental trials would have needed to be conducted in the same phase of menstrual cycle, and verified with resting concentrations of ovarian hormones. However, this was not possible within

the budget available for this study. Accordingly, it is recommended that the study presented here be repeated in female athletes. The Auckland University of Technology Ethics Committee (AUTEC) approved all experimental procedures (AUTEC reference number 21/121, Appendix 1), and participants provided written, informed consent.

Whitham et al. (2007) studied the combined and independent effects of hyperthermia and exercise on the extracellular heat shock protein 72 (HSP72) response in humans, and found that the combination of exercise and hyperthermia significantly increased extracellular HSP72 concentration. Using 50% of the between-group effect size for post-exercise extracellular HSP72 concentration observed by these researchers (Whitham et al., 2007), it was *a priori* calculated that a total sample size of 10 participants would be required to observe a between-group difference ($P < 0.05$) in post-exercise extracellular HSP72 concentration with 80% statistical power. This smaller effect size was utilised as smaller between-group differences in post-exercise thermoregulatory variables were expected in this study (~1°C difference in T_{re} based on Maunder et al. (2020), compared to the ~2°C difference in Whitham et al. (2007)).

Table 2. Participant characteristics

	Overall cohort (N = 10)	Seasonal subgroups	
		Winter (N = 4)	Summer (N = 6)
Age (years)	30.7 ± 7.5	34.5 ± 7.3	28.2 ± 7.1
Height (cm)	180.9 ± 2.9	182.9 ± 1.4	179.6 ± 2.9
Body mass (kg)	75 ± 5.7	73.8 ± 6.4	75.7 ± 5.8
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)*	4.4 ± 0.6	4.5 ± 0.9	4.3 ± 0.3
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)*	59.2 ± 6.8	61.2 ± 8.1	57.6 ± 5.9
Power at VT_1 (W)	204 ± 46	222 ± 68	194 ± 30
Heart rate at VT_1 (bpm)	137 ± 11	139 ± 15	136 ± 9
Overall training volume ($hr \cdot wk^{-1}$)	9 ± 3	8 ± 4	9 ± 2
Cycling training volume ($hr \cdot wk^{-1}$)	6 ± 2	7 ± 1	5 ± 2
Peak fat oxidation ($g \cdot min^{-1}$)	0.52 ± 0.23	0.54 ± 0.26	0.50 ± 0.23

Values are presented as mean ± standard deviation. Abbreviations: $\dot{V}O_{2peak}$ = peak oxygen uptake, VT_1 = the first ventilatory threshold. * Missing data of $\dot{V}O_{2peak}$ values at overall cohort (N = 9) and summer subgroup (N = 5) due to technical difficulty.

3.2 Study design

This experimental study adopted an acute, randomised, counterbalanced cross-over design. Within ~3 weeks, each participant reported to the Sports Physiology Laboratory at the Sports Performance Research Institute New Zealand on three occasions, including a characterisation trial and two experimental trials (Figure 1). The characterisation trial began with participants providing written, informed consent, and completing a health screening questionnaire to ensure that participants had no pre-existing contraindications to the exercise trials, and met the inclusion criteria (Section 3.1). Following a successful health screen, an incremental exercise test was conducted for determination of $\dot{V}O_{2peak}$, and the heart rate (HR) at the first ventilatory threshold (VT_1), which was used for individualising the exercise intensity in the experimental trials (Section 3.3.1). The two

experimental trials consisted of a 90-minute bout of cycling exercise at a specified HR equivalent to 95% of VT_1, in ~60% relative humidity (rH) and either 18 or 33˚C (Section 3.3.2). The order in which participants completed 90-minute cycling trials in temperate (18˚C) and heat (33˚C) environments was randomised with a counterbalanced design using a Microsoft Office Excel random number generator.

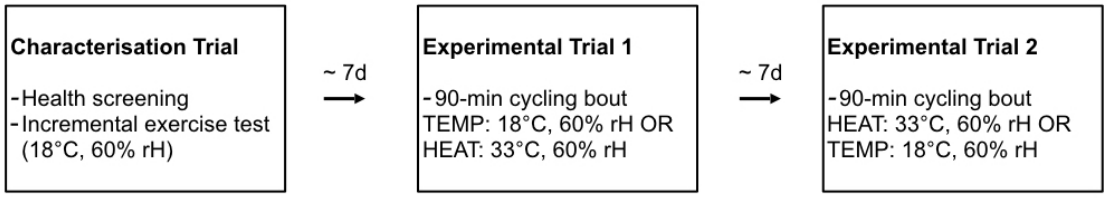


Figure 1. Experimental overview.

All characterisation and experimental trials were commenced at ~7:00 am (± 2 hours), and each participant arrived at the laboratory at the same time of day to avoid potential circadian effects. In each visit, participants arrived after an overnight fast, having refrained from caffeine, alcohol, and intense exercise for 24 hours. These pre-trial controls were necessary since previous research has observed effects of these variables on the primary outcome measures of this study. Caffeine ingestion before exercise might have an ergogenic effect on endurance performance (Graham & Spriet, 1995; Hodgson et al., 2013; Spriet et al., 1992), alter substrate metabolism (Costill et al., 1978), increase circulating adrenaline concentrations (Graham et al., 2000; Graham & Spriet, 1995; Greer et al., 2000), and influence a rise in core temperature when exercising in hot conditions (Hanson et al., 2019). Acute alcohol intake might influence changes in thermoregulatory responses to exercise in both temperate and hot environments (Fellows et al., 1984; Graham, 1981). Also, alcohol ingestion might alter circulating metabolites, and therefore substrate metabolism during exercise (Heikkonen et al., 1998; Siler & Neese, 1998), although this has not always been shown (Smith et al., 2021). Furthermore, alcohol ingestion might also reduce aerobic performance the following day

(~18-h after the ingestion) (Shaw et al., 2022). High-intensity exercise might increase total energy expenditure and alter substrate oxidation within 24-h post-exercise (Treuth et al., 1996). Failure to properly standardise these factors between-trials could therefore mask the effect of the experimental manipulation, environmental temperature, on metabolic responses to exercise. Consequently, the pre-trial controls were established to minimise these possible impacts on the primary outcome measures of this study.

In order to minimise seasonal effects which might influence the primary outcomes of this study, all laboratory trials were intended to be completed during the non-summer months in Auckland, New Zealand. Four participants completed their trials during the winter months (between June and August) in 2021. However, data collection was interrupted by the nationwide lockdown for COVID-19, announced on 17 August 2021. After lockdown restrictions were eased, data collection was resumed, and therefore six of the ten participants were tested during the warmer months (between January and April) in 2022. However, it is possible that participants tested in the summer months were partially acclimated to heat. Several studies have reported effects of seasonal acclimatisation on thermoregulatory responses, which might reduce the effects of acute environmental heat stress on physiological responses and impact the primary outcomes of this study. Lei et al. (2021) observed effects of seasonal acclimatisation during 60-min cycling at 40% of maximal oxygen uptake ($\dot{V}O_{2max}$), and reported that both whole body sweat rate and mean skin temperature were higher in summer (the post-acclimatisation trial) compared to the identical exercising trial previously conducted in winter (the pre-acclimatisation trial). However, no main effect between the pre- and post- acclimatisation trials (P = 0.77), or season × timepoint interaction was observed (P = 0.60) was observed for core temperature in this study (Lei et al., 2021). Therefore, subgroup analyses were performed to assess seasonal effects on the primary outcome measures of this study.

3.3 Experimental procedures

3.3.1 Characterisation trial

Participants arrived the laboratory at ~7:00 am for the characterisation trial following an approximate period of ~8-12 h fast, having refrained from caffeine, alcohol, and intense exercise for 24 h. On arrival, participants had the opportunity to read and discuss the participant information sheet (Appendix 2), ask questions, and provide written informed consent (Appendix 3). Participants then completed a health screening questionnaire (Appendix 4) to ensure that they had no pre-existing contraindications to exercise trials (Section 3.1).

After the pre-testing procedures, participant height and body mass were first measured in cycling clothes using a stadiometer and an electronic weighing scale (HW-2000KGL, A&D Company, Limited, Japan). An incremental exercise test then commenced in the temperate environment (18.3 ± 1.4˚C, 60-80% rH). Participants mounted a laboratory ergometer (Excalibur Sport, Lode, Groningen, NET), and cycling commenced at 95 W. Expired gases were collected continuously using metabolic cart (TrueOne2400, ParvoMedics, Sandy, UT, US), which was calibrated with a 3-L calibration syringe (Series 5530, Hans Rudolph, Inc., USA) and a standard gas cylinder comprised of 4% CO_2, 16% O_2 in N_2 (LSA80038D, BOC Limited, New Zealand) for flow volume and gas concentrations, respectively. Heart rate was also measured continuously using a chest-strap HR monitor (TICKR, Wahoo, Taiwan). The power output was increased by 35 W every 3 minutes until the respiratory exchange ratio reached 1.0. After this point, the duration of each stage was shortened to 1 minute until task failure. The first phase of this protocol was designed to allow determination of VT_1 and the peak fat oxidation rate, and the second phase was designed to achieve $\dot{V}O_{2peak}$ rapidly (Achten et al., 2002). Convective air flow was provided using a pedestal fan (GCPF340, Goldair, China).

After cessation of the incremental exercise test, $\dot{V}O_{2peak}$ was determined to ensure eligibility for study participation based on $\dot{V}O_{2peak}$, and the VT_1 was also identified to

individualise the exercise intensity in the experimental trials. The $\dot{V}O_{2peak}$ was identified as the highest 15-s average value for $\dot{V}O_2$ during the incremental exercise test. The VT_1 was identified as the first increase in the ventilatory equivalent for oxygen ($VE{\cdot}VO_2^{-1}$) without changes in ventilatory equivalent for carbon dioxide ($VE{\cdot}VCO_2^{-1}$) (Lucía et al., 2000).

3.3.2 Experimental trials

Approximately seven days following the characterisation trial, participants arrived at the laboratory for the first experimental trial, having adhered to the same pre-trial instructions described above (Section 3.3.1), and made written records of their diet for 48 h and training for 7 d (Appendices 5–6), such that these could be repeated in advance of the second experimental trial. On arrival, a 6-mL pre-exercise blood sample was drawn from an antecubital vein using the venepuncture technique. Pre-exercise body mass was measured and then participants self-inserted a rectal thermometer ~10 cm beyond the anal sphincter in privacy for determination of rectal temperature (T_{re}). A skin temperature thermistor was subsequently taped over the mid-belly of the vastus lateralis ~15 cm above the patella, and covered with 2 pieces of 2-mm neoprene in order to insulate the skin underneath from the ambient environment for continuous observation of insulated skin temperature (T_{ins}), which was used for estimation of muscle temperature (T_{mus}) (Brajkovic et al., 2006). With the use of corrective equations, a previous study has validated this insulated skin method against direct, indwelling measurement as an accurate assessment of the vastus lateralis temperature during resting and cycling exercise (Flouris et al., 2015). No mean differences ($P > 0.05$) and very high correlation coefficients ($P < 0.001$) between the actual and the estimated muscle temperature values during resting, exercise, and recovery periods were observed. Therefore, this non-invasive technique was utilised in the present thesis (Figure 2). Participants then sat

comfortably for 5 min for measurement of resting estimation T_{mus} and T_{re} in the laboratory environment (~18˚C, 60-80% rH).

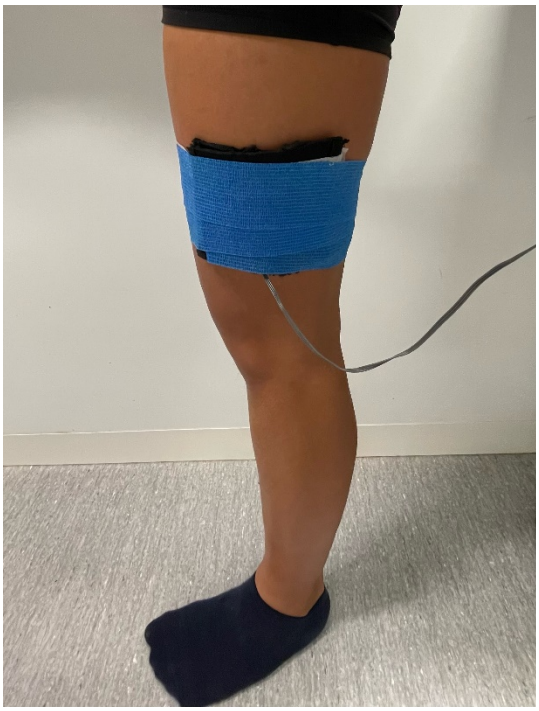


Figure 2. Application of the insulated skin technique for observation and calculation of the *vastus lateralis* temperature during resting and exercise.

Participants then entered the environmental chamber, which was set at either 18 or 33˚C, with 60% rH. Participants were fitted with a chest-strap HR monitor (TICKR, Wahoo, Taiwan) for continuous observation of HR during exercise, and then mounted their own road bicycle, which was connected to a calibrated, direct-drive indoor trainer (Kickr, Wahoo Fitness, Atlanta, USA). Recent research has shown that the Wahoo KICKR trainer provides valid and reliable measurements of power output (Hoon et al., 2016; Zadow et al., 2018). Zadow et al. (2016) reported that at the power output of 250–700 W and cadences of 80–120 rpm, the KICKR had acceptable accuracy with a small mean bias of –1.1% and narrow limits of agreement (LoA) (95% LoA –3.6% to 1.4%) when compared with the power output of a dynamic calibration rig ([CALRIG] Flinders University, Dynamic Calibrator 34118, Adelaide, Australia). A 90-minute bout of cycling then commenced. During the first 5-min of cycling, participants were asked to

progressively increase their HR to a specified target equivalent to 95% (± 2 $b \cdot min^{-1}$) of the VT_1 HR determined in the incremental exercise test, and then maintain their target HR until the exercise cessation. Participants were reminded of this if their HR drifted outside the target range. Participants had *ad libitum* access to plain water throughout the cycling trial. Convective air flow (~3.2 $m \cdot s^{-1}$) was provided by an industrial fan (FS-75, FWL, Auckland, NZ). Expired gases were obtained for 4 min every 15 min. Rating of perceived exertion (RPE) on a scale of 6 to 20 (Borg, 1982), thermal comfort on a 1-to-10 scale, and thermal sensation on a 1-to-14 scale, which were adapted from an previous work (Gagge et al., 1967), were also assessed every 15 min (Appendices 7–9). Throughout the experimental trial, T_{re} was monitored to ensure that it did not exceed 39.5˚C (Silva et al., 2019). This safeguard was necessary for reducing possibility of participants developing exertional heat illnesses during the trial (Casa et al., 2015). No participant reached this temperature.

At exercise cessation, a second 6-mL antecubital venous blood sample was obtained using venepuncture technique, and total water consumption was recorded through weighing of drink bottles. Participants subsequently removed the HR monitor, skin temperature thermistor, and rectal thermometer in privacy. Participants dried their skin using a towel, and post-exercise body mass was measured in order to estimate the magnitude of sweat loss during the trial, accounting for fluid consumption. Participants returned to the laboratory ~7 days later to perform the remaining experimental trial, having adhered to the same pre-trial instructions described above (Section 3.2), and repeated their 48-h diet and 7-day training records in advance.

3.4 Analyses

3.4.1 Gas analysis

In this present study, indirect calorimetry was utilised for estimating substrate oxidation rates during exercise. This method involves collection of expired gases, analyses of flow rate and gas contents, and estimations of oxygen uptake ($\dot{V}O_2$), and carbon dioxide production ($\dot{V}CO_2$), which allows the calculation of whole-body energy expenditure (Haugen et al., 2007). Furthermore, the rates of carbohydrate and fat oxidation can be separately calculated according to basic stoichiometric equations detailing $\dot{V}O_2$ and $\dot{V}CO_2$ associated with each substrate (Eq. 1) (Jeukendrup & Wallis, 2005).

Glucose stoichiometric equation: $C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$

Fatty acid stoichiometric equation: $C_{17.2702}H_{32.7142}O_2 + 24\ O_2 \rightarrow 17.3\ CO_2 + 16.4\ H_2O$

Equation 1. Stoichiometric equations of glucose and fatty acid

In the incremental exercise test, $\dot{V}O_2$ and $\dot{V}CO_2$ from the last 1 min of each stage were used to estimate substrate oxidation rates using standard stoichiometric equations (Eq. 2) (Jeukendrup & Wallis, 2005). Peak fat oxidation rate (PFO, $g \cdot min^{-1}$) was then identified as the highest fat oxidation rate in each individual during the test. *In the experimental trials*, expired gases from the final three minutes of each 4-min sampling time point were averaged and used for calculation of whole-body rates of energy expenditure, carbohydrate oxidation, and fat oxidation using standard equations (Eq. 2). The first minute of each sampling timepoint was discarded to minimise any potential

hyperventilatory effect associated with reorganising the headgear, and to ensure that participants had resettled into their comfortable cycling position.

$$\text{Energy expenditure } (kcal \cdot min^{-1}) = (0.550 \times \dot{V}CO_2) + (4.471 \times \dot{V}O_2)$$

$$\text{Carbohydrate oxidation } (g \cdot min^{-1}) = (4.210 \times \dot{V}CO_2) - (2.962 \times \dot{V}O_2)$$

$$\text{Fat oxidation } (g \cdot min^{-1}) = (1.695 \times \dot{V}O_2) - (1.701 \times \dot{V}CO_2)$$

Equation 2. Estimates of whole-body rates of energy expenditure, carbohydrate oxidation, and fat oxidation, where both carbon dioxide production ($\dot{V}CO_2$), and oxygen uptake ($\dot{V}O_2$) are in $L \cdot min^{-1}$.

3.4.2 Thermoregulatory analysis

Mean values obtained from rectal thermometer and skin temperature thermistor during the 30-second prior to each measurement time-point were defined as T_{re} and insulated skin temperature (T_{ins}). The T_{ins} was used for estimation of the vastus lateralis temperature (T_{mus}) in line with previous work (Eq. 3) (Flouris et al., 2015).

$$\text{Estimated } T_{mus} \text{ at rest} = (0.597 \times T_{ins}) - (0.439 \times T_{ins}Lag_2) + (0.554 \times T_{ins}Lag_3) - (0.709 \times T_{ins}Lag_4) + 14.767$$

$$\text{Estimated } T_{mus} \text{ during exercise} = (0.599 \times T_{ins}) - [(0.311 \times T_{ins}Lag_4) + 15.63]$$

Equation 3. Estimates of insulated skin temperature over the vastus lateralis (T_{ins}) at rest and during exercise, where $T_{ins}Lag_2 = T_{ins} - T_{ins}$ two min beforehand, etc.

3.4.3 Plasma analysis

Venous blood samples were stored on ice in pre-chilled ethylenediaminetetraacetic acid tubes until trial completion. In order to allow the correction of plasma concentrations for changes in plasma volume across the trial (Eq. 4) (van Beaumont et al., 1972), a small sample of whole blood was pipetted into two duplicate capillary tubes before spinning for 3-min using a micro-haematocrit centrifuge (Haematospin 1400, Hawksley& Sons, Ltd., England), and then the proportion of red blood cells was manually measured (the coefficient of variation [CV], 1.55 ± 1.85%). Plasma was then extracted from the remaining whole blood by centrifugation in 4°C for 10 min using a *Heraeus Megafuge 16* Centrifuge (D-37520 Osterode, Thermo Fisher Scientific, Inc., Germany), and stored at -80°C for further analysis.

$$\text{Change in PV (\%)} = \frac{100}{100 - Hct_{pre}} \times \frac{100 \times (Hct_{pre} - Hct_{post})}{Hct_{post}}$$

Equation 4. Calculation of changes in plasma volume, where PV = plasma volume, Hct_{pre} = haematocrit pre-exercise, Hct_{post} = haematocrit post-exercise

3.4.4 Heat shock protein-70 concentration analysis

Frozen plasma samples were defrosted on ice at room temperature. Heat shock protein-70 concentration, via an enzyme-linked immunosorbent assay (Human HSP70 ELISA Kit, Thermo Fisher Scientific, Inc., US), was determined using a commercially available kit according to the manufacturer's instructions. Samples were assayed in duplicate, and the CV was ~13.0%.

3.4.5 Statistical analysis

Statistical analysis was performed with GraphPad Prism Version 9.3.1 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD) unless otherwise stated. The normality of data distributions were assessed using the Shapiro-Wilk test, which is considered as an appropriate test for small sample sizes (N < 50) (Rahman & Govindarajulu, 1997). Within- and between-trial differences in physiological variables were assessed using two-way, repeated measures analyses of variance, with temperature and time as factors. Simple comparisons were made using paired t-tests (or the Wilcoxon signed-rank test for non-normal distributions). Bivariate linear correlations (Pearson's product-moment correlation coefficients or Spearman's rank-order correlation coefficients, depending on the distribution of the data) were used to assess relationships between primary outcome measures (heat stress-induced changes in mean carbohydrate oxidation rate and post-exercise plasma HSP70 concentrations) and heat stress-induced changes in dehydration percentage, sweat rate, mean power output, mean T_{re}, and mean estimated T_{mus}. In order to identify which of these variables explained variation in the primary outcome measures, stepwise linear regression models were constructed in R using the 'stepAIC' function. The dependent variables for these models were heat stress-induced changes in mean carbohydrate oxidation rate and end-exercise plasma HSP70 concentrations. The independent variables were heat stress-induced changes in dehydration percentage, sweat rate, mean power output, mean T_{re}, and mean estimated T_{mus}. The model selection process identified the most predictive and succinct combination of variables using the Akaike Information Criterion (AIC). Collinearity was checked using the 'performance' function. All modelling was performed in R (v4.4.0) with RStudio (v1.4.1717). The level of statistical significance was set at $P \leq 0.05$.

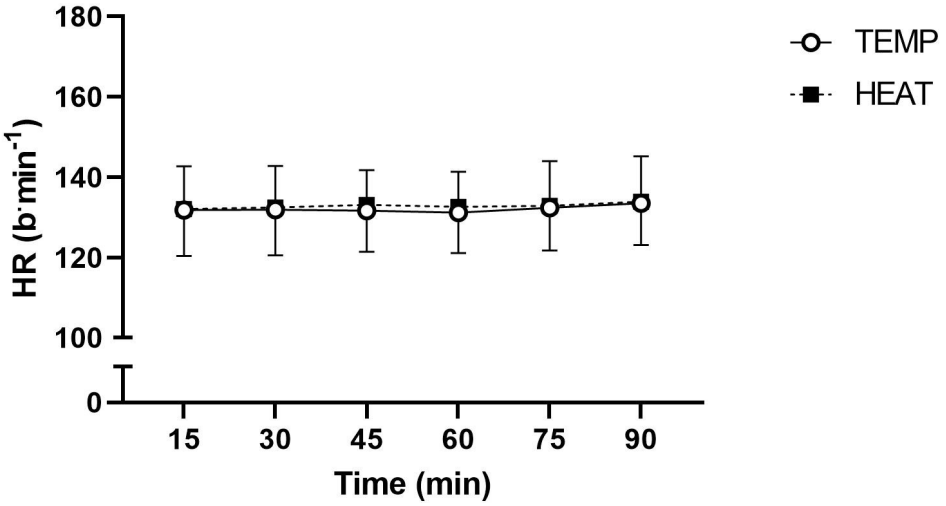
Chapter 4: Results

4.1 Characterisation of the experimental trials

4.1.1 Heart rate

A main effect of time was observed for HR (P = 0.011, Figure 3a), whereby a small increase in HR at 90 min vs. 15 min was observed (132 ± 11 vs. 134 ± 11 beats·min^{-1}, at 15 and 90 min, respectively, P = 0.007). No main effect of temperature (P = 0.229), or time × temperature interaction (P = 0.528), was observed for HR. Mean HR was not significantly different between-trials (P = 0.628, Figure 3b); in all cases, the individual difference in mean HR between-trials was not larger than 4 beats·min^{-1}.

(a)



(b)

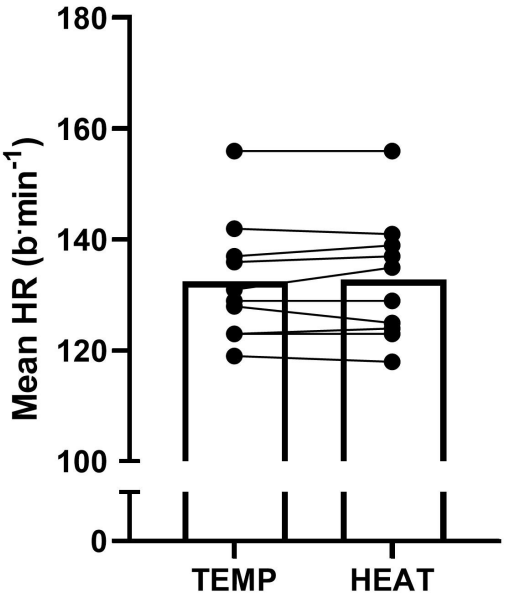
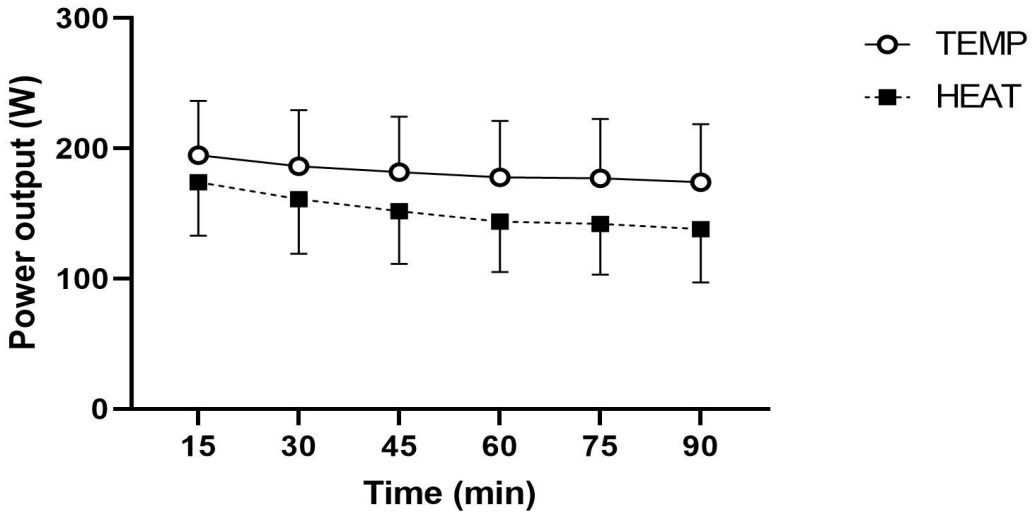


Figure 3. Heart rates (HR) during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). (a) Heart rate at each timepoint throughout the 90-min exercise trials (beats·min^{-1}), and (b) mean heart rates during the 90-min exercise trials (beats·min^{-1}). In 3a, a solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT. In 3b, bars indicate group mean values, and dots identify individual mean values.

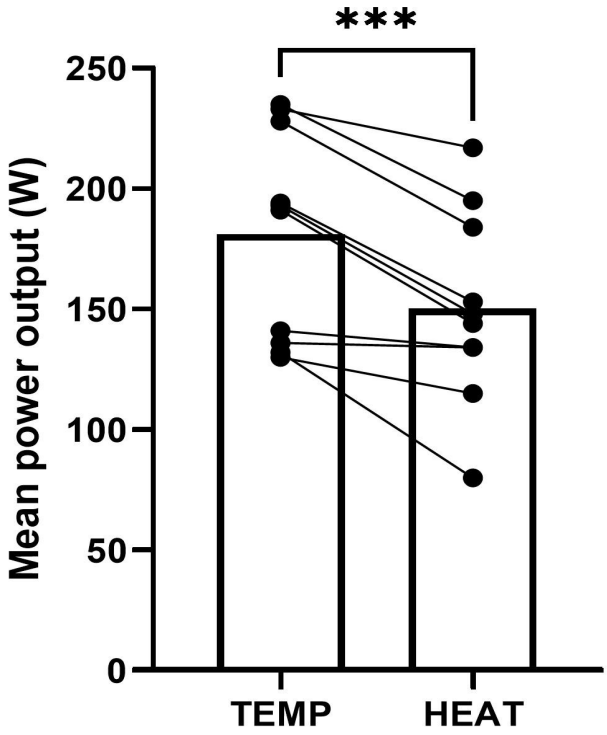
4.1.2 Power output

A main effect of time was observed for power output, whereby power output significantly decreased as exercise progressed ($P < 0.0001$, Figure 4a). A main effect of temperature was observed, whereby power output was significantly lower in HEAT ($P = 0.001$). A significant time × temperature interaction was observed ($P = 0.01$), whereby the reduction in power output over time (15 vs. 90 min) was greater in HEAT (11 ± 7 and 22 ± 11%, in TEMP and HEAT, respectively, $P = 0.005$). Mean power output was significantly different between-trials ($P = 0.0005$), whereby it was lower in HEAT (17 ± 11%). Specifically, mean power output was numerically lower in HEAT in all participants (Figure 4b). Subgroup analyses revealed no significant effect of season ($P = 0.516$), or temperature × season interaction ($P = 0.272$), for mean power output (Figure 4c).

(a)


300
200
100
0
Power output (W)
15
30
45
60
75
90
Time (min)
TEMP
HEAT

(b)


***
250
200
150
100
50
0
Mean power output (W)
TEMP
HEAT

(c)

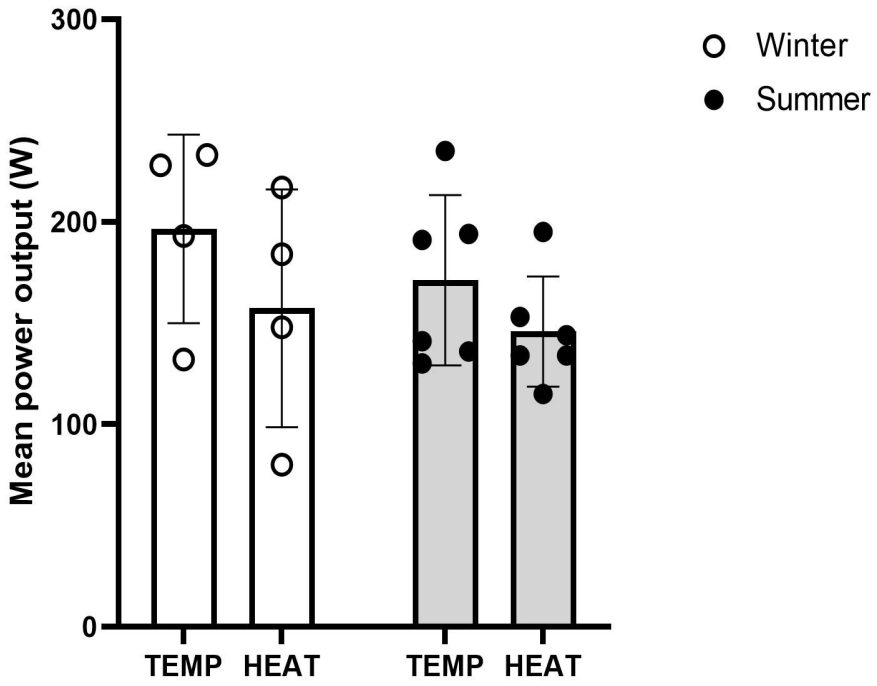


Figure 4. Power output (W) during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). (a) Power output (W) at each timepoint throughout the 90-min exercise trials, (b) mean power output (W) during the 90-min exercise trials, and (c) mean power output (W) of the winter (N = 4) and summer (N = 6) sub-groups. In 4a, a solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT. In 4b and 4c, bars indicate group mean values and dots identify individual mean values.

4.2 Thermoregulation

4.2.1 Rectal temperature

A main effect of time was observed for T_{re}, whereby T_{re} significantly increased with exercise duration (1.1 ± 0.4 and 1.2 ± 0.6˚C, from rest to 90 min in TEMP and HEAT, respectively, $P < 0.0001$, Figure 5). No main effect of temperature ($P = 0.494$), or time × temperature interaction ($P = 0.581$), was observed for T_{re}.

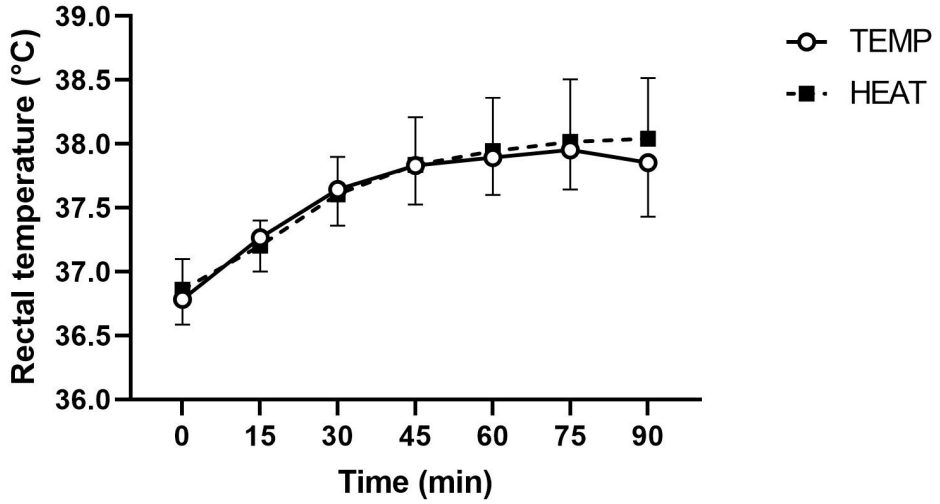


Figure 5. Rectal temperature (˚C) at each timepoint during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). Rectal temperature (˚C) data is missing for one participant due to technical difficulties (N = 9). A solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT.

4.2.2 Estimated muscle temperature

A main effect of time was observed for estimated T_{mus}, whereby estimated T_{mus} significantly increased as exercise progressed (P < 0.0001, Figure 6). A main effect of temperature was observed, whereby the end-exercise estimated T_{mus} was greater in HEAT (1.0 ± 0.4˚C, P = 0.0002). A significant time × temperature interaction was observed (P = 0.017), whereby the increase in estimated T_{mus} was greater in HEAT (3.8 ± 0.7 and 4.5 ± 0.9˚C, in TEMP and HEAT, respectively, P = 0.003).

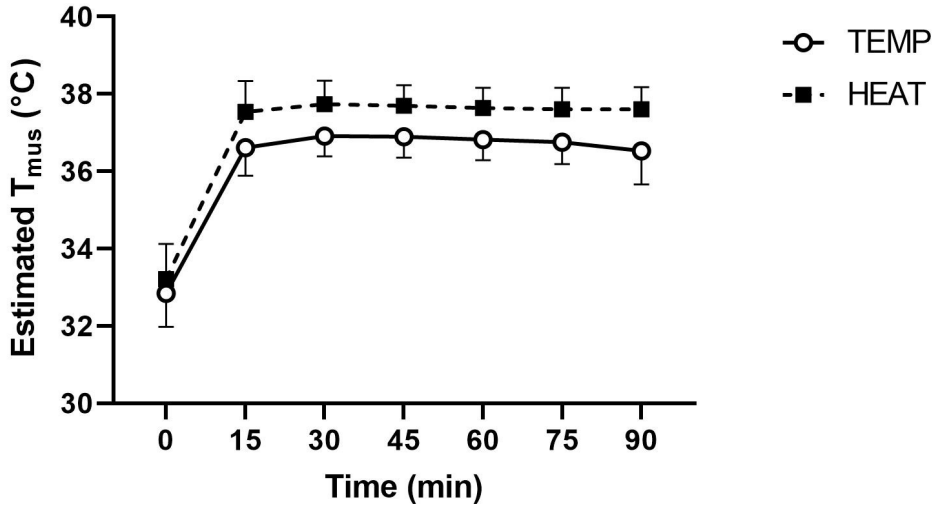


Figure 6. Estimated muscle temperature (˚C) at each timepoint during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). The 0 min timepoint represents estimated muscle temperature (˚C) at rest, which was observed in the laboratory conditions (~18˚C, 60-80% relative humidity). A solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT.

4.2.3 Sweat rate and dehydration

Mean sweat rate was significantly different between-trials, whereby it was greater in HEAT (0.6 ± 0.3 and 1.1 ± 0.4 $L \cdot h^{-1}$, in TEMP and HEAT, respectively, $P = 0.0003$, Figure 7). Mean dehydration was significantly different between-trials, whereby it was greater in HEAT (0.9 ± 0.7 and 1.4 ± 0.6%, in TEMP and HEAT, respectively, $P = 0.003$, Figure 8).

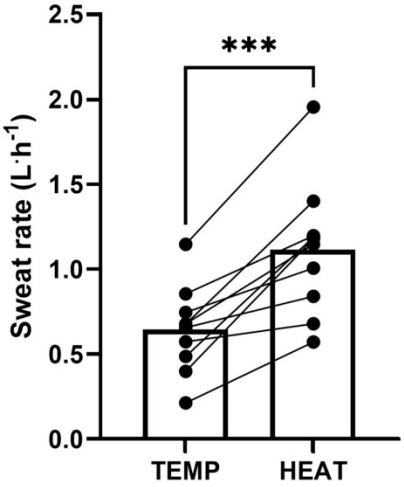


Figure 7. Sweat rate ($L \cdot h^{-1}$) during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). Bars indicate group mean values and dots identify individual mean values.

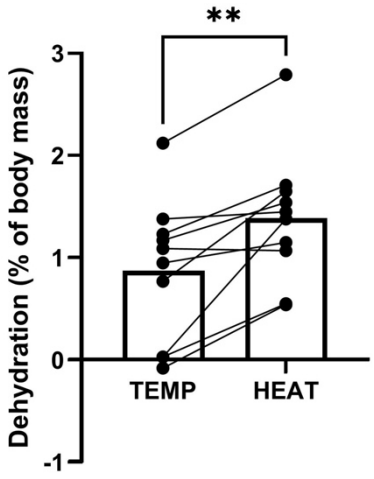


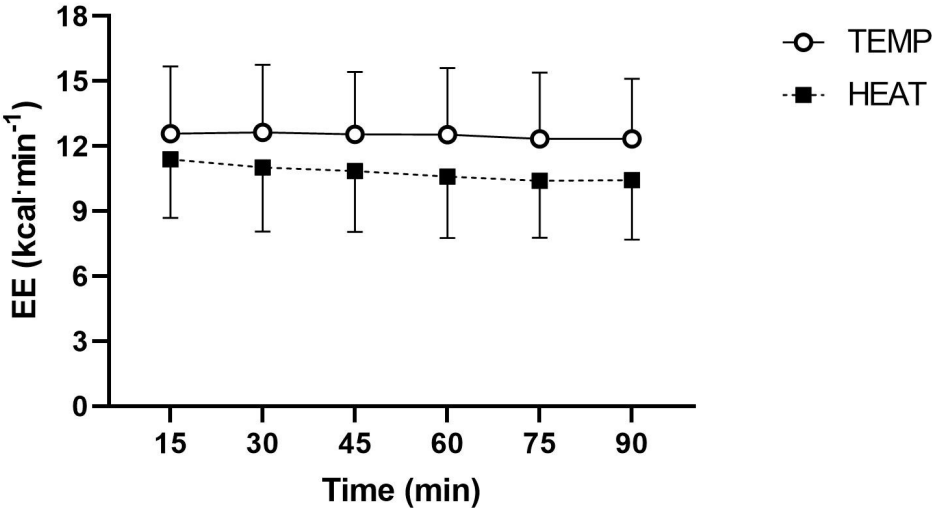
Figure 8. Dehydration (% of body mass) during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). Bars indicate group mean values and dots identify individual mean values.

4.3 Substrate oxidation rates

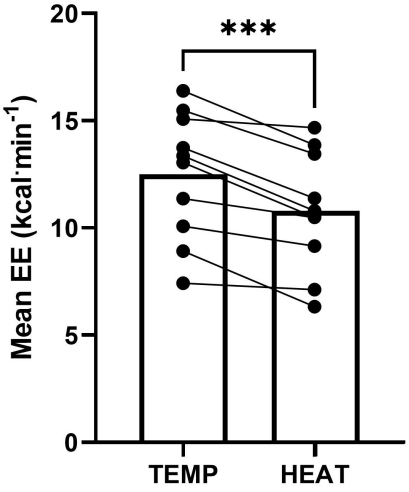
4.3.1 Whole-body energy expenditure (EE)

A main effect of time was observed for whole-body EE (P = 0.002), whereby it significantly decreased as exercise progressed (12.0 ± 2.9 vs. 11.4 ± 2.9, at 15 and 90 min, respectively, P = 0.026, Figure 9a). A main effect of temperature was observed, whereby whole-body EE was significantly lower in HEAT (P = 0.0003). No time × temperature interaction was observed (P = 0.128). Mean whole-body EE was significantly different between-trials (P = 0.0003), whereby it was lower in HEAT (14 ± 8%). Specifically, mean whole-body EE was numerically lower in HEAT in all participants (Figure 9b). Subgroup analyses revealed a main effect of temperature for whole-body EE (P = 0.0006, Figure 9c). However, no main effect of season (P = 0.543), or temperature × season interaction (P = 0.447) was observed for mean whole-body EE.

(a)



(b)



(c)

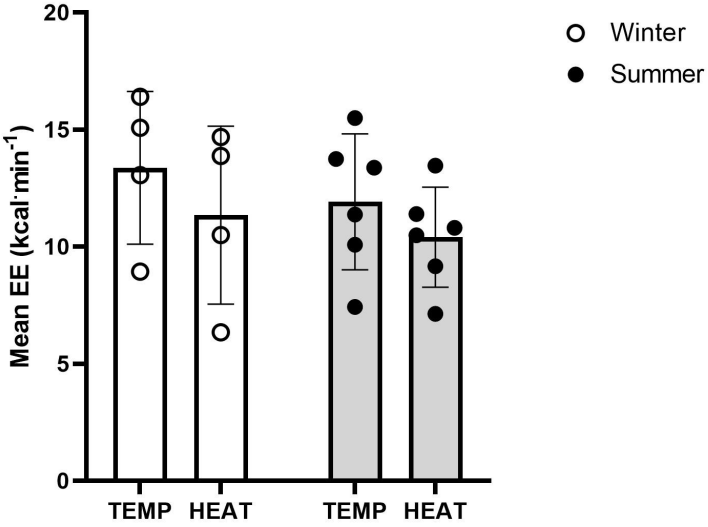
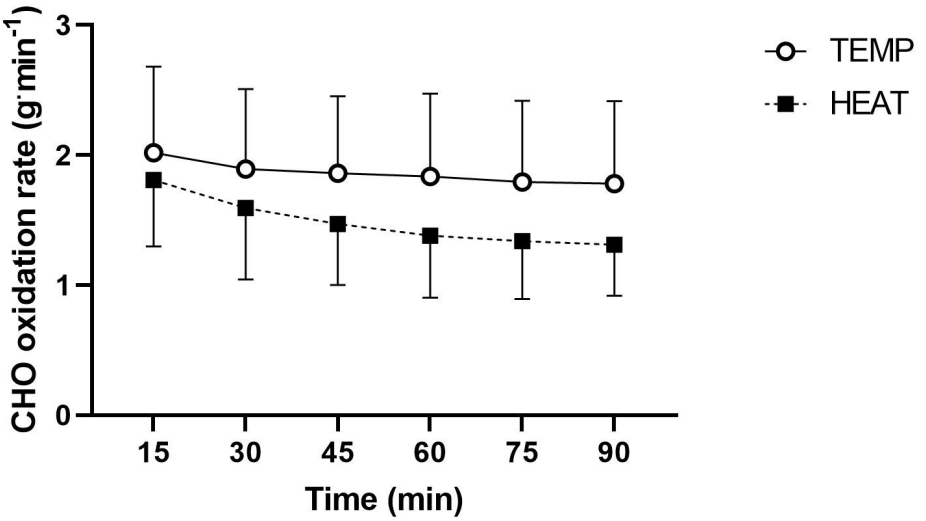


Figure 9. Energy expenditure (kcal·min^{-1}) during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). (a) Energy expenditure (kcal·min^{-1}) at each timepoint throughout the 90-min exercise trials, (b) mean energy expenditure (kcal·min^{-1}) during the 90-min exercise trials, and (c) mean energy expenditure (kcal·min^{-1}) of the winter (N = 4) and summer (N = 6) sub-groups. In 9a, a solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT. In 9b and 9c, bars indicate group mean values and dots identify individual mean values.

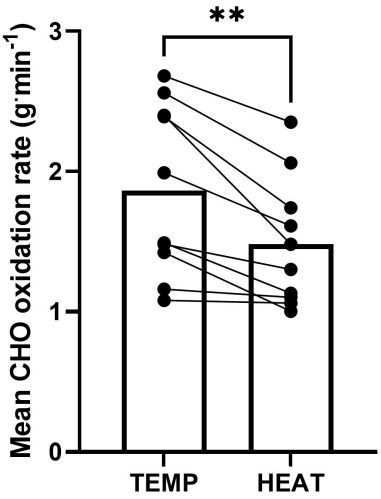
4.3.2 CHO oxidation

A main effect of time was observed for CHO oxidation, whereby it significantly decreased as exercise progressed (P = 0.003, Figure 10a). A main effect of temperature was observed, whereby CHO oxidation was significantly lower in HEAT (P = 0.002). A significant time × temperature interaction was observed (P = 0.018), whereby the reduction in CHO oxidation over time (15 vs. 90 min) was greater in HEAT (9.6 ± 21.3 and 27.1 ± 10.1%, in TEMP and HEAT, respectively, P = 0.003). Mean CHO oxidation was significantly different between-trials, whereby it was lower in HEAT (19 ± 11%, P = 0.002, Figure 10b). Specifically, mean CHO oxidation was lower in HEAT in all participants. Subgroup analyses revealed a main effect of temperature for CHO oxidation (P = 0.003, Figure 10c). However, no main effect of season (P = 0.58), or temperature × season interaction (P = 0.91) was observed for mean CHO oxidation.

(a)



(b)



(c)

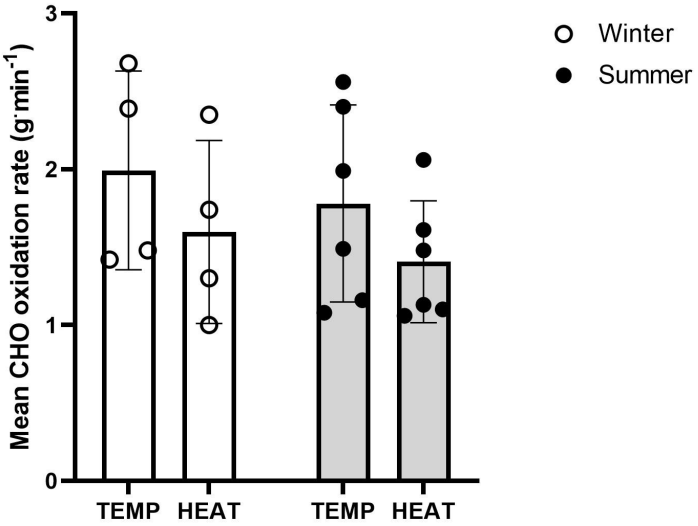
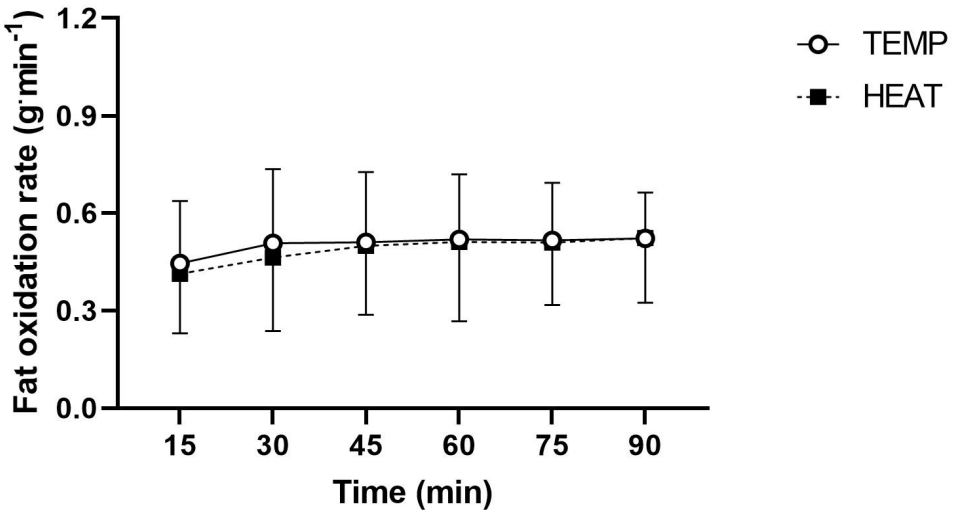


Figure 10. Carbohydrate oxidation rates ($g \cdot min^{-1}$) during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). (a) Carbohydrate oxidation rates ($g \cdot min^{-1}$) at each timepoint throughout the 90-min exercise trials, (b) mean carbohydrate oxidation rates ($g \cdot min^{-1}$) during the 90-min exercise trials, and (c) mean carbohydrate oxidation rates ($g \cdot min^{-1}$) of the winter (N = 4) and summer (N = 6) sub-groups. In 10a, a solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT. In 10b and 10c, bars indicate group mean values and dots identify individual mean values.

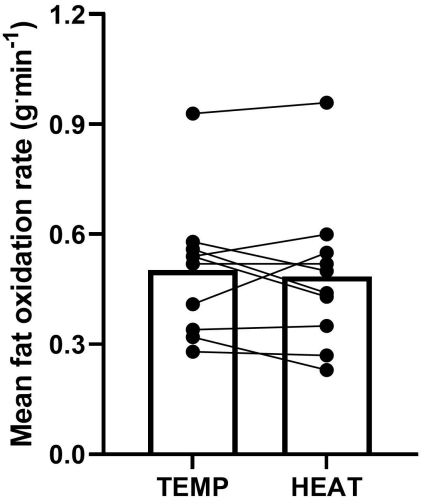
4.3.3 Fat oxidation

A main effect of time was observed for fat oxidation (P = 0.0002), whereby it significantly increased as exercise progressed (0.43 ± 0.18 and 0.52 ± 0.17 g·min^{-1}, at 15 and 90 min, P = 0.0011, Figure 11a). No main effect of temperature (P = 0.543), or time × temperature interaction (P = 0.725), was observed for fat oxidation. Mean fat oxidation was not significantly different between-trials (P = 0.535, Figure 11b). Subgroup analyses indicated no main effect of temperature (P = 0.462), season (P = 0.779), or temperature × season interaction (P = 0.91) was observed for mean fat oxidation (Figure 11c).

(a)



(b)



(c)

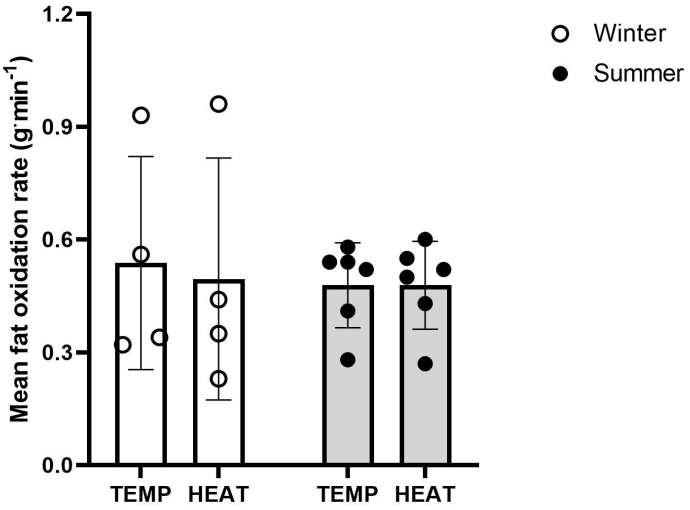
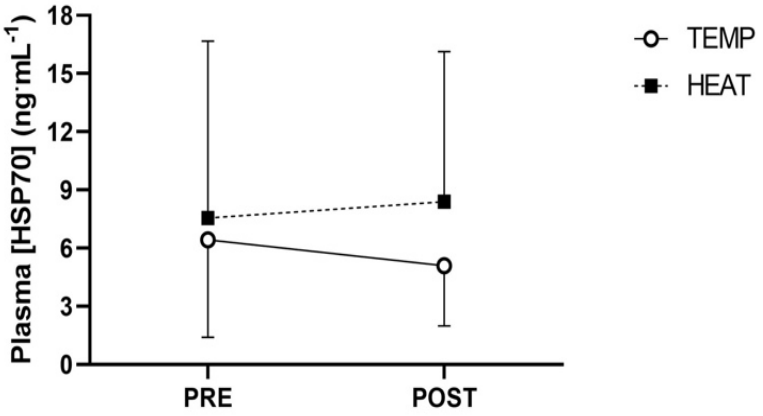


Figure 11. Fat oxidation rates ($g \cdot min^{-1}$) during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT). (a) Fat oxidation rates ($g \cdot min^{-1}$) at each timepoint throughout the 90-min exercise trials, (b) mean fat oxidation rates ($g \cdot min^{-1}$) during the 90-min exercise trials, and (c) mean fat oxidation rates ($g \cdot min^{-1}$) of the winter (N = 4) and summer (N = 6) sub-groups. In 11a, a solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT. In 11b and 11c, bars indicate group mean values and dots identify individual mean values.

4.4 Plasma heat shock protein 70 concentrations

No main effect of time (P = 0.796), temperature (P = 0.510), or time × temperature interaction (P = 0.301), was observed for plasma heat shock protein 70 concentration (Figure 12a). Subgroup analyses indicated no main effect of temperature (P = 0.306), season (P = 0.255), or temperature × season interaction (P = 0.379) was observed for post-exercise plasma heat shock protein 70 concentration (Figure 12b).

(a)



(b)

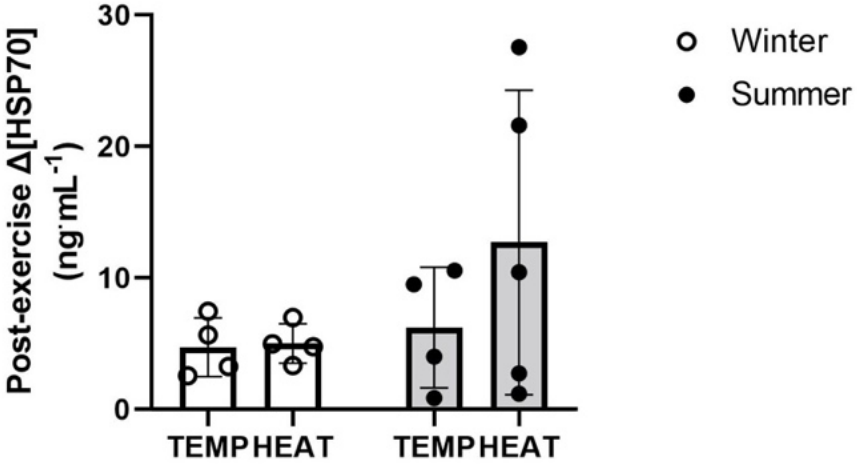


Figure 12. Plasma heat shock protein 70 concentrations ($ng \cdot mL^{-1}$) in 18˚C (TEMP) and 33˚C (HEAT). (a) Mean pre-exercise and post-exercise plasma heat shock protein 70 concentrations ($ng \cdot mL^{-1}$) in TEMP (N = 8) and HEAT (N = 9) trials, and (b) post-exercise plasma heat shock protein 70 concentrations ($ng \cdot mL^{-1}$) of the winter (N = 4) and summer (N = 4 and 5, in TEMP and HEAT, respectively) sub-groups. In 12a, a solid line with clear markers indicates data obtained from TEMP, and a dashed line with solid markers indicate data obtained from HEAT. In 12b, bars indicate group mean values and dots identify individual values.

4.5 Perceptual responses

4.5.1 Rating of perceived exertion (RPE)

A main effect of time was observed for RPE, whereby it significantly increased as exercise progressed (15 vs. 90 min, P = 0.0003, Figure 13). No main effect of temperature (P = 0.09), or time × temperature interaction (P = 0.761), was observed for RPE.

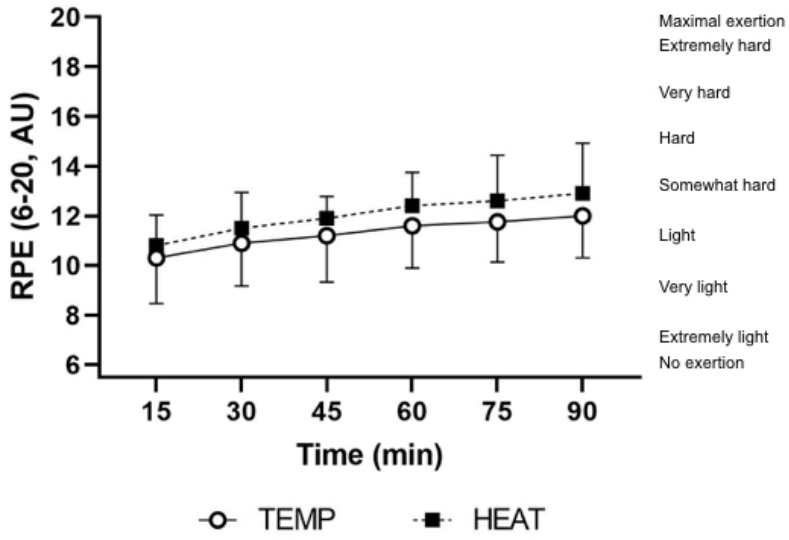


Figure 13. Rating of perceived exertion (RPE) (6-20, AU) at each timepoint during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT).

4.5.2 Thermal comfort

No main effect of time was observed for thermal comfort (P = 0.116), but a significant effect of temperature was observed, whereby thermal comfort was significantly higher in HEAT (P = 0.001, Figure 14). A significant time × temperature interaction was observed (P = 0.034), whereby the change in thermal comfort over time was greater in HEAT (-0.1 ± 0.7 and 1 ± 1.6 AU, at TEMP and HEAT, P = 0.128).

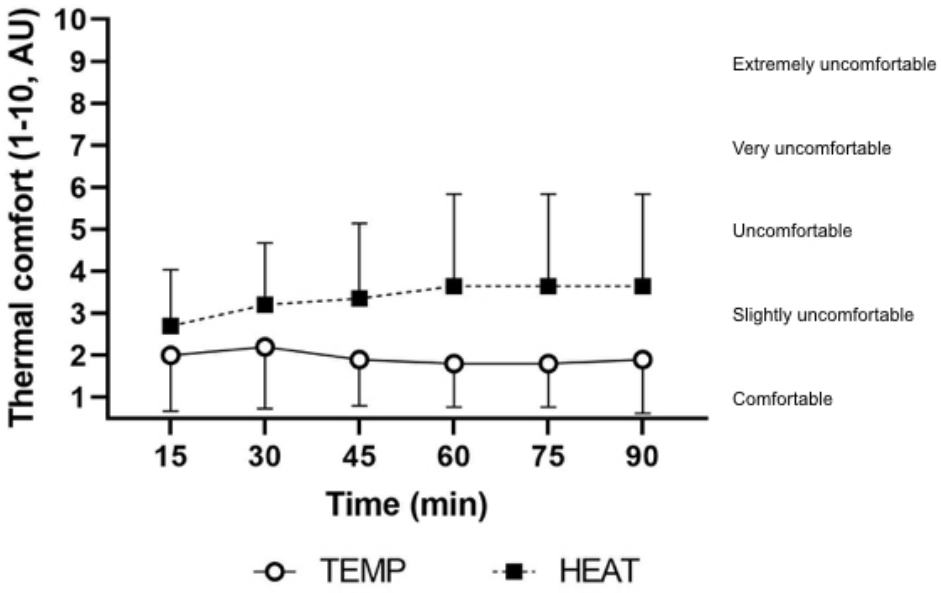


Figure 14. Thermal comfort (1-10, AU) at each timepoint during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT).

4.5.3 Thermal sensation

No main effect of time was observed for thermal comfort (*P* = 0.097), but a significant effect of temperature was observed, whereby thermal sensation was significantly higher in HEAT (*P* = 0.0002, Figure 15). No time × temperature interaction was observed for thermal sensation (*P* = 0.892).

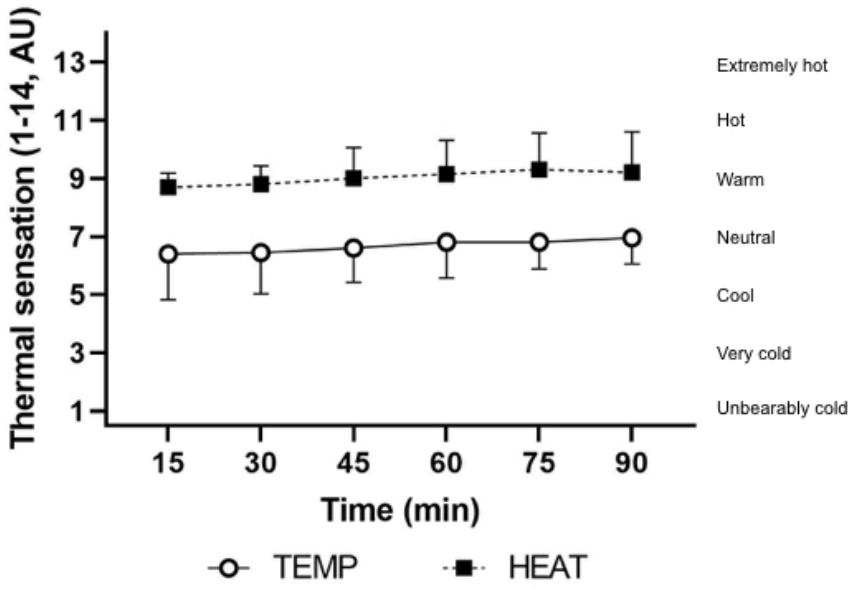


Figure 15. Thermal sensation (1-14, AU) at each timepoint during 90 min of moderate-intensity exercise with a target HR of 95% of the first ventilatory threshold in 18˚C (TEMP) and 33˚C (HEAT).

4.6 Bivariate correlations and regression models

Significant positive correlations between heat stress-induced change in mean CHO oxidation rate and heat stress-induced changes in mean power output and sweat rate were observed. However, no significant correlation was observed between heat stress-induced change in CHO oxidation rate and heat stress-induced changes in dehydration, mean T_{re}, or mean estimated T_{mus} (Table 3). A significant positive correlation between heat stress-induced in change in mean power output and heat stress-induced change in sweat rate was observed ($r = 0.64$, $P = 0.04$).

No significant correlations were observed between heat stress-induced change in post-exercise plasma HSP70 concentration and heat stress-induced changes in mean power output, dehydration, sweat rate, mean T_{re}, or mean estimated T_{mus} (Table 3).

A simple linear regression model containing heat stress-induced changes in mean power output, dehydration, sweat rate, mean T_{re}, and mean estimated T_{mus} as input variables did not significantly predict variation in heat stress-induced change mean CHO oxidation rate (Adjusted $R^2 = 0.41$, $P = 0.29$, Figure 16). Stepwise selection of this model retained only heat stress-induced increase in sweat rate (Adjusted $R^2 = 0.68$, $P = 0.004$). Similarly, a simple linear regression model containing heat stress-induced changes in mean power output, dehydration, sweat rate, mean T_{re}, and mean estimated T_{mus} as input variables did not significantly predict variation in heat stress-induced change end-exercise plasma HSP70 concentration (Adjusted $R^2 = -0.43$, $P = 0.73$, Figure 17). Stepwise section of this model retained only heat stress-induced increases in mean estimated T_{mus} and sweat rate, but the stepwise still did not predict variation in heat stress-induced change end-exercise plasma HSP70 concentration (Adjusted $R^2 = 0.22$, $P = 0.24$).

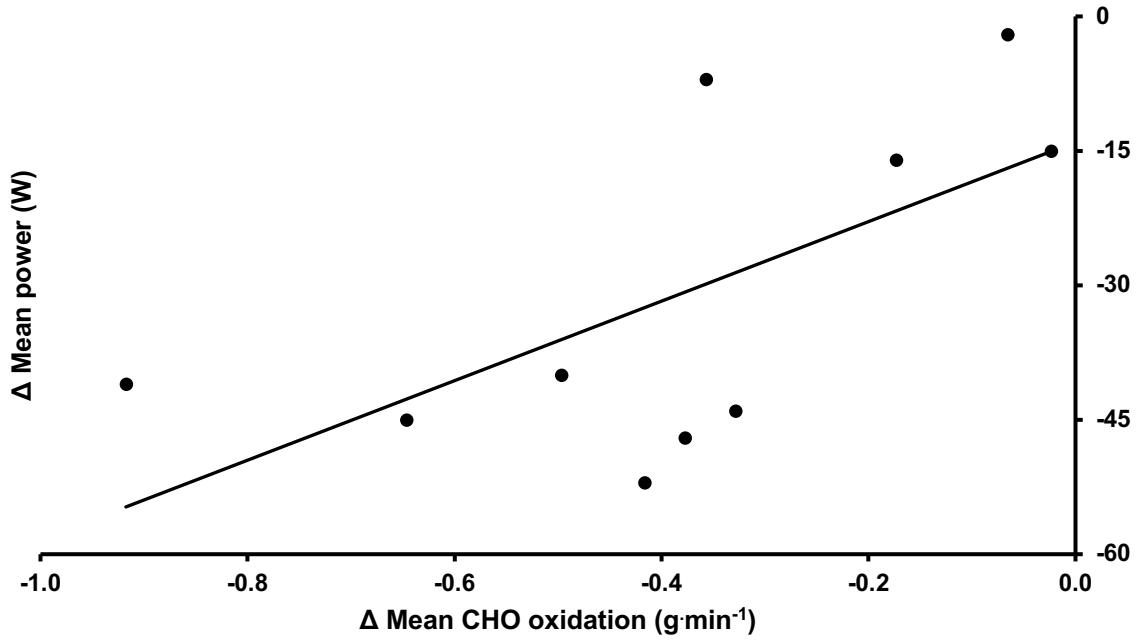
Table 3. Bivariate linear correlations between primary outcome measures (heat stress-induced changes in mean carbohydrate oxidation rate and post-exercise plasma HSP70 concentration) and input variables (heat stress-induced changes in mean power output, dehydration, sweat rate, mean rectal temperature, and mean estimated muscle temperature). Data are expressed with 95% confidence intervals. Significant correlations are in bold.

	Δ Mean CHO oxidation rate ($g \cdot min^{-1}$) **(N = 10)**	**Δ Post-exercise plasma HSP70 concentration ($ng \cdot mL^{-1}$)** **(N = 8)**
Δ Mean power (W)	**r = 0.64** **(0.01, 0.91)** ***P* = 0.05**	r_s = - 0.45 (-0.88, 0.37) *P* = 0.27
Δ Dehydration (% of body mass)	r = 0.42 (-0.29, 0.83) *P* = 0.23	r_s = -0.19 (-0.79, 0.59) *P* = 0.66
Δ Sweat rate ($L \cdot h^{-1}$)	**r = 0.85** **(0.49, 0.96)** ***P* = 0.002**	r_s = -0.40 (-0.86, 0.42) *P* = 0.33
Δ Mean T_{re} (˚C)	r = - 0.37 (-0.83, 0.39) *P* = 0.33 *	r_s = 0.17 (-0.61, 0.78) *P* = 0.69
Δ Mean estimated T_{mus} (˚C)	r = 0.08 (-0.58, 0.68) *P* = 0.83	r_s = 0.60 (-0.19, 0.92) *P* = 0.13

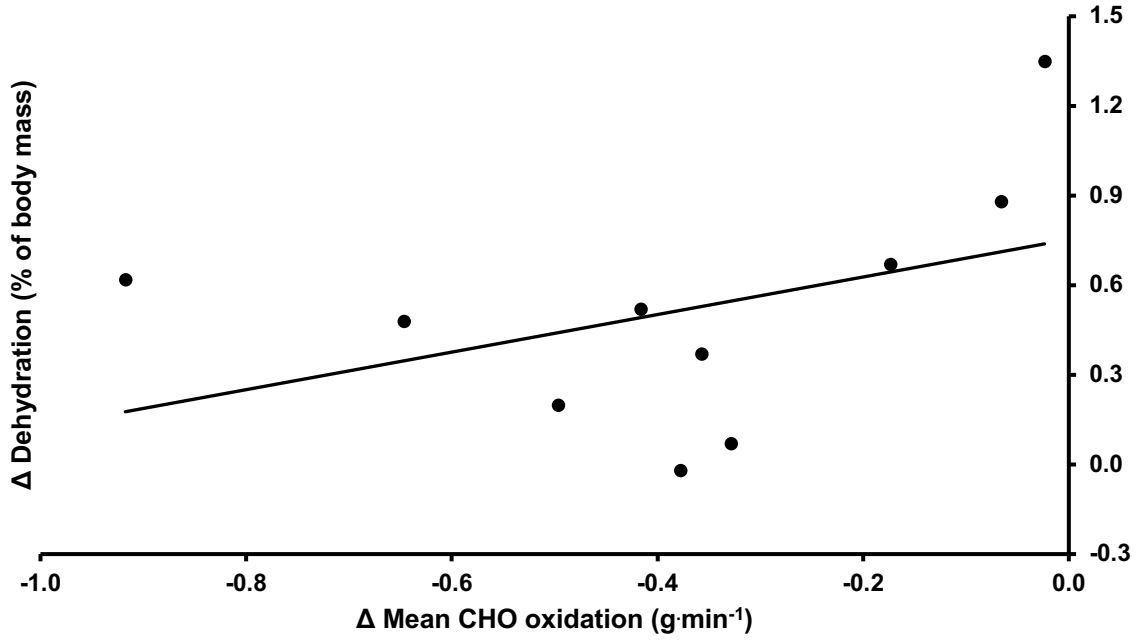
Δ represents the difference between the two experimental trials (HEAT – TEMP)

Abbreviations: CHO oxidation = Carbohydrate oxidation, HSP70 = Heat shock protein 70, T_{re} = Rectal temperature, estimated T_{mus} = Estimated muscle temperature, r = Pearson's product-moment correlation coefficients, r_s = Spearman's rank-order correlation coefficients, and * Missing data due to technical difficulty (N = 9)

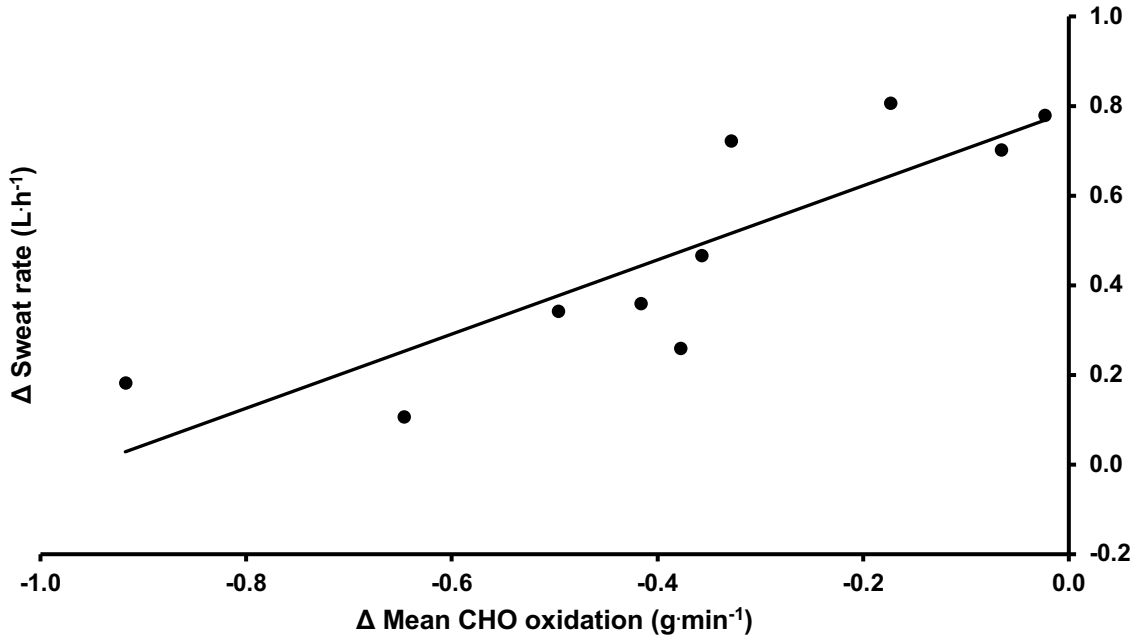
(a)


Δ Mean power (W)
0
-15
-30
-45
-60
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
Δ Mean CHO oxidation (g·min-1)

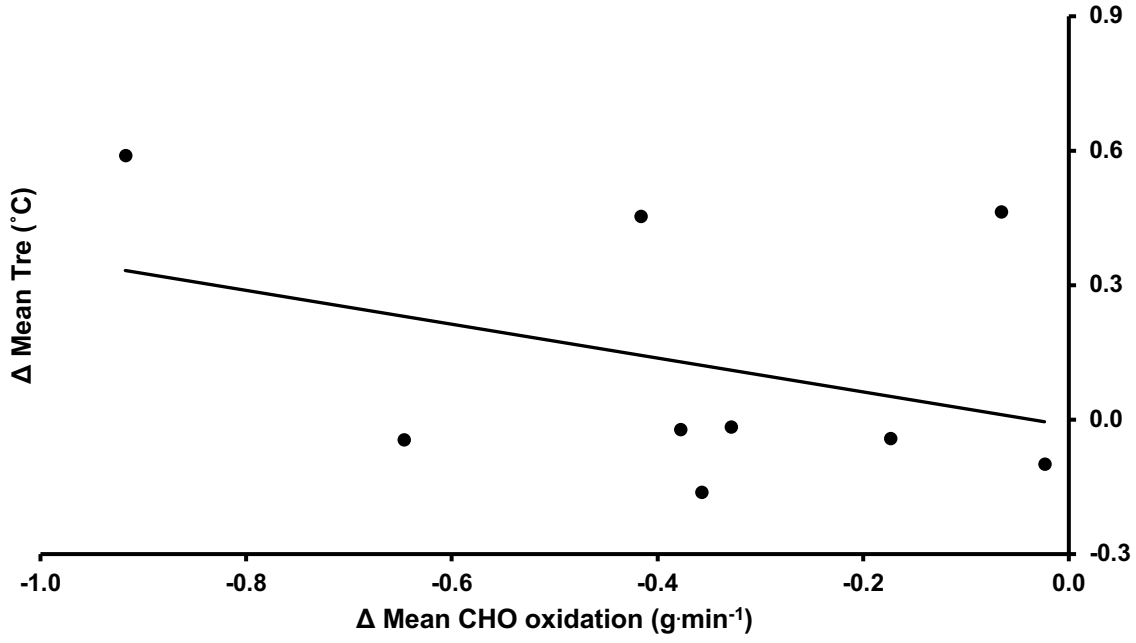
(b)


Δ Dehydration (% of body mass)
1.5
1.2
0.9
0.6
0.3
0.0
-0.3
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
Δ Mean CHO oxidation (g·min-1)

(c)


Δ Sweat rate (L·h-1)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
Δ Mean CHO oxidation (g·min-1)

(d)


Δ Mean Tre (˚C)
0.9
0.6
0.3
0.0
-0.3
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
Δ Mean CHO oxidation (g·min-1)

(e)

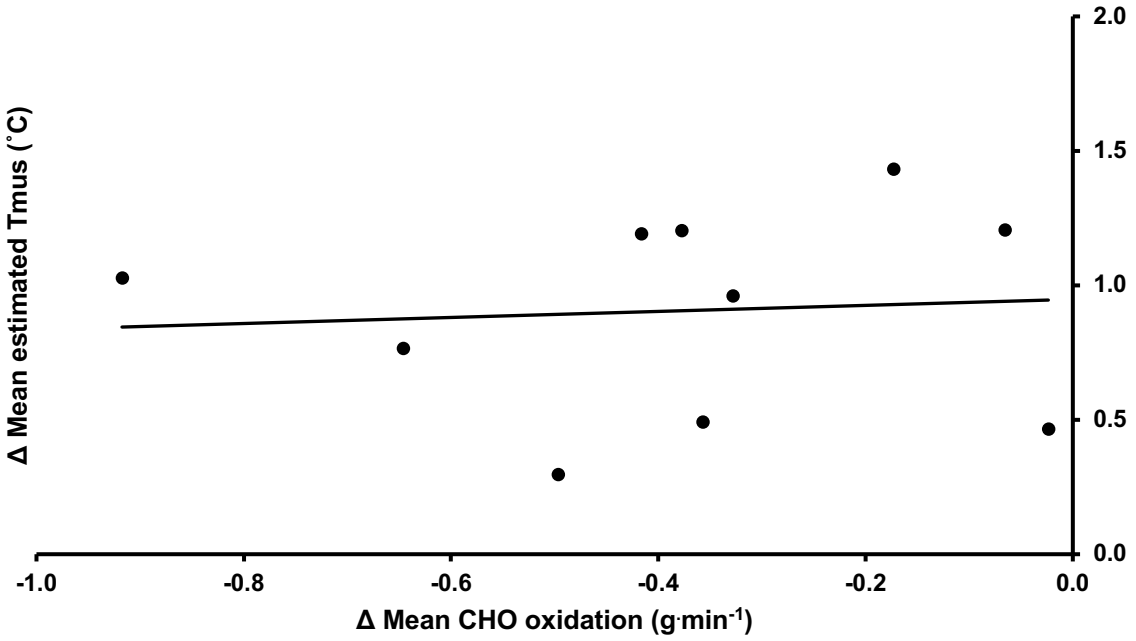
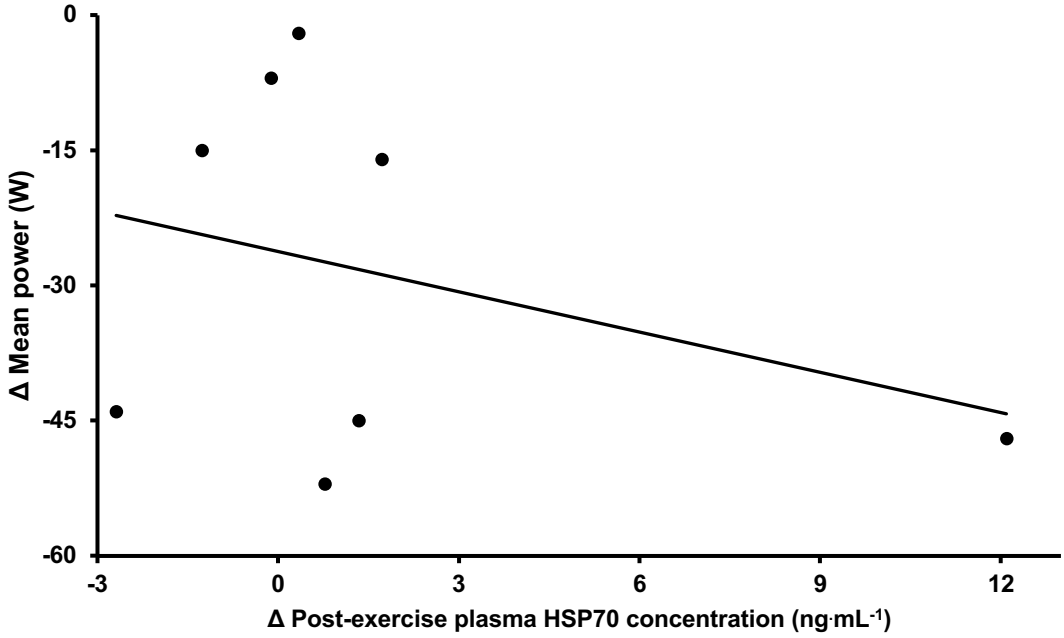
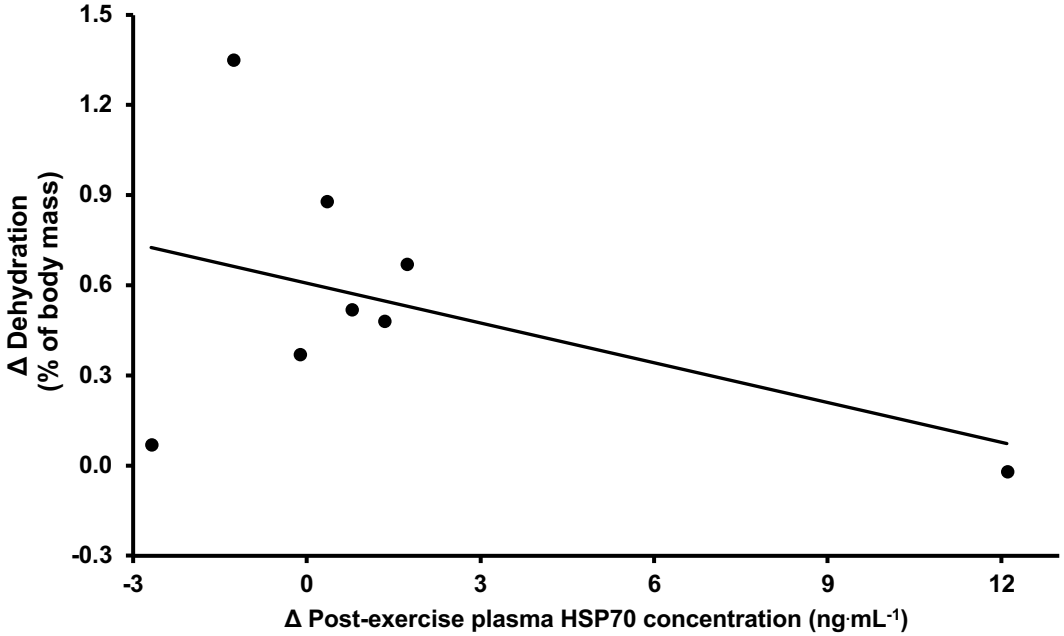


Figure 16. Bivariate linear correlations between heat stress-induced change in mean carbohydrate oxidation rate ($g \cdot min^{-1}$) and heat stress-induced changes in (a) mean power output (W), (b) dehydration (% of body mass), (c) sweat rate ($L \cdot h^{-1}$), (d) mean rectal temperature (˚C), and (e) mean estimated muscle temperature (˚C). Δ represents the difference between the two experimental trials (HEAT – TEMP).

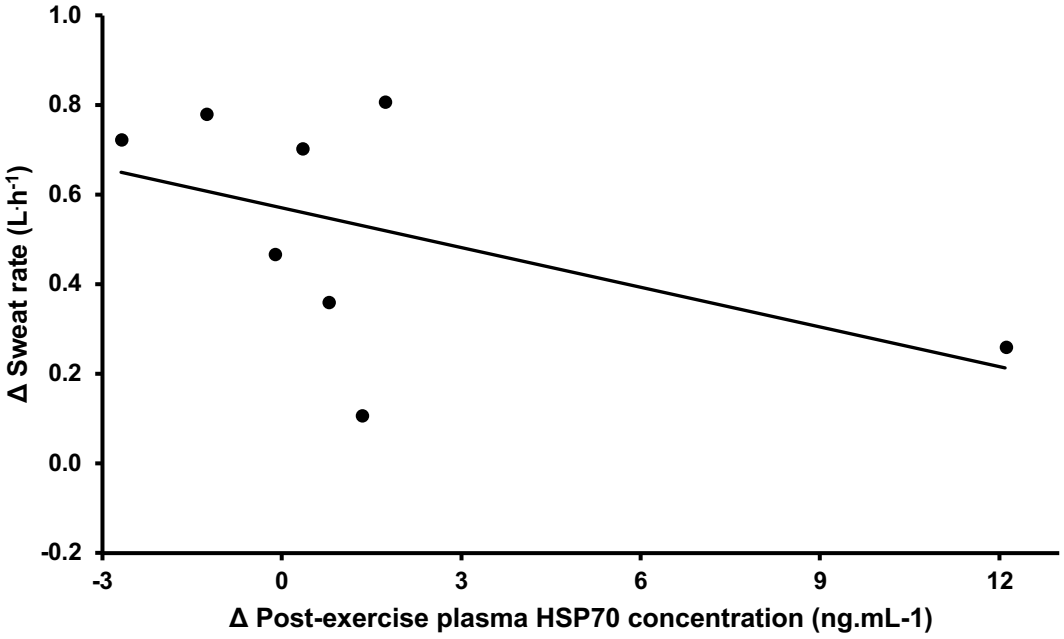
(a)


0
-15
-30
-45
-60
Δ Mean power (W)
-3
0
3
6
9
12
Δ Post-exercise plasma HSP70 concentration (ng·mL-1)

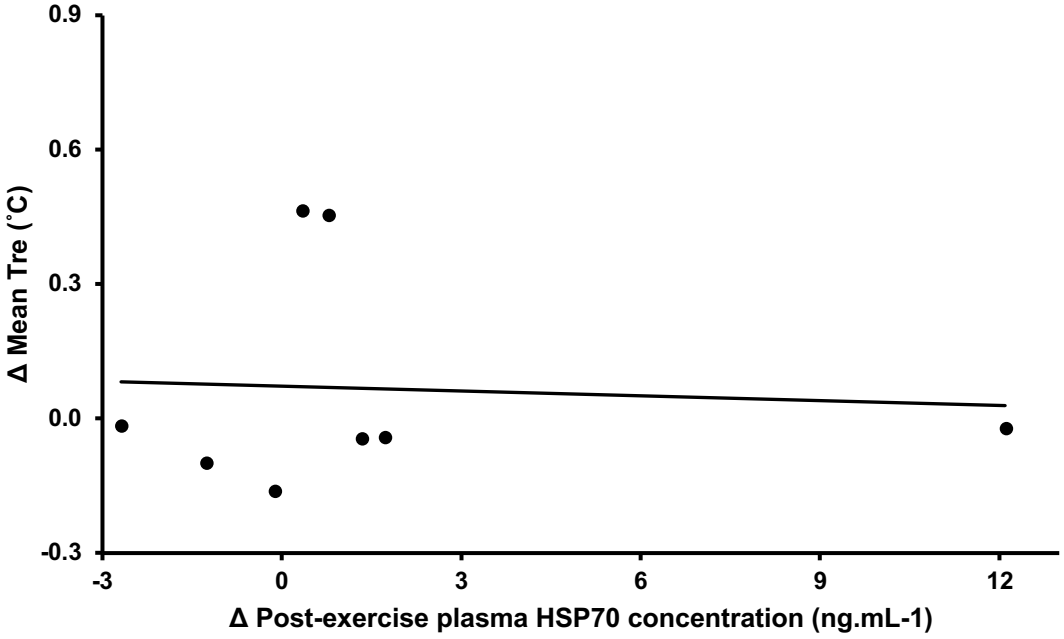
(b)


1.5
1.2
0.9
0.6
0.3
0.0
-0.3
Δ Dehydration (% of body mass)
-3
0
3
6
9
12
Δ Post-exercise plasma HSP70 concentration (ng·mL-1)

(c)


1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Δ Sweat rate (L·h-1)
-3
0
3
6
9
12
Δ Post-exercise plasma HSP70 concentration (ng.mL-1)

(d)


0.9
0.6
0.3
0.0
-0.3
Δ Mean Tre (°C)
-3
0
3
6
9
12
Δ Post-exercise plasma HSP70 concentration (ng.mL-1)

(e)

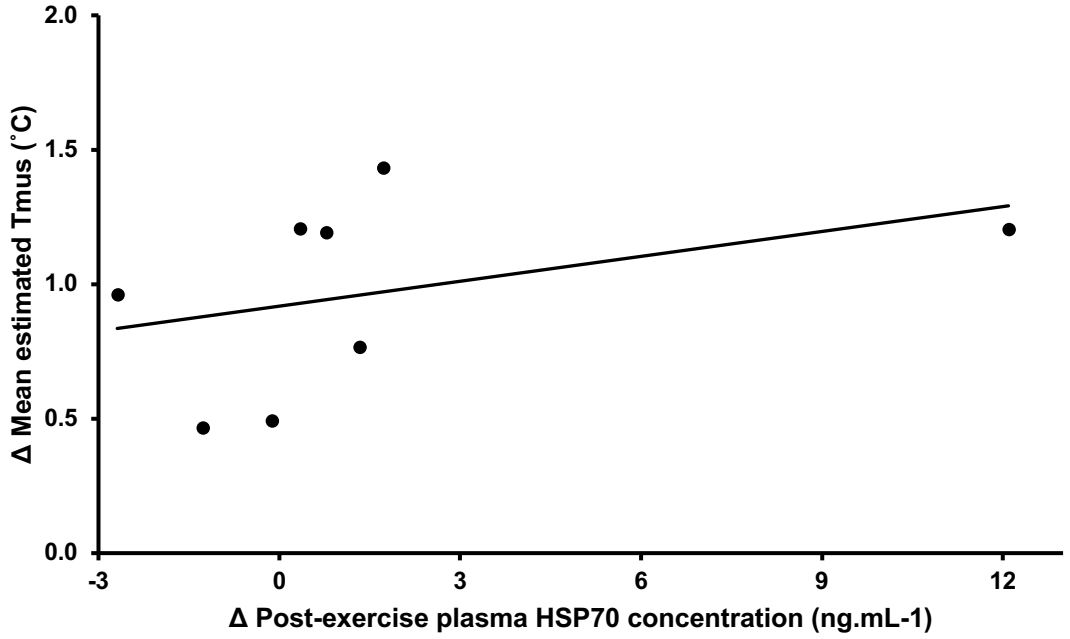


Figure 17. Bivariate linear correlations between heat stress-induced change in post-exercise plasma heat shock protein 70 concentration ($ng \cdot ml^{-1}$) and heat stress-induced changes in (a) mean power output (W), (b) dehydration (% of body mass), (c) sweat rate ($L \cdot h^{-1}$), (d) mean rectal temperature (˚C), and (e) mean estimated muscle temperature (˚C). Δ represents the difference between the two experimental trials (HEAT – TEMP).

Chapter 5: Discussion

The purpose of the present thesis was to determine the effect of moderate environmental heat stress on substrate oxidation rates and plasma HSP70 expression in response to heart rate-matched prolonged moderate-intensity cycling exercise. The primary findings were that (i) whole-body CHO oxidation rates were lower, but whole-body fat oxidation rates were not altered during moderate-intensity endurance exercise in HEAT, compared to equivalent exercise in TEMP (33 vs. 18˚C), with these effects likely due to a reduction in power output and associated decrease in EE in HEAT, and (ii) moderate-intensity endurance exercise did not increase plasma HSP70 expression in either HEAT or TEMP. Collectively, these data contribute to our understanding of how environmental temperature is likely to influence substrate oxidation rates and plasma HSP70 expression in an ecologically-valid model of endurance exercise.

In this present study, the experimental design was successfully administered as heart rate was statistically matched in TEMP and HEAT (Figure 3). As exercise intensity was regulated in relation to HR thresholds (95% of the HR associated with VT_1), the reduced power output in HEAT vs. TEMP was not unexpected. Indeed, a decrease in mean power output was observed in all participants in HEAT (-17 ± 11 % from mean power output in TEMP, $P = 0.001$, Figure 4). Lower power output achieved during endurance exercise – at a given HR – in HEAT may be explained by an increase in peripheral blood flow required to regulate temperature via sweating (Febbraio, 2001), which is supported by the observed exercise-induced increase in sweat rate in HEAT (87 ± 63%, $P = 0.0003$, Figure 7). Under mildly hyperthermic conditions, a progressive increase in peripheral blood flow associates with greater cardiac output and reduced stroke volume, and therefore results in an increase in HR during exercise (Trinity et al., 2010). Thus, participants had to reduce the absolute work rate to maintain the HR target during endurance exercise in HEAT.

In the present thesis, RPE increased significantly as exercise duration progressed, but was not different between TEMP and HEAT (Figure 13), suggesting that the individual's perceived exertion during endurance exercise appears to be similar between-trial despite a difference in the achieved absolute work rates, and an addition of thermal stress. Moreover, thermal comfort and thermal sensation data demonstrate that participants felt less comfortable (P = 0.001, Figure 14), and perceived their body temperatures to be warmer when exercised in HEAT (P = 0.0002, Figure 15). Therefore, along with the reduction in mean power output, these data collectively indicate that the moderate environmental heat stress used in the present experimental model did indeed exert a physiological and perceptual effect on the participants.

It is acknowledged that six of the ten participants were tested during the warmer months, and it is plausible that these athletes might be more naturally acclimated to hot temperatures, and therefore be affected relatively less by the heat stress experimentally employed than those athletes tested in the winter months (Lei et al., 2021). In order to assess if there were seasonal effects on the primary outcome measures of this study, subgroup analyses were performed. As the present results show that no between-subgroup difference was observed in any variables (Figure 4c, Figure 9c, Figure 10c, Figure 11c, Figure 12c), it could be assumed that the seasonal effects on the primary outcome measures are negligible in this present study. Therefore, in this discussion section, I am confident to elaborate on the findings of this study from the whole cohort of ten participants.

In alignment with my hypothesis, the present data suggests that whole-body CHO oxidation rates are likely lower when moderate-intensity endurance exercise is performed under moderate environmental heat stress, if the intensity is matched using heart rate (1.86 ± 0.61 vs. 1.48 ± 1.46 $g \cdot min^{-1}$, TEMP vs. HEAT, P = 0.002, Figure 10b). However, these findings appear to be in opposition to previous research demonstrating increased carbohydrate metabolism during endurance exercise performed under environmental heat stress (≥ 30˚C) compared to equivalent exercise in temperate

conditions (Febbraio et al., 1994a; Febbraio et al., 1994b; Hargreaves et al., 1996a; Jentjens et al., 2002; Maunder et al., 2020). It is acknowledged that the aforementioned investigations have studied effects of environmental heat stress at matched absolute, external work rates (i.e., 65-70% $\dot{V}O_{2peak}$ (Febbraio et al., 1994a; Febbraio et al., 1994b; Hargreaves et al., 1996a), 55% maximum power output (Jentjens et al., 2002), the absolute power outputs eliciting at VT_1 and VT_2 (Maunder et al., 2020)), while in this present study, exercise intensity was prescribed as a specified HR target equivalent to 95% (± 2 $b \cdot min^{-1}$) of the VT_1 HR. To my knowledge, this is the first study that has compared substrate oxidation rates during endurance cycling exercise at matched internal work rates, or the same HR performed in TEMP (18˚C) and HEAT (33˚C) environments.

Indeed, the bivariate correlational analysis indicates that the observed heat stress-induced decrease in whole-body CHO oxidation rate may be explained by the associated reduction in power output in HEAT (Table 3). The lower absolute power output in HEAT likely reduced the demand for ATP synthesis, as evidenced by the observed heat stress-induced reduction in whole-body EE (12.50 ± 2.96 vs. 10.78 ± 2.75 $g \cdot min^{-1}$, TEMP vs. HEAT, P = 0.0003, Figure 9b). Interestingly, the present data suggests whole-body fat oxidation rates during moderate-intensity exercise were unaffected by environmental temperature (P = 0.535, Figure 11b). This aligns with previous research reporting that whole-body fat oxidation rates were not affected by environmental heat stress (28, 34, 40 vs. 18˚C) during 20-min moderate-intensity cycling exercise (P > 0.05) (Maunder et al., 2020). Therefore, the observed heat stress-induced reduction in whole-body EE associated with the lower power output during moderate-intensity endurance exercise performed in HEAT is explained by reduced CHO oxidation and maintained fat oxidation rates, with the assumption that protein oxidation contributing to total energy expenditure during exercise is negligible.

Furthermore, heat stress-induced changes in sweat rate may also explain the observed heat stress-induced decrease in whole-body CHO oxidation rate (Table 3). This aligns

with available evidence indicating that exercise-induced dehydration stimulates various physiological responses (e.g., increases in HR and core temperature), and alters substrate metabolism in a greater magnitude compared to the same exercise performed with euhydration (Logan-Sprenger et al., 2015; Logan-Sprenger et al., 2012). The present data demonstrates that sweat rate was significantly greater in HEAT (0.6 ± 0.3 and 1.1 ± 0.4 $L \cdot h^{-1}$, TEMP vs. HEAT, P = 0.0003, Figure 7). Evaporation of sweat facilitates heat dissipation during exercise, and consequently the observed heat stress-induced increase in sweat rate was likely to reflect greater rate of heat dissipation during endurance exercise in HEAT. However, T_{re} increased with endurance exercise similarly in the two environments (1.1 ± 0.4 and 1.2 ± 0.6˚C, in TEMP and HEAT, respectively, P < 0.0001, Figure 5), and reached 37.8 ± 0.4 and 38.0 ± 0.5˚C, at the end of exercise in TEMP and HEAT, respectively. These data indicate that heat storage induced by endurance exercise was relatively similar between the two environments. Plausibly, participants who had a larger sweat response to environmental heat stress could dissipate heat more efficiently in HEAT, and therefore produce more metabolic heat for the same T_{re} response during endurance exercise, resulting in a more subtle reduction in power output in HEAT vs. TEMP. Therefore, athletes with greater between-environment difference in sweat rate had lower reduction in power output, and therefore lower reduction in CHO oxidation rates (Figure 16c).

Secondly, in this model of moderate-intensity cycling exercise regulated by heart rate, an exercise-induced increase in plasma HSP70 expression was not observed in either TEMP or HEAT (Figure 12a). This finding contrasts with my hypothesis, which was that heat exposure would further increase exercise-induced plasma HSP70 expression, despite the likely, and observed, reduction in power output (HEAT vs. TEMP). Indeed, based on the results of bivariate correlational analysis, heat stress-induced change in plasma HSP70 expression was unlikely to associate with heat stress-induced reduction in power output during moderate-intensity cycling exercise regulated by heart rate (Table 3). As mentioned above, endurance exercise combined with exposure to environmental

heat stress resulted in reductions in power output (Figure 4b) and EE (Figure 9b), which may have also blunted any potential for heat stress-induced stimulation of HSP70 expression. Consideration of the addition of an experimental trial in a hot environment at the same absolute power output as achieved in TEMP would have been useful to confirm these speculations.

Many published studies have reported a significant increase in extracellular HSP72 concentration in unacclimated individuals following endurance exercise performed in temperate (18-24˚C) (Fehrenbach et al., 2005; Whitham et al., 2007) and hot environments (35-40˚C) (Gibson et al., 2014; Magalhães et al., 2010; Marshall et al., 2006; Périard et al., 2012; Whitham et al., 2007), but exercise-induced increase in plasma HSP70 accumulation did not occur in the present investigation (Figure 12a). It is worth acknowledging that the aforementioned studies have mainly focused on the expression of extracellular *HSP72* in response to various experimental protocols, while the enzyme-linked immunosorbent assay kit used in this present study quantified the concentration of the whole HSP70 family (including HSP72, HSP73, GRP75 and GRP78 isoforms). This could at least partially explain greater plasma HSP70 concentrations (~6-9 $ng \cdot mL^{-1}$) observed in this present thesis, compared with the other studies measuring extracellular HSP72 (~1-6 $ng \cdot mL^{-1}$) (Fehrenbach et al., 2005; Magalhães et al., 2010; Marshall et al., 2006; Périard et al., 2012; Whitham et al., 2007). As existing evidence has demonstrated that the other members of the HSP70 family, apart from HSP72, are unlikely to be stimulated by environmental heat stress (Kregel, 2002; Locke, 1997; Welch & Suhan, 1986), it is plausible that the capture of HSP70 may have obscured an effect of endurance exercise on plasma HSP72 expression. Additionally, the CV of the assay performed in this present investigation was ~13.0%, which may also be contribute to the lack of observed plasma HSP70 accumulation during endurance exercise in this present study.

In TEMP, the exercise-induced change in plasma HSP70 concentration was not statistically significant (6.43 ± 5.04 vs. 5.48 ± 3.44 $ng \cdot mL^{-1}$, at pre- vs. post-exercise in

TEMP, respectively, N = 8, Figure 12a). This finding contrasts to previous research reporting a significant increase in plasma HSP72 concentration in response to endurance exercise performed in temperate conditions (Fehrenbach et al., 2005; Whitham et al., 2007). Existing evidence indicates that the extracellular HSP72 response to endurance exercise performed in temperate conditions is related to exercise intensity and duration (Fehrenbach et al., 2005), with the addition of internal thermal stress, evidenced by an increase in core temperature, promoting further increases extracellular HSP72 responses (Marshall et al., 2006). In this present study, exercise intensity was prescribed as a specified HR target equivalent to 95% (± 2 $b \cdot min^{-1}$) of the VT_1 HR, thus was maintained in the moderate domain throughout the entire protocol (Jamnick et al., 2020). Expired gases collected during TEMP indicated the intensity corresponded to 58.8 ± 5.4 $\%\dot{V}O_{2peak}$. This is relatively similar to studies reporting increased concentrations of plasma HSP72 after endurance exercise intensity prescribed at ~60% $\dot{V}O_{2peak}$ (Fehrenbach et al., 2005; Whitham et al., 2007), suggesting that moderate exercise intensities may be sufficient to stimulate extracellular HSP72 expression. Therefore, it appears that the exercise intensity used in the present investigation is unlikely to explain my finding that plasma HSP70 expression was not increased by exercise. However, it should be acknowledged that the mentioned investigations were conducted with differences in exercise duration and exercise modality (cycling vs. treadmill or under-water running), which might explain the lack of plasma HSP70 accumulation during endurance exercise in this present study.

Insufficient exercise duration may explain the lack of stimulation of extracellular HSP70 (Figure 12a), as the aforementioned studies included longer exercise protocols (90 vs. 120 min) (Fehrenbach et al., 2005; Whitham et al., 2007). Existing evidence demonstrates that at the similar absolute work-rates, the magnitude of plasma HSP70 concentration is more pronounced with an increase in running duration (Fehrenbach et al., 2005). This study reported that an outdoor running bout of 260 ± 39 min at ~65% $\dot{V}O_{2peak}$ resulted in a more than 2.5-fold increase in extracellular HSP72 expression

during exercise, compared with a 120-min treadmill exercise at ~60% $\dot{V}O_{2peak}$ ($P < 0.05$). Therefore, these data suggest exercise duration plays an important role in the release of HSP72 into the circulation during endurance exercise (Fehrenbach et al., 2005). A longer exercise duration may further increase heat stress-induced physiological strain relevant to HSP72 accumulation, which may include elevated core and/or local temperatures (Cuthbert et al., 2019; Morton et al., 2006), or decreased carbohydrate availability (Dalgaard et al., 2022; Febbraio & Koukoulas, 2000; Febbraio et al., 2002b). Plausibly, an exercise-induced increase in plasma HSP70 expression may have been observed in this present study if exercise duration was ≥ 120 min.

Furthermore, the cycling modality used in this present study may also explain the lack of stimulation of extracellular HSP70 expression. Although increases in extracellular HSP72 concentration have been observed following treadmill running (Fehrenbach et al., 2005) and deep-water running exercise (Whitham et al., 2007), a similar experimental study employing cycling exercise (Gibson et al., 2014) appeared to demonstrate relevant findings to this present study (Figure 12a). This study reported that 90-min cycling exercise at 50% $\dot{V}O_{2peak}$ in 20˚C did not increase plasma HSP72 expression (-1.9% from pre-exercise plasma HSP70 concentration, $P > 0.05$) (Gibson et al., 2014). Available evidence demonstrates that less muscle mass is activated during cycling exercise, compared to running exercise (Bijker et al., 2002; Brownstein et al., 2022). Specifically, the majority of force production during cycling exercise is generated from the quadriceps, while the hamstrings and the gastrocnemius are more comparatively important for force production in running exercise (Bijker et al., 2002). Accordingly, it is possible that lower metabolic demand is required for cycling than running exercise at the same relative work rates.

Interestingly, exposure to heat stress combined with endurance exercise did not evoke a significant increase in post-exercise plasma HSP70 expression in this present study (7.56 ± 9.11 vs. 9.29 ± 9.18 $ng \cdot ml^{-1}$, at pre- vs. post-exercise in HEAT, respectively, P = 0.796, N = 9, Figure 12a). As endurance exercise was performed at the same HR, lower

external power output was observed in HEAT (Figure 4b). Data from expired gas collection demonstrated that participants exercised at the relative work rate corresponding to 50.0 ± 6.2 %$\dot{V}O_{2peak}$ in HEAT. In a similar study in cycling, Gibson et al. (2014) reported similar findings; specifically that plasma HSP72 concentration did not significantly increase at the end of 90-min cycling exercise at 50% $\dot{V}O_{2peak}$ in 30˚C (25.7% from pre-exercise plasma HSP70 concentration, $P > 0.05$).

Previous studies reporting increased extracellular HSP70 concentrations in response to a single bout of endurance exercise in hot conditions have typically utilised a greater magnitude of environmental heat stress than the present investigation (35-40 vs. 33˚C) (Gibson et al., 2014; Magalhães et al., 2010; Marshall et al., 2006; Périard et al., 2012; Whitham et al., 2007). Endurance exercise performed in a higher environmental temperature might further elicit thermoregulatory responses (Gibson et al., 2014; Marshall et al., 2006; Périard et al., 2012), which might further stimulate the release of HSP72 into the circulation. Specifically, research indicates that the elevated core temperature attained during moderate-intensity endurance exercise (50-60% $\dot{V}O_{2peak}$) may contribute to the upregulated circulating HSP72 (Gibson et al., 2014; Périard et al., 2012). In this present investigation, exercise-induced changes in core temperature were similar between the two environments. Indeed, both exercise-induced elevation of T_{re} (1.1 ± 0.4 and 1.2 ± 0.6˚C, in TEMP and HEAT, $P < 0.0001$) and end-exercise T_{re} (37.8 ± 0.4 and 38.0 ± 0.5 ˚C, in TEMP and HEAT, Figure 5) were not significantly different between the two environments. This lower metabolic heat production when moderate-intensity endurance exercise was performed in hot conditions may be explained by the heat stress-induced reduction in power output (Figure 4a, Figure 4b) associated with the observed decrease in whole-body EE in HEAT (Figure 9a, Figure 9b). Although the combination of endurance exercise and moderate environmental heat stress (33˚C) resulted in a significant increase in T_{re} to 38.0 ± 0.5˚C at the end of exercise ($P < 0.0001$, Figure 5), this is well below a proposed minimum endogenous criteria of T_{re} (≥ 38.5˚C) for the stimulation of HSP70 to be released into the circulation (Amorim et al., 2008).

Therefore, it is possible that the apparently absent effect of moderate environmental heat stress on extracellular HSP70 expression during endurance exercise might possibly be due to insufficient thermal stress in this present study.

5.1 Practical applications

In terms of practical applications, the results of this present thesis (Figure 10a, Figure 10b) suggest that moderate-intensity endurance exercise – regulated by heart rate – in combination with exposure to heat stress (33˚C) has the potential to reduce whole-body CHO oxidation rates associating with the reduction in external work rate, compared to the equivalent exercise performed under temperate conditions. Practitioners who are working with athletes exposed to hot environments incidentally (e.g., during summer months) or purposefully (e.g., heat acclimation training) may take these variables into account when programming training schedules and considering nutrition. For example, carbohydrate refeeding following training performed under heat stress may not need to be as aggressive as previously thought, given that, if power output does decrease, it appears likely that the CHO oxidation associated with exercise may actually be lower than had that session been performed in temperate conditions. Furthermore, exposure to heat stress during moderate-intensity exercise need not be avoided if one of the training goals is to elicit a high rate of fat metabolism, assuming power output is allowed to fall, as whole-body fat oxidation rates appear to be similar in HEAT and TEMP (Figure 11a, Figure 11b). Lastly, many previous studies (Bates & Miller, 2008; Sawka & Montain, 2000; Trinity et al., 2010), combined with the data from this present thesis (Figure 7, Figure 8), demonstrate that endurance exercise performed in hot conditions results in augmented sweat rates and greater dehydration (% of body mass) compared to temperate environments. The present study indicates this remains the case even when intensity is regulated by HR, and therefore at a lower power output. Therefore, this might be considered in hydration strategies.

5.2 Limitations

Nevertheless, the experimental model employed in this present study does have some limitations that should be considered. The first primary limitation relates to the failure to provoke HSP70 response to endurance exercise in both TEMP and HEAT environments. As previously acknowledged, exposure to environmental heat stress appeared to reduce the external power output, and therefore total energy expenditure during endurance exercise. This may have obstructed other exercise-induced stimuli that potentially activate HSP70 expression. To minimise any potential effects of reductions in external power output and total energy expenditure on plasma HSP70 expression, the addition of an experimental trial in HEAT with the exercise intensity matched the absolute power output as achieved in TEMP would have been useful to confirm these speculations. Secondly, existing evidence has indicated that adrenaline has a stimulatory effect on both carbohydrate metabolism (Febbraio et al., 1994a; Febbraio et al., 1996b; Hargreaves et al., 1996b), and HSP70 release (Whitham et al., 2006) in exercising humans. Therefore, a plasma adrenaline concentration analysis, which was not performed in this present study due to budgetary limitations, would broaden understanding of the effects of environmental heat stress on substrate metabolism and extracellular HSP70 expression, and may have been useful for explaining the primary outcomes of the present thesis.

5.3 Conclusion

In conclusion, moderate-intensity cycling exercise regulated by heart rate in a hot environment (33˚C) reduced whole-body CHO oxidation rates, but did not alter fat oxidation rates compared to the equivalent exercise performed in a temperate environment (18˚C). This metabolic change was associated with the lower external power output and greater heat dissipation when endurance exercise combined with

environmental heat stress. However, exercise-induced increases in plasma HSP70 expression did not occur in either the temperate or hot environment. These data should be considered by practitioners working with endurance athletes who are exposed to moderate environmental heat stress, either incidentally or purposefully, during training and/or competition.

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Appendices

Appendix 1: Ethics approval

Auckland University of Technology Ethics Committee (AUTEC)

Auckland University of Technology
D-88, Private Bag 92006, Auckland 1142, NZ
T: +64 9 921 9999 ext. 8316
E: ethics@aut.ac.nz
www.aut.ac.nz/researchethics

2 June 2021

Ed Maunder
Faculty of Health and Environmental Sciences

Dear Ed

Re Ethics Application: **21/121 The effect of environmental temperature on substrate oxidation and extracellular heat shock proteins in response to heart rate matched moderate-intensity cycling in endurance trained males**

Thank you for providing evidence as requested, which satisfies the points raised by the Auckland University of Technology Ethics Committee (AUTEC).

Your ethics application has been approved for three years until 2 June 2024.

Standard Conditions of Approval

1. The research is to be undertaken in accordance with the Auckland University of Technology Code of Conduct for Research and as approved by AUTEC in this application.
2. A progress report is due annually on the anniversary of the approval date, using the EA2 form.
3. A final report is due at the expiration of the approval period, or, upon completion of project, using the EA3 form.
4. Any amendments to the project must be approved by AUTEC prior to being implemented. Amendments can be requested using the EA2 form.
5. Any serious or unexpected adverse events must be reported to AUTEC Secretariat as a matter of priority.
6. Any unforeseen events that might affect continued ethical acceptability of the project should also be reported to the AUTEC Secretariat as a matter of priority.
7. It is your responsibility to ensure that the spelling and grammar of documents being provided to participants or external organisations is of a high standard and that all the dates on the documents are updated.

AUTEC grants ethical approval only. You are responsible for obtaining management approval for access for your research from any institution or organisation at which your research is being conducted and you need to meet all ethical, legal, public health, and locality obligations or requirements for the jurisdictions in which the research is being undertaken.

Please quote the application number and title on all future correspondence related to this project.

For any enquiries please contact ethics@aut.ac.nz. The forms mentioned above are available online through http://www.aut.ac.nz/research/researchethics

(This is a computer-generated letter for which no signature is required)

The AUTEC Secretariat
Auckland University of Technology Ethics Committee

Cc: Ppg0795@autuni.ac.nz; Andrew Kilding

Appendix 2: Participant information sheet

Date Information Sheet Produced:

2nd June 2021

Project Title

The effect of environmental temperature on substrate oxidation and extracellular heat shock proteins in response to heart-rate matched moderate-intensity cycling in endurance trained males

An Invitation

Kia ora! I'm Pam, Thanchanok Charoensap. I am a Master of Sport and Exercise student at AUT's Sports Performance Research Institute New Zealand supervised by Dr. Ed Maunder and Prof. Andrew Kilding. I am delighted to extend you an invitation to participate in my research project, which is focusing on how environmental heat stress affects our metabolism during prolonged exercise at the target heart rate.

What is the purpose of this research?

The purpose of this study is to investigate the effect of a hot environment, which is simulated in our environmental chamber at AUT Millennium, on human metabolism in response to moderate-intensity prolonged exercise at a target heart rate. We want to figure out how our metabolism during exercise is impacted by moderate heat-stress. This study will form part of my Master of Sport and Exercise. We intend to write-up the study for publication in an academic journal.

How was I identified and why am I being invited to participate in this research?

You have been identified, and so are being invited to participate in this research, after responding to my recruitment advertisement. You are a 20-to-50-year old endurance-trained male taking part in cycling and/or triathlon events with ≥ 3 years training experience, and habitual training volume of 5-to-10 cycling hours per week. I am looking for male athletes who have been free of viral infections (>1 month) and musculoskeletal injuries (>3 months), not suffered from cardiovascular disease, never experienced exertional heat stress illness, and have not been engaged in specific heat acclimatisation training within the last 6 months. Unfortunately, you cannot take part in this project if you are a student of either of my supervisors (Dr. Ed Maunder of Prof. Andrew Kilding). These criteria will be formally assessed through a health screening questionnaire during the first laboratory visit.

How do I agree to participate in this research?

In order to accept my invitation to participation, you will need to get in touch by email (ppg0795@aut.ac.nz) to briefly discuss the study and ensure your suitability as a participant. If you are interested in participating, we will arrange your first visit to our laboratory at AUT Millennium on the North Shore (17 Antares Place, Rosedale, Auckland, New Zealand). During this visit, we will discuss in more detail about what participating in the study will involve, and you will be provided opportunity to ask questions relating to your participation. If you are still keen to take part in the research, you will then have the opportunity to provide written informed consent. Your eligibility will be further assessed using a health screening questionnaire. If this is passed, you will then be asked to perform an incremental exercise test using our laboratory ergometer. If you achieve our inclusion criteria of a VO_2max of greater than 50 $mL \cdot kg^{-1} \cdot min^{-1}$, we will then schedule your next two laboratory visits, which involve 90 minutes of moderate-intensity cycling at either 18 or 33˚C.

Your participation in this research is voluntary (it is your choice) and whether or not you choose to participate will neither advantage nor disadvantage you. You are able to withdraw from the study at

any time. If you choose to withdraw from the study, then you will be offered the choice between having any data that is identifiable as belonging to you removed or allowing it to continue to be used. However, once the findings have been produced, removal of your data may not be possible.

What will happen in this research?

As part of this study, we will ask you to report to our laboratory at AUT Millennium on three occasions, including the initial trial I described above, and two experimental trials. These three laboratory visits will occur over a three-week period, ~1 week apart. As mentioned above, the first visit will involve discussion about what the study involves, provision of informed consent, eligibility assessment using a health screening questionnaire, and incremental cycling test. For this visit we will ask you to come to our laboratory first thing in the morning after a 10-hour overnight fast, having refrained from caffeine, alcohol, and intense exercise for 24 hours. After discussing the study and providing informed consent, you will be asked to respond to a health screening questionnaire to ensure your eligibility for the remainder of the study. You will then complete an incremental exercise test for determination of your aerobic fitness (VO_2max), physiological thresholds, and carbohydrate and fat metabolism during exercise. In order to be eligible for further participation, you must have a VO_2max of greater than 50 mL/kg/min. If successful, you will then be provided with blank diaries to record physical activity for 7 days and diet for 48 hours in advance of the first experimental trial, such that this can be repeated in advance of the second experimental trial.

Approximately seven days after the first visit, we will ask you to return to our laboratory in order to complete the first experimental trial, which will involve 90 minutes of cycling exercise on your own road bicycle connected to our indoor smart trainer, at either 18 or 33˚C. You will be asked to report to the laboratory having fasted for 10-hours overnight, refrained from caffeine, alcohol, and intense exercise for 24 hours, and made written records of your diet for 48-h, and a 7-day training diary prior to your arrival. Before the exercise begins, we will measure your height and weight, which will be used to determine your pre-exercise body mass, fit you with a heart rate monitor, and then ask you to self-insert a rectal thermometer in a private bathroom. The placement of the rectal thermometer might also cause very mild discomfort, and it will allow us to closely monitor your core temperature throughout the exercise. We will then collect an ~10 mL blood sample from a forearm vein using a venepuncture (Figure 1). The venepuncture will be performed by Dr. Ed Maunder, who is well-trained and experienced. This venepuncture procedure is very safe, and may cause only a very modest amount of transient discomfort as the needle is inserted. Then, we will tape a skin temperature thermistor onto your thigh for measuring the skin temperature above your working quadriceps muscles. You will then enter the environmental chamber, and the exercise will begin.

During the exercise itself, you will be asked to pedal in order to maintain a target heart rate throughout the 90-minute of moderate-intensity cycling. The target heart rate will be ~95% of your lactate threshold heart rate (as determined in the first laboratory visit), and will therefore be of moderate-intensity. We will collect your expired gas samples (Figure 2) for 4 minutes every 15 minutes to assess your metabolism. During the trials, you will be able to drink as much water as you like. At the end of exercise, another ~10 mL blood sample will be taken using the venepuncture technique. By comparing the pre- and post-exercise blood samples, we will gain insight into how the environmental temperature impacted your physiological response to the exercise. You will then be provided with a towel to wipe off your sweat before checking your post-exercise body mass, so that we can calculate how much fluid you have lost during the trial.

For the second experimental trial (your third laboratory visit), you will be asked to report to the laboratory ~7 days later, having adhered to the same pre-trial instructions and repeated your 48-h diet and 7-day training records in advance. During this trial, the same procedures will be repeated, but the exercise will be performed in the other environmental temperature (i.e. either 18 or 33˚C).

After each trial, we will prepare a consulting room, where you can rest, eat, and drink before leaving our laboratory, if you would like to. Also, private shower and changing facilities are available here at AUT Millennium.

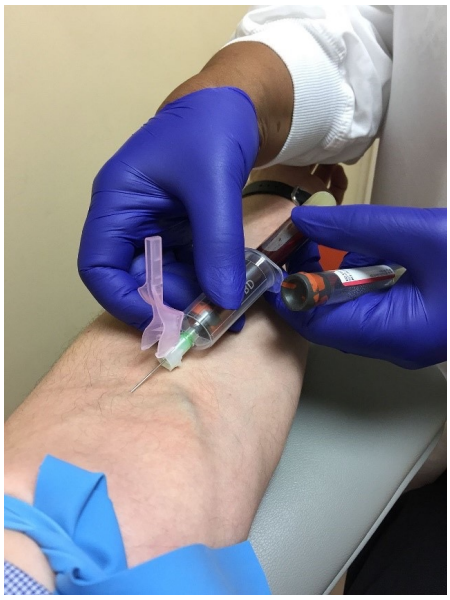

Figure 1. Venepuncture technique, which will be performed twice in each experimental trial (your second and third visits to our laboratory).

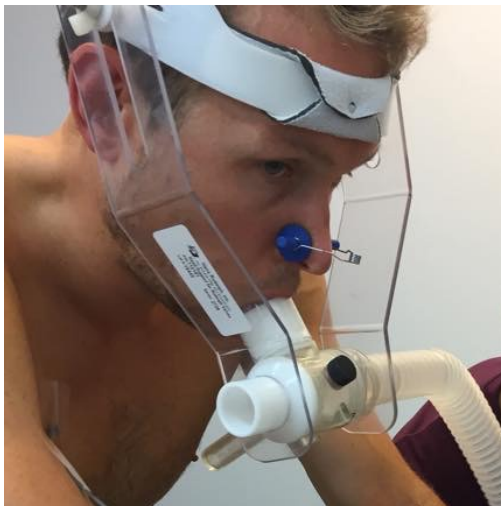

Figure 2. Expired gas collection during cycling exercise

What are the discomforts and risks?

As you are an endurance-trained cyclist, the discomforts and risks associated with this study participation will be minimal. The exercise itself will not be anything you do not do on a regular basis. The placement of the rectal thermometer may cause a very modest amount of discomfort. You will insert the thermometer yourself in a completely private room (i.e. a locked bathroom). Venepuncture is a very safe procedure and should not cause more than mild discomfort upon insertion, that will be very similar to when getting your blood taken at your GP clinic or hospital. In some instances, a small bruise can form around the venepuncture site. If this occurs, this is likely to heal in a couple of days or so and should not cause any discomfort.

How will these discomforts and risks be alleviated?

If at any point, you feel discomfort or pain beyond what you are comfortable with, please let me know so that we can make adjustments and/or stop the test immediately.

What are the benefits?

By participating in this study, you will be provided written reports of your individual test results. These reports will give you insight into your performance and exercise physiology, such as your aerobic fitness level (VO_2max), your physiological thresholds, how well you metabolise fat and carbohydrate during exercise, and how well you cope with heat. This data is generally only accessible to paying customers in commercial laboratories, such as in our Sports Performance Clinic at AUT Millennium (see http://www.autmillennium.org.nz/clinics/sports-performance-clinics/). We will also be happy to provide our expertise regarding training and nutrition for your specific events. If you like, we can also send you a short report of the main findings of the study as a whole at its completion.

From my perspective, this study will form a major part of my Master of Sport and Exercise here at AUT. We also intend to write the study up and publish it in a peer-reviewed academic journal. The results of this study will be interesting to exercise physiologists as the effect of environmental heat stress on metabolism during prolonged exercise at matched heart rates are not clear.

What compensation is available for injury or negligence?

In the unlikely event of a physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

How will my privacy be protected?

Only myself, Dr. Maunder, and Prof. Kilding will be able to trace your participation back to you, nobody else. You will not be identifiable in any documentation associated with this study, including my thesis, and a peer-reviewed academic journal. Both signed consent form and health screening questionnaire be stored in a locked cabinet in the Sports Performance Research Institute New Zealand (SPRINZ) postgraduate office, and destroyed by shredding after ten years. The data generated as a result of your participation will be given a unique code (e.g., STUDY001), and only myself, Dr. Maunder, and Prof. Kilding will have access to this coding system. After the process of data analysis, the de-identified data will be stored on a password-protected hard drive and stored securely in the locked SPRINZ ethics cupboard.

What are the costs of participating in this research?

As mentioned above, this study will take place over a course of three weeks. You will be asked to visit to our laboratory here at AUT Millennium on 3 occasions, and each of these will take between 1 and 2 hours.

What opportunity do I have to consider this invitation?

You are under no pressure to respond to this invitation. Please feel free to take your time considering for participation in this study. However, we will only be testing a limited number of participants, and so eventually you may no longer be able to take part if the study has concluded.

Will I receive feedback on the results of this research?

Yes, you will if you are interested in your personal results and feedback. I will write you a detailed report on all of the factors related to your performance, including your aerobic fitness level (VO_2max), your ventilatory thresholds, how well you metabolise fat and carbohydrate during exercise, and how

well you cope with heat. Also, at the end of the study, I will be able to provide you with a short summary of the results we found.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor, *Dr. Ed Maunder, ed.maunder@aut.ac.nz (Make sure you include the international prefix if the participants are likely to be calling from overseas.)*

Concerns regarding the conduct of the research should be notified to the Executive Secretary of AUTEC, *ethics@aut.ac.nz* , (+649) 921 9999 ext 6038.

Whom do I contact for further information about this research?

Please keep this Information Sheet and a copy of the Consent Form for your future reference. You are also able to contact the research team as follows:

Researcher Contact Details:

Thanchanok Charoensap

AUT Millennium, 17 Antares Place, Rosedale, Auckland, 0632

ppg0795@aut.ac.nz

Project Supervisor Contact Details:

Dr. Ed Maunder

AUT Millennium, 17 Antares Place, Rosedale, Auckland, 0632

ed.maunder@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee on 2nd June 2021, AUTEC Reference number 21/121.

Appendix 3: Consent form

Project title: **The effect of environmental temperature on substrate oxidation and extracellular heat shock proteins in response to heart-rate matched moderate-intensity cycling in endurance trained males**

Project Supervisor: ***Dr. Ed Maunder***

Researcher: ***Thanchanok Charoensap***

- o I have read and understood the information provided about this research project in the Information Sheet dated 2nd June 2021
- o I have had an opportunity to ask questions and to have them answered.
- o I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without being disadvantaged in any way.
- o I understand that if I withdraw from the study then I will be offered the choice between having any data or tissue that is identifiable as belonging to me removed or allowing it to continue to be used. However, once the findings have been produced, removal of my data may not be possible.
- o I am not suffering from heart disease, high blood pressure, any respiratory condition (mild asthma excluded), any illness or injury that impairs my physical performance, or any infection, and I have never had exertional heat stroke.
- o I have not been engaged in specific heat acclimatisation training in the last six months.
- o I agree to self-insert a rectal thermometer in privacy in the two experimental trials of this study.
- o I agree to provide blood samples twice for each experimental trial of this study.
- o I agree to take part in this research.
- o I wish to receive a summary of the research findings (please tick one): Yes o No o
- o I wish to have my blood samples returned to me in accordance with right 7 (9) of the *Code of Health and Disability Services Consumers' Rights* (please tick one): Yes o No o

Participant's signature: .....

Participant's name: .....

Participant's Contact Details (if appropriate):

.....

.....

.....

.....

Date:

Note: The Participant should retain a copy of this form.

Approved by the Auckland University of Technology Ethics Committee on 2nd June 2021, AUTEC Reference number 21/121.

Appendix 4: Health screening questionnaire

Project title:	**The effect of environmental temperature on substrate oxidation and extracellular heat shock proteins in response to heart-rate matched moderate-intensity cycling in endurance trained males**
Project Supervisor:	***Dr. Ed Maunder***
Researcher:	***Thanchanok Charoensap***

Please answer to <u>all</u> following questions.

1. What is your gender? …….
2. What is your age? ……years
3. In what endurance sport do participate? (Identify more than one if applicable)
   ……
4. How would you classify your current performance level? (Select only one)
   - ☐ Recreational (train but do not participate in competition)
   - ☐ Amateur (enter races but do not expect to win)
   - ☐ High-level amateur (qualify and compete at National Championship level)
   - ☐ Elite non-professional (qualify and compete at the international level)
   - ☐ Professional
5. Approximately how many hours per week do you normally train (e.g., over the last two months)? ……hours/week
6. Do you have ≥ 3 years of cycling experience? ☐ Yes ☐ No
7. Approximately how many hours per week do you cycle (e.g., over the last two months)? ……hours/week
8. Have you recently (≤6 months) undertaken specific heat acclimatisation/heat training? ☐ Yes ☐ No
9. Do you suffer from any chronic disease impacting for maximal exercise (e.g., cardiovascular disease, kidney disease, etc.)? ☐ Yes ☐ No
10. Are you taking medication for blood pressure or your heart? ☐ Yes ☐ No
11. Have you had chest pain during physical activity? ☐ Yes ☐ No
12. Have you experienced chest pain when not doing exercise in the last month? ☐ Yes ☐ No
13. Have you ever lost balance because of dizziness? ☐ Yes ☐ No
14. Have you ever fallen into unconsciousness? ☐ Yes ☐ No
15. Have you had a feverish illness in the last month? ☐ Yes ☐ No
16. Are you suffering with any lower-limb injury (e.g., knee pain, sprained ankle, etc.)? ☐ Yes ☐ No
17. Have you experienced any musculoskeletal injury in the past three months? ☐ Yes ☐ No
18. Have you previously had exertional heat stroke? ☐ Yes ☐ No

Approved by the Auckland University of Technology Ethics Committee on 2nd June 2021, AUTEC Reference number 21/121.

Appendix 5: 48-hour dietary diary

Please record your diet and drinks for 48 hours prior to your second laboratory visit, and have this repeated in advance of your third laboratory visit.

	Day 1	Day 2
** NOTE*		* Please refrain from caffeine and alcohol for 24 hours prior to your laboratory visit
BREAKFAST		
SNACK		
LUNCH		
SNACK		
DINNER		* Please keep an overnight fast before reporting to laboratory
PLAIN WATER		

Approved by the Auckland University of Technology Ethics Committee on 2nd June 2021, AUTEC Reference number 21/121.

Appendix 6: 7-day physical activity diary

Please record your physical activities for 7 days prior to your second laboratory visit, and have this repeated in advance of your third laboratory visit.

	MORNING	AFTERNOON	EVENING	*NOTE
Day 1				
Day 2				
Day 3				
Day 4				
Day 5				
Day 6				
Day 7				* Please refrain from intense exercise

Approved by the Auckland University of Technology Ethics Committee on 2nd June 2021, AUTEC Reference number 21/121.

Appendix 7: Rating of perceived exertion (RPE)

6. No exertion
7. Extremely light
8.
9. Very light
10.
11. Light
12.
13. Somewhat hard
14.
15. Hard
16.
17. Very hard
18.
19. Extremely hard
20. Maximal exertion

Appendix 8: Thermal comfort

How comfortable do you feel with the temperature of your body?

1.0 Comfortable

2.0

3.0 Slightly uncomfortable

4.0

5.0 Uncomfortable

6.0

7.0 Very uncomfortable

8.0

9.0 Extremely uncomfortable

10.0

Appendix 9: Thermal sensation

How does the temperature of your body feel?

1.0 Unbearably cold

2.0 Extremely cold

3.0 Very cold

4.0 Cold

5.0 Cool

6.0 Slightly cold

7.0 Neutral

8.0 Slightly warm

9.0 Warm

10.0 Very warm

11.0 Hot

12.0 Very hot

13.0 Extremely hot

14.0 Unbearably hot