Metabolic implications of environmental heat stress for endurance athletes

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Abstract

Carbohydrates and fats are the primary substrates used to fuel energy metabolism during exercise, and endogenous carbohydrate availability may be an influential physiological characteristic in some endurance events. Substrate metabolism during exercise is regulated by various factors such as exercise intensity and duration, nutrient availability, and training status. Importantly in the context of the many endurance competitions and training camps that take place under environmental heat stress, substrate metabolism during acute exercise, as well as metabolic adaptation to endurance training, may also be impacted by environmental heat stress. Specifically, previous research has observed increased carbohydrate metabolism to support a given bout of exercise performed under heat stress compared to temperate conditions, which may have specific implications for acute exercise responses and metabolic training adaptations. However, the majority of studies investigating the acute effect of heat stress on substrate metabolism during endurance exercise have employed relatively extreme combinations of exercise and heat stress, thus making it difficult to make inferences regarding the specific conditions under which acute heat stress is likely to influence substrate metabolism during endurance exercise. Furthermore, despite significant discussion, systematic evaluation of the effect of heat stress on metabolic training adaptations has so-far not been conducted. Therefore, the purpose of this thesis was to improve the understanding of the metabolic implications of environmental heat stress for endurance athletes, with respect to both acute heat exposure, and the adaptive metabolic response to serial training sessions performed under heat stress. Accordingly, in the present thesis two main research questions are investigated: (i) in what situations might environmental heat stress be expected to impact substrate metabolism during acute endurance exercise? and (ii) does environmental heat stress impact the adaptive metabolic response to endurance exercise training?

From an acute perspective, studies in thesis provide evidence that exercise intensity appears to regulate the effect of heat stress on substrate oxidation rates, with greater shifts towards carbohydrate metabolism seen at higher absolute workloads in 35°C (Acute Study 1). However, when matched for temperature-specific ventilatory thresholds, the reduced absolute workloads achieved under heat stress at individual physiological thresholds may actually necessitate reduced carbohydrate use, at least at low-intensities (Acute Study 1). The magnitude of the environmental heat stress also appears to be important for the metabolic perturbation, with higher temperatures evoking greater stimulatory effects on carbohydrate metabolism at given absolute workloads, and lowering the absolute workload at which metabolic perturbations are

observed (Acute Study 2). From a training adaptation perspective, a case study of elite Ironman triathletes undertaking a three-week heat stress training camp in Kailua-Kona, Hawaii demonstrated that elite endurance athletes appear capable of undertaking successful heat stress training camps despite the apparent physiological strain experienced when exercising under environmental heat stress (Training Study 1). In a laboratory setting, heart rates observed at given physiological thresholds appear to be consistent between 18 and 35°C, and therefore may provide a useful for tool for regulating training load and intensity when beginning a heat stress training camp (Training Study 2). These findings were used in the design of a three-week, laboratory-based training intervention in which endurance training performed in 33°C evoked greater improvements in pre-loaded 30-min time-trial (TT) performance than a matched training programme performed in 18°C, with some of this effect explained by positive effects of maximal *vastus lateralis* citrate synthase activity, and therefore mitochondrial protein content (Training Study 3).

Overall, this thesis adds to existing literature by providing evidence for the specific combinations of exercise-heat stress likely to impact acute substrate oxidation rates, and the metabolic and performance adaptations observed when training under environmental heat stress. Specifically, the data presented in this thesis suggests practitioners working with endurance athletes performing acute bouts of exercise under heat stress can expect minimal heat stress-effects on substrate oxidation rates unless the exercise intensity or environmental temperature is high (~40°C), and that executing a well-controlled heat stress training camp may provide a useful stimulus for endurance performance-related adaptations.

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.”

Ed Maunder, November 2020

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“To see what is in front of one’s nose needs a constant struggle.”

George Orwell

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List of abbreviations

ACC	p-acetyl-CoA carboxylase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine 5’-monophosphate-activated protein kinase
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
AU	Arbitrary units
CaMK	Calcium/calmodulin-dependent protein kinases
CHO	Carbohydrate
COX I-IV	Electron transport chain complex I-IV
CREB	cAMP response element-binding protein
CV	Coefficient of variation
EE	Energy expenditure
ERR-α	Oestrogen-related receptor-α
ES	Effect size
Fat_{max}	Exercise intensity at the maximum rate of fat oxidation
GCN5	General control of amino acid synthesis 5
GLP-1	Glucagon-like peptide-1
HA	Heat acclimation
HIGH	The second ventilatory threshold in temperate conditions
HR_{max}	Maximum heart rate
HRV	Heart rate variability

HSF1	Heat shock factor 1
HSP	Heat shock protein
IET	Incremental exercise test
IL-6	Interleukin-6
LOW	The first ventilatory threshold under environmental heat stress
LT	Lactate threshold
MBI	Magnitude-based inferences
MEF-2	Myocyte enhancer factor 2
MFO	Maximum rate of fat oxidation
MOD	The first ventilatory threshold in temperate conditions
NHT	Null-hypothesis test
NRF1	Nuclear respiratory factor 1
NRF2	GA binding protein transcription factor α-subunit 60 kDa
OBLA	4 $mmol.L^{-1}$ blood lactate concentration
p38 MAPK	p38 mitogen-activated protein kinase
PBS	Phosphate-buffered saline
PCAF	P300/CBP-associated factor
PCr	Phosphocreatine
Perm1	PGC-1- and ERR-induced regulator in muscle 1
PGC-1α	Peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PPAR-δ	Peroxisome proliferator-activated receptor nuclear receptor-δ
PROL	Prolonged exercise
RER	Respiratory exchange ratio

rMSSD	Square root of the mean sum of the squared differences between R–R intervals
SEE	Standard error of the estimate
SIC	Smallest important change
SIRT1	Sirtuin 1
TE	Typical error
TFAM	Mitochondrial transcription factor A
T_{mus}	Muscle temperature
T_{re}	Rectal temperature
TT	Time-trial
$\dot{V}CO_2$	Rate of carbon dioxide production
$\dot{V}E$	Rate of ventilation
VEGF	Vascular endothelial growth factor
VIG	The second ventilatory threshold under environmental heat stress
$\dot{V}O_2$	Rate of oxygen consumption
$\dot{V}O_{2max}$	Maximum rate of oxygen consumption
VT_1	The first ventilatory threshold
VT_2	The second ventilatory threshold
W_{max}	Maximum work rate achieved during an incremental test

Ethical approval

All of the original research studies conducted as part of this thesis were approved by the Auckland University of Technology Ethics Committee and in accordance with the Declaration of Helsinki. The ethical approval codes for these studies were 17/409 (16/01/2018), 18/101 (22/03/2018), 18/186 (06/06/2018), and 19/146 (07/06/2019).

List of publications associated with this thesis

Peer-reviewed journal articles

1. **Maunder E**, Kilding AE, Plews DJ. Substrate metabolism during Ironman triathlon: Different horses on the same courses. *Sports Med* 48: 2219-2226, 2018.
    a. **Maunder E,** Kilding, AE, Plews DJ. Authors' reply to Webster and Smith: Comment on: "Substrate metabolism during Ironman triathlon: Different horses on the same courses". *Sports Med* 48*:* 2423-2424, 2018.
2. **Maunder E**, Plews DJ, Kilding, AE. Contextualising maximal fat oxidation during exercise: Determinants and normative values. *Front Physiol* 9: 599, 2018.
3. Sandford GN, **Maunder E.** Commentaries on viewpoint: Relative exercise intensity should be quantified by physiological and mechanical thresholds. *J Appl Physiol* 125: 676-682, 2018.
4. **Maunder E**, Plews DJ, Merien F, Kilding AE. Exercise intensity regulates the effect of heat stress on substrate oxidation rates during exercise. *Eur J Sport Sci* 20: 935-943, 2020.
5. **Maunder E**, Kilding AE, Stevens CJ, Plews DJ. Heat stress training camps for endurance sport: A case study of successful monitoring in two Ironman triathletes. *Int J Sports Physiol Perform* 15: 146-150, 2020.
6. **Maunder E**, Plews DJ, Merien F, Kilding AE. Stability of heart rate at physiological thresholds between temperate and heat stress environments in endurance-trained males. *Int J Sports Physiol Perform* (in press), 2020.

Conference abstracts and posters

1. **Maunder E**, Plews DJ, Kilding AE. Heat stress effects on substrate oxidation rates during incremental cycling. Sport and Exercise Science New Zealand, 26-27 October 2018.
2. **Maunder, E**, Plews DJ, Merien, F, Kilding AE. Stability of heart rate at lactate thresholds in temperate and heat stress environments. Sport and Exercise Science New Zealand, 27-29 November 2019.

1 Introduction and background

Elite endurance sport is characterised by the need to sustain high external workloads over long distances (427, 428), and elite endurance athletes are defined by their ability to sustain high metabolic rates over prolonged periods (296, 298). High metabolic rates during prolonged endurance exercise are produced primarily by oxidation of carbohydrates and fatty acids (290, 412), which results in energy liberation for resynthesis of adenosine triphosphate (180). The metabolic profile of an endurance athlete is impacted by many variables that have been the focus of research in exercise physiology for many years, such as exercise intensity (290, 412) and duration (412, 496, 498), nutrition (3), training status (291), and environmental conditions (118).

The physiological impact of environmental heat stress on endurance athletes has been a focus of interest in the literature, given many endurance athletes train and/or compete in hot environments (71, 403). When prolonged exercise is performed under environmental heat stress (>30°C), greater core temperatures (369), peripheral blood flow (160, 434), evaporative sweat loss (242, 474), dehydration (195), heart rates (369, 418), and circulating catecholamines (121) are observed compared to the same exercise performed in temperate conditions. From a metabolic standpoint, prolonged exercise performed under environmental heat stress appears to effect a shift in substrate metabolism in favour of increased carbohydrate (CHO) and decreased fat oxidation (118). Mechanistically, it appears that the environmental heat stress-induced shift toward CHO metabolism during prolonged exercise is related to increases in core temperature (123), muscle temperature (119, 460), and circulating catecholamines (120, 212, 213, 234, 495), as well as greater levels of hypohydration (178, 287, 288), and may be exacerbated in fed-state conditions given environmental heat stress appears to blunt exogenous CHO oxidation during exercise (242).

Studies demonstrating a stimulatory effect of heat stress on CHO metabolism during prolonged exercise have been conducted at single relative exercise intensities, and the literature as a whole covers a narrow range of intensities and short durations (104, 121, 122, 124, 177, 242, 307). Both exercise intensity and exercise duration have the potential to impact the effect of heat stress on substrate metabolism during exercise, given the greater metabolic heat production occurring at higher intensities and the longer time for thermoregulatory perturbations to become apparent with longer durations, but the design of previous studies does not allow for conclusions to be made regarding the regulatory effects of these important variables. Similarly, several studies have not observed heat stress-related changes in acute substrate metabolism during exercise, and in these studies it appears the heat stress stimulus was insufficient to

induce meaningful changes in thermoregulatory variables to exert metabolic effects (1, 118, 148, 455, 514). This therefore suggests a minimum magnitude internal and/or external heat stress is required before heat stress elicits stimulatory effects on CHO metabolism during exercise. However, no studies have investigated the magnitude of the internal or external heat stress required for these effects, with existing studies assessing the effect of heat stress on substrate metabolism in a single heat stress condition (1, 104, 121, 122, 124, 148, 177, 242, 307, 455, 514, 516). Therefore, from the existing literature, it is not possible to identify the combinations of exercise and heat stress in which acute metabolic shifts are likely to be observed. Specifically, the influence of exercise intensity and duration, and the grading of heat stress-effects to environmental temperature, have not been systematically investigated. Research into the regulatory effect of these variables may provide useful information for practitioners working with endurance athletes when considering if a specific combination of acute exercise and environmental heat stress has implications for substrate metabolism during a training session or competition.

It is plausible, though not yet comprehensively studied, that the additional physiological strain associated with performing endurance training under environmental heat stress may augment the stimulus for metabolic training adaptations (183). This hypothesis is based on plausible interactions between observed exercise-heat stress responses and the signalling pathways implicated in mitochondrial adaptations to training, and specifically the transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) often touted as the ‘master regulator’ of mitochondrial biogenesis (171, 176, 513). Firstly, it can be hypothesised that exercise performed under heat stress may increase PGC-1α phosphorylation and therefore activation. This could occur via heat stress effects on muscle osmolality, and therefore activation of upstream factor p38 mitogen-activated protein kinase (p38 MAPK) (448), and muscle glycogenolysis (118) and interleukin-6-induced (461) activation of upstream factor adenosine 5’-monophosphate-activated protein kinase (AMPK) (392). Secondly, exercise-heat stress may increase PGC-1α mRNA expression via the increased expression of heat shock proteins (285, 352, 470) and circulating catecholamines expression (13, 57, 79, 336) observed when exercise is performed under heat stress. The increased heat shock protein expression observed with exercise-heat stress may also assist with the chaperoning of newly-synthesised nuclear-encoded mitochondrial proteins from the nucleus to the mitochondria (518), and a direct effect of lactate, which circulates in blood and resides in muscle at greater concentrations during exercise-heat stress (104, 121, 122, 124, 192, 242, 514, 517), has also been proposed in mitochondrial adaptation (469). However, whilst the potential for an interaction between heat stress and adaptive signalling pathways may

be compelling, mitochondrial adaptations to an endurance training intervention performed under heat stress have not been investigated. Therefore, it is also not possible to determine if endurance training performed under environmental heat stress is likely to augment the metabolic adaptations to training observed during a period of equivalent training in temperate conditions. Research into the repeated exposure effects of environmental heat stress during endurance training on metabolic adaptation is warranted, given many endurance athletes train in hot environments (71, 403).

The purpose of this thesis was therefore to improve the understanding of the metabolic implications of environmental heat stress for endurance athletes, with respect to both acute heat exposure, and the adaptive metabolic response to serial training sessions performed under heat stress. Given the number of endurance events taking place under environmental heat stress, and the increasing interest in use of environmental heat as an additional training stress to drive adaptation, the data and discussion within this thesis has strong implications for endurance athletes, coaches, and applied exercise physiologists. Specifically, better understanding of the specific combinations of exercise and environmental temperature under which heat stress-induced alterations to substrate metabolism during endurance exercise are more likely to be seen may allow more specific organisation of training to induce the desired metabolic response, nutrition to support training, and pacing strategies to preserve endogenous fuel stores, whilst understanding how heat stress interacts with the adaptive metabolic response to endurance training may help inform athletes and practitioners as to when and if they might undertake a heat stress training camp. Accordingly, the specific research questions investigated in this thesis are:

1. Does the intensity of exercise impact the acute effect of environmental heat stress on whole-body CHO oxidation rates?
2. Is the acute effect of environmental heat stress on substrate oxidation rates during exercise graded to the environmental temperature?
3. Does the duration of exercise impact the acute effect of environmental heat stress on whole-body CHO oxidation rates?
4. How are heat stress training camps performed by elite endurance athletes in the real-world?
5. Are heart rates at individual physiological thresholds consistent between temperate and heat stress environments?
6. Does environmental heat stress exert an additive effect on adaptations to mitochondria and endurance performance in humans?

1.1 Structure and organisation of the thesis

This thesis is set out into seven chapters, including this initial overview Chapter 1 (Table 1). In Chapter 2 I provide a narrative overview that sets out the importance of substrate metabolism in endurance sport, with evidence for this assertion provided in theoretical models of substrate use during Ironman triathlon at different performance levels, and its applied relevance contextualised with normative values for fat oxidation in typical exercise measures taken in the laboratory. In Chapter 3 I further this narrative overview to discuss the potential for both acute exposure to heat stress and repeated training performed under heat stress to influence acute and adaptive responses relevant to substrate metabolism during exercise. In Chapter 4 I describe and rationalise some of the major methodologies used in the subsequent original studies conducted as part of this thesis.

Chapter 5 is a series of three experimental studies, all adopting acute, cross-over designs, designed to add to the literature and interrogate the overall question "*In what situations might environmental heat stress be expected to impact substrate metabolism during acute endurance exercise?*" Chapter 6 is a series of three studies, one a case study describing the real-world practices of two elite endurance athletes, one an acute cross-over study, and one a randomised controlled trial training intervention seeking to add to the literature and interrogate the overall question "*Does environmental heat stress impact the adaptive metabolic response to endurance exercise training?*" The case study describes the conduct of real-world endurance athletes during an apparently successful heat stress training camp, and this, in combination with the acute cross-over study providing some evidence that the heart rates observed at key physiological thresholds appears to be matched between temperate and heat stress environments, were used in the design of the training study. In Chapter 7, I aimed to summarise the findings of the present thesis to answer the question "*What are the metabolic implications of environmental heat stress for endurance athletes?*" References and appendices are then provided at the end.

Table 1. Specific content of each chapter of this thesis

Chapter	Title	Research questions/purpose
1	Introduction and background	Initial overview of the contents of this thesis
2	Literature review: Substrate metabolism and the endurance athlete	Narrative literature review making the case for endogenous carbohydrate availability as an important variable in ultra-endurance sport, including a model of substrate use during an Ironman triathlon, and its applied relevance with normative exercise fat oxidation values
3	Literature review: How does acute and repeated exposure to heat stress impact metabolic responses to exercise?	Two-part narrative literature review discussing how acute exposure to environmental heat stress can impact substrate metabolism during exercise, and formulation of a hypothesis for how exposure to environmental heat stress during endurance exercise training might impact metabolic adaptations
4	Research methods and methodology	Overview of some of the key methods used in the experimental studies of this thesis
5	Acute studies	A series of three experimental studies investigating the situations in which environmental heat stress might be expected to impact substrate metabolism during acute endurance exercise, divided into three components: 1. Does the intensity of exercise impact the acute metabolic effect of environmental heat stress? 2. Is the acute metabolic effect of environmental heat stress on substrate metabolism during exercise graded to the environmental temperature? 3. Does the duration of exercise impact the acute metabolic effect of environmental heat stress?
6	Training studies	A series of three studies investigating the use of environmental heat stress to augment the adaptive metabolic response to endurance exercise training, divided into three parts: 1. How are heat stress training camps performed by elite endurance athletes in the real-world? 2. Are heart rates at individual physiological thresholds consistent between temperate and heat stress environments? 3. Does environmental heat stress exert an additive effect on the adaptive metabolic response to endurance training in humans?
7	Discussion	What are the metabolic implications of environmental heat stress for endurance athletes?

2 Literature review: Substrate metabolism and the endurance athlete

2.1 Introduction to endurance exercise metabolism

During prolonged exercise, CHO and fat are the primary substrates oxidised to resynthesise adenosine triphosphate (ATP) and support energy metabolism (290, 412), with oxidation of amino acids unlikely to play a major role (~5% of energy expenditure) (152, 211, 278). Humans store the majority of endogenous CHO as glycogen in skeletal muscle (35, 36) and the liver (365, 366), with minor quantities residing in adipose tissue, brain, and kidney cells (39, 330, 378, 409). Additionally, ~4 g circulates as glucose in the blood (494). The total endogenous CHO pool is finite, typically <3000 kcal (162), with ~80% in skeletal muscle and ~10-15% in the liver (236). In contrast, human fat storage is vast, and primarily situated in subcutaneous and visceral adipose tissue cells as triacylglycerol (143), although intramuscular triacylglycerol is also present (496). Given that 1 g of fat provides ~9.75 kcal of energy (246), it can be estimated that a lean 70-kg individual with 10% body fat has ~68,250 kcal of endogenous fat energy. Theoretically, this amount of energy might be sufficient to complete more than six full-distance Ironman triathlons (260).

These inter-substrate disparities in human energy storage have implications during prolonged exercise. Whereas endogenous fat stores are effectively unlimited in the context of exercise, prolonged exercise of sufficient length and intensity will eventually deplete working skeletal muscle glycogen to very low concentrations (7, 35, 36, 189, 217, 218); ~2-7% (36, 189, 217, 218) or ~10-25% (7, 35, 36) of pre-exercise content, depending on the exercise protocol and analytical techniques administered. Furthermore, hepatic glycogen content is also substantially reduced after prolonged exercise (51, 73, 163, 245, 389, 464), and has been indirectly associated with exercise capacity (73, 320). Mechanistically, depletion of muscle glycogen content to these low, but crucially not zero, concentrations has been linked to fatigue in the so-called ‘localisation hypothesis’ (374, 376). Depletion of muscle glycogen sequestered in the intramyofibrillar compartment has been specifically associated with impaired fatigue resistance (361) and tetanic Ca^{2+} handling (359, 375), implicating a role for these stores in excitation-contraction coupling, and therefore a role of their depletion in muscle fatigue. This explanation for muscle fatigue is made particularly compelling by the observed preferential utilisation of intramyofibrillar glycogen during exercise, resulting in even lower intramyofibrillar compared to whole-muscle glycogen concentrations at fatigue (306, 360), and may serve to explain why fatigue during prolonged exercise can occur before whole-muscle glycogen concentrations approach

zero. How depleted intramyofibrillar glycogen content might impair excitation-contraction coupling is not known, although it has been speculated that this is mediated by structural dependence on the presence of glycogen at the sarcoplasmic reticulum and/or ATP deficiency at the triadic junction due to localised metabolic dependence on intramyofibrillar glycogen (376). The metabolic explanation is plausible in spite of observed fatigue with low glycogen and high ATP concentrations (27, 78, 361, 463) due to the restricted access to the triadic junction, which can leave it out of equilibrium with global muscle ATP concentrations (377). In line with a metabolic link between intramyofibrillar glycogen content and skeletal muscle function, recent studies performed using mechanically skinned rat skeletal muscle appear to show some dependence of Na^+,K^+-ATPase activity on glycogenolytic ATP production via experimental inhibition of the glycogenolysis pathway (238). Whether hepatic glycogen exerts a limiting influence over performance of prolonged exercise has not been confirmed, although associational (73) and indirect (320) relationships with exercise capacity have been inferred.

Importantly, however, the metabolic mechanisms behind fatigue during prolonged exercise may change when exercise intensity increases. The aforementioned relationship between glycogen depletion and skeletal muscle contractile function refers to prolonged, moderate-intensity exercise whereby glycogen is depleted to very low concentrations after multiple hours (7, 35, 36, 189, 217, 218). When exercise intensity increases to outputs only sustainable for shorter periods (<60 min), the energy requirement exceeds the capacity of skeletal muscle mitochondria to provide (almost) all necessary ATP via oxidative metabolism, resulting in greater utilisation of alternative metabolic pathways such as conversion of pyruvate to lactate and an inability to achieve steady-state values for whole-body oxygen consumption, blood lactate concentration, and muscle pH (44, 116, 248, 251, 400, 481). From a metabolic standpoint, fatigue in this intensity domain is believed to occur when these homeostatic disturbances reach the individual's limit of tolerance (44, 248, 250, 482). Interestingly, emerging work may provide a link between glycogen depletion and fatigue during prolonged endurance exercise that includes brief periods of higher-intensity work, as is seen during events such as road cycling (295, 428). Experimental models demonstrated exacerbated impairments in skeletal muscle force production associated with the altered skeletal muscle potassium balance seen during high-intensity exercise when the muscle glycogen concentration was low (64). Whilst caution is required as this work has not yet been replicated in exercising humans, the implication of these results might be that muscle glycogen sparing during prior periods of moderate-intensity work could partially attenuate the fatiguing effect of altered skeletal muscle

potassium in response to subsequent higher-intensity work. This may have relevance to an endurance athlete taking part in a stochastic event such as road cycling.

Thus, the mix of substrates oxidised during prolonged exercise (>90 min), and the capacity for oxidative metabolism, are important considerations for endurance athletes given with the possibility of fatigue associated with depletion of stored CHO, and the fatigue associated with metabolic fluxes greater than can be sustained by full oxidative breakdown of energy substrates. Exercise and performance physiologists must therefore seek to understand an endurance athlete's metabolic profile in order to effectively plan training interventions and prepare for competition.

2.2 Substrate metabolism and endurance performance: What are the metabolic qualities sought after in an endurance athlete?

The traditional physiological models of endurance exercise suggest performance is determined by the maximum rate of oxygen consumption, the percentage of maximum oxygen consumption that can be sustained, and the economy of movement (29, 326). However, given the implications of endogenous CHO availability for muscle fatigue during prolonged exercise, it is quite possible that this should also be considered a key determinant of performance during long duration endurance competition. I will now briefly describe the traditional physiological determinants of endurance performance, before making a case for why endogenous CHO availability should also be considered in the context of long duration endurance competition.

2.2.1 Maximum oxygen uptake

Measurement of maximum oxygen uptake ($\dot{V}O_{2max}$) has been a cornerstone of exercise physiology since its conceptual founding by A.V. Hill in the 1920s (193, 194). $\dot{V}O_{2max}$ refers to the maximum rate at which an individual can uptake, transport, and consume oxygen, and is typically measured during an incremental exercise assessment of progressively increasing intensity to failure (29, 399). Given the requirement for high rates of oxidative metabolism to maintain high absolute workloads during endurance sport, it is unsurprising that a high $\dot{V}O_{2max}$ is a characteristic feature of elite endurance athletes (297, 428, 468, 477), and many studies have observed positive relationships between $\dot{V}O_{2max}$ and endurance performance (67, 87, 90, 112, 115, 139, 303, 326). Studies failing to observe this relationship have often been conducted in more homogenous populations (86, 221, 342), where this relationship may be masked by differences in other physiological determinants of endurance performance (25, 29, 326). Therefore, having a high $\dot{V}O_{2max}$ is a highly desirable metabolic quality for an endurance athlete.

$\dot{V}O_{2max}$ is highly trainable, with an abundance of studies reporting an increase in response to exercise training (61, 150, 270, 280). Physiologically, there is data to suggest $\dot{V}O_{2max}$ is limited at the level of oxygen delivery. Greater oxygen consumption has been observed in the knee extensors during maximal knee extension exercise compared to the same muscles during maximal cycling using the gold standard saline infusion thermodilution technique (345), and several investigators have reported lower *in vivo* oxygen consumption of the quadriceps during maximal cycling than the maximal mitochondrial capacity of the same muscle *in vitro* (52, 53). This suggests that, during maximal whole-body exercise, the capacity of skeletal muscle to consume oxygen is greater than the rate it can be provided. This is supported by a recent meta-analysis reporting a significant correlation between training-induced increases in $\dot{V}O_{2max}$ and maximum cardiac output (340). Thus, training interventions concerned with increasing $\dot{V}O_{2max}$ in well-trained endurance athletes performing exercise with large muscle-mass should be designed with the intention of inducing positive adaptations to oxygen delivery.

2.2.2 Physiological thresholds

The ‘anaerobic threshold’ is a widely discussed concept in exercise physiology (295, 296, 298, 356, 395, 468, 477), and refers to the maximum metabolic steady-state that an athlete can achieve; that is, the highest workload at which steady-state values can be reached for physiological variables such as blood lactate concentration, oxygen consumption, and muscle H^+ concentration (116). The anaerobic threshold therefore defines the boundary between steady-state and non-steady-state metabolism, what some have called the ‘heavy’ and ‘severe’ exercise intensity domains (248). For an endurance athlete, a high work rate at the anaerobic threshold is therefore essential in order to be able to maintain high work rates throughout an event without eliciting prohibitive fatigue. Indeed, the work rate at anaerobic threshold has been positively correlated with endurance performance in a range of events (14, 34, 41, 49, 326, 328, 357). Various concepts exist for the identification of this ‘anaerobic threshold’, most notably (i) the lactate threshold, which involves collection of blood samples during an incremental exercise test and subsequent construction of a blood lactate concentration vs. work rate curve (233), (ii) the ventilatory threshold, which involves collection of expired gas samples during an incremental exercise test and identification of perturbations in a range of ventilatory parameters (296, 437), (iii) the maximal lactate steady-state, which involves repeated constant work rate exercise trials to identify the highest power output or pace at which ‘stable’ blood lactate concentrations exists from 10 to 30 min (249, 258), and (iv) critical power, which involves repeated constant work rate exercise trials to exhaustion and subsequent construction of the individual power-

duration relationship and identification of the asymptotic work rate (248, 250). Profiling of the anaerobic threshold is useful in defining exercise intensity domains and therefore setting training intensity zones, as knowledge of this threshold allows a coach to predict the physiological response to a training session. For instance, high-intensity interval training is programmed specifically such that it takes place above the anaerobic threshold in order to disturb homeostasis and produce the largest possible adaptive stimulus (127).

Physiologically, the classic model links the maximum metabolic steady-state, and therefore work rate at anaerobic threshold, to mitochondrial protein content and enzyme function (29, 200). This model argues that metabolic flux is driven, at least in part, by adenosine diphosphate (ADP) accumulation in skeletal muscle cells (229). Endurance training creates more mitochondria in skeletal muscle cells (166, 167, 209, 299, 339, 436, 458), meaning that the ADP accumulation induced by a given absolute workload is 'shared' by a greater number of mitochondria. This theoretically means that a lower absolute rate of oxidative flux is required through each individual mitochondrion to meet the ATP synthesis demands of the workload, resulting in lower overall ADP concentrations before the required rate of oxidative phosphorylation is stimulated (29, 200). Lower ADP concentrations would then be expected to attenuate stimulation of phosphofructokinase, glycolysis, and lactate formation, or creatine kinase and phosphocreatine degradation (29, 200, 254). Thus, with greater mitochondrial protein content and enzyme activity in skeletal muscle cells, higher steady-state rates of oxidative phosphorylation are achievable, allowing greater submaximal workloads to be maintained with less flux through the pyruvate-lactate or phosphocreatine (PCr) metabolic pathways, and therefore less H^+ accumulation or PCr degradation. This therefore manifests as a higher maximum metabolic steady-state and work rate at anaerobic threshold.

A second, less discussed, physiological threshold commonly conceptualised by exercise physiologists working with endurance athletes is the 'aerobic threshold'. The aerobic threshold typically refers to the highest work rate achieved without a discernable change in blood lactate concentration from rest (or the ventilatory equivalent for oxygen) (444–446, 468). The aerobic threshold seems to define the boundary between exercise that will and will not elicit cardiac autonomic disturbance (445), and therefore knowledge of whether training sessions are taking place above or below the aerobic threshold has implications for the time-course of recovery. Accordingly, the aerobic threshold may be used to define the upper limit for 'low' intensity training sessions designed to result in minimal physiological strain and rapid recovery (444, 446, 468). Indeed, there is now suggestion that endurance athletes may

look to conduct ~80% of their training below the aerobic threshold, in order to maximise availability for training and therefore overall training volume (395, 444, 446, 477). Several original studies have shown favourable adaptive outcomes with this 'polarised' training intensity distribution (257, 354, 465).

2.2.3 Exercise economy

Exercise economy refers to the energetic efficiency of human metabolism to turn metabolic work into mechanical work, and therefore the speed/power elicited by a given rate of energy expenditure. In runners, this is typically expressed as running economy, either in units of oxygen uptake (24–26) or energy expenditure (132, 134) per distance covered. In cyclists, due to the difficulty associated with measuring speed in the laboratory, this is typically expressed as gross cycling efficiency, or the percentage of energy expenditure that is translated into mechanical power on a cycle ergometer (192, 346, 347). The importance of exercise economy is clear in the context of the previous section on physiological thresholds (Chapter 2.2.2). Physiological thresholds refer to the maximum rate of energy expenditure that can be sustained by an athlete (116). Exercise economy therefore determines the external work rate that this threshold rate of energy expenditure translates to.

Running economy appears to be determined by an array of factors including the Achilles tendon moment arm (342, 405, 438), gait pattern (74, 332, 344, 430, 473, 507), lower-leg stiffness (131, 134, 410), and body composition (43). Interventions shown to improve running economy include, unsurprisingly, run-specific training (140, 467) and gait retraining (344), but also plyometric training interventions (379, 433, 459), possibly mediated through beneficial effects on Achilles tendon stiffness (133). Crucially, associations between running economy and performance are evident in the literature (86, 326), and, interestingly, there is some speculation that excellent running economy may be a distinguishing feature that contributes to the dominance of East African runners (272, 341, 426, 505).

Cycling is, as a purely concentric action, a very different movement to running (467). Less is known about the determinants of gross cycling efficiency, with mixed observations regarding relationships with physiological attributes such as type I muscle fibre percentage (203, 300) or even performance standard and training status (202, 204, 205, 346, 453). That is not to say, however, that within an individual athlete improving gross cycling efficiency will not translate into a performance improvement when all other physiological attributes are held constant. Interestingly, recent discussion suggests a physiological trade-off may exist between gross cycling efficiency and overall aerobic capacity, or $\dot{V}O_{2max}$, with this theoretical trade-off

plausibly residing at complex I in the mitochondria (135, 364). Recently performed enzyme-constrained metabolic modelling suggested bypass of the complex I phase of oxidative phosphorylation is favoured during periods of high-energy demand due to greater rates of ATP synthesis, but with lower efficiency of substrate metabolism (364). Drawing on longitudinal laboratory testing data from an elite cyclist who eventually exhibited the highest-ever measured $\dot{V}O_{2max}$, with gross cycling efficiency measures decreasing as $\dot{V}O_{2max}$ increased (415), authors of the aforementioned metabolic modelling paper (364) posited that training regimens focused on augmenting oxygen delivery to skeletal muscle and therefore $\dot{V}O_{2max}$ may decrease efficiency via effects, or more specifically lack of effects, on complex I (135). This hypothesis may explain the seeming lack of effect of training status on gross cycling efficiency (202, 204, 205, 346, 453). However, as yet no firm inferences can be made from this discussion, given training-induced adaptations to complex I metabolic activity have not yet been studied, and the possibility that training-induced changes in maximum oxygen delivery and any complex I 'bypass threshold' could plausibly adapt in unison given the mitochondrial protein synthesis and biogenesis observed in response to endurance training (166, 167, 209, 299, 339, 436, 458).

2.2.4 Endogenous carbohydrate availability

As described above, another physiological factor likely of importance to performance in long-duration endurance sport is endogenous CHO availability (Chapter 2.1). Finite endogenous CHO stores can be depleted to very low concentrations after endurance exercise of sufficient duration and intensity (7, 35, 36, 189, 217, 218), and this glycogen depletion may be mechanistically associated with muscle fatigue (374, 376). Indeed, CHO is a major fuel source during exercise, with the absolute rate of CHO oxidation increasing with rising relative intensity (290, 412), decreasing with elevations in training status (291), and decreasing over time during prolonged bouts (412, 496, 498). Thus, strategies to protect endogenous CHO availability during endurance competition might be considered by endurance athletes concerned with fatigue attributable to glycogen depletion.

Accordingly, an abundance of researchers have investigated the performance-enhancing effects of nutritional strategies designed to either boost (i) pre-exercise CHO stores (35, 50, 196) or (ii) spare endogenous CHO oxidation during exercise through provision of exogenous substrate (95, 240, 420, 489). Indeed, ingestion of a high CHO diet in advance of an exercise capacity test has been shown to enhance pre-exercise endogenous CHO availability and extend endurance (35), while ingestion of exogenous CHO during exercise is known to reduce endogenous CHO oxidation rates (240, 487, 489), which has consistently been shown to translate into a performance benefit in long

duration exercise (>2 h) (16, 69, 95, 101, 269, 275, 324, 370, 420, 462). Thus, this large body of literature supports the notion that endogenous CHO availability should be considered a key parameter of endurance performance.

The evidence cited above comprehensively demonstrates the importance of endogenous CHO availability for performance in well-controlled, laboratory studies designed to simulate endurance sport (16, 69, 95, 101, 269, 275, 324, 370, 420, 462). Given the invasive methodologies required to study human metabolism during endurance exercise, research into the implications of endogenous CHO availability for endurance sport has almost exclusively been confined to laboratory simulations (420, 462), and are therefore limited with regard to their application to real-world endurance sport or ability to isolate prolonged endogenous CHO availability as the mechanism through which performance is improved. For example, short-duration endurance performance likely not limited by endogenous CHO availability (<1 h) has also been shown to improve with CHO ingestion under certain circumstances (32, 243). Indeed, there is suggestion that CHO ingestion may exert ergogenic effects on short-duration performance via central mechanisms following exposure to receptors in the oral cavity, as simply rinsing the mouth with CHO-containing solutions has been shown to improve performance (70, 75). So, to try and understand the specific relevance of endogenous CHO availability for performance in an ultra-endurance event, I modelled the energetic and substrate-specific cost of completing the Ironman World Championship course in Kaliua-Kona, Hawaii (this work has now been published in *Sports Medicine* (312, 313)).

2.2.4.1 Substrate metabolism during Ironman triathlon: Endogenous carbohydrate availability as a potential limiting factor

To do this, I constructed models for elite (~8 h) and top-amateur (~9 h) finishers (Table 2). Publically available data concerning Lionel Sanders' performance in the 2017 Kona World Ironman Championship was identified (350). Sanders came second and completed the course in 8:04:07, with 0:53:41 to complete the 3.9-km swim, 4:14:19 to complete the 180.2-km cycle at a mean power of 313 W, and 2:51:53 to complete the 42.2-km run. Best *estimates* of exercise economy, or energy cost, from existing literature were applied to these values to generate a rate of energy expenditure ($kcal.min^{-1}$) and absolute caloric cost (kcal) for each phase, and for the Ironman as a whole. Specifically, swim economy was estimated as ~283.3 $kcal.km^{-1}$ based on data in national-level Italian swimmers during a 2-km pool swim (520), gross efficiency of cycling was estimated as 23%, the value reported in a recent case study of a Grand Tour-winning cyclist at a similar power output (31), and running economy (~84.6 $kcal.km^{-1}$) was estimated as the average from a cohort of highly-endurance trained male runners ($\dot{V}O_{2max}$ = 75.5 ± 5.2 $mL.kg^{-1}.min^{-1}$) 2 $km.h^{-1}$ below lactate turn-point,

which equated to a mean speed of 15 $km.h^{-1}$ (Sanders' mean running speed was 14.7 $km.h^{-1}$) (447).

For the top-amateur model, second supervisor Daniel J. Plews' 2015 Kona Ironman performance was used. D.J.P. completed the course in 9:05:46, with 0:59:13 to complete the swim, 4:58:26 to complete the cycle at a mean power output of 225 W, and 3:08:07 to complete the run, therefore finishing 46th overall (including professionals). This time would have placed sixth in the female competition, so might be used as model of elite female performance. Due to the paucity of data available for swimming economy, the same values as for Sanders were assumed. Gross cycling efficiency was estimated as 21.0% in the top-amateur model using data from the 235-W stage of a 2016 step test performed by D.J.P. Running economy for the top-amateur model was estimated as the mean of a cohort of well-trained ($\dot{V}O_{2max}$ = 66.5 ± 5.6 $mL.kg^{-1}.min^{-1}$, ~80.3 $kcal.km^{-1}$ (132)) males running at 85% of the speed at lactate threshold, which equated to 13.4 $km.h^{-1}$ on average. A body mass of 75 kg was assumed.

Subsequently, wide lower and upper estimates of average fat oxidation rate were inputted to approximate the CHO requirements of each phase and the overall Ironman, assuming 9.75 $kcal.g\ fat^{-1}$, 4.07 $kcal.g\ CHO^{-1}$, and a negligible contribution from protein oxidation (246). The *lower* estimated fat oxidation rate in the elite cohort was taken as the mean *maximum* fat oxidation value in a cohort of male triathletes (141), and this was reduced accordingly in the top amateur model. Furthermore, the authors' own laboratory measures confirm D.J.P.'s fat oxidation rate resides in the speculated top-amateur range during cycling. I acknowledge and emphasise that this model is a postulated *estimate*, and full details regarding the models are available in the online spreadsheet (312). Those seeking to use this approach to model the metabolic costs of finishing an Ironman triathlon in any specific athlete are encouraged to adjust values as they see fit or in accordance with measured values. Notably, the estimated energy costs in this model are supported by similar mean measured values during an Ironman triathlon in eight males (10,036 ± 931 kcal) finishing in 10.9-13.0 hours (260).

Table 2. Theoretical model of the metabolic costs associated with performing an elite, top-amateur, and lower-amateur Ironman triathlon

Phase	Intensity	Energy expenditure	Mean FO ($g.min^{-1}$)	Fat (g)	Mean CO ($g.min^{-1}$)	CHO (g)
Elite						
Swim	1.20 $m.s^{-1}$	20.4 $kcal.min^{-1}$	Lower: 0.6	32	3.57	192
		1094 kcal	Upper: 1.2	64	2.13	115
Cycle	313 W	19.5 $kcal.min^{-1}$	Lower: 0.6	153	3.35	853
		4959 kcal	Upper: 1.2	305	1.92	487
Run	14.7 $km.h^{-1}$	20.8 $kcal.min^{-1}$	Lower: 0.6	103	3.67	631
		3572 kcal	Upper: 1.2	206	2.23	383
Total	8:04:07	20.1 $kcal.min^{-1}$	Lower: 0.6	288	3.49	1675
		9626 kcal	Upper: 1.2	576	2.05	985
Top amateur						
Swim	1.09 $m.s^{-1}$	18.5 $kcal.min^{-1}$	Lower: 0.5	30	3.34	198
		1094 kcal	Upper: 1.1	65	1.91	113
Cycle	225 W	15.4 $kcal.min^{-1}$	Lower: 0.5	149	2.57	768
		4580 kcal	Upper: 1.1	328	1.14	339
Run	13.5 $km.h^{-1}$	18.0 $kcal.min^{-1}$	Lower: 0.5	94	3.23	607
		3288 kcal	Upper: 1.1	207	1.79	337
Total	9:05:46	16.6 $kcal.min^{-1}$	Lower: 0.5	273	2.88	1573
		9062 kcal	Upper: 1.1	600	1.44	788

Abbreviations: CHO = carbohydrate, CO = carbohydrate oxidation, FO = fat oxidation.

This modelling approach suggests fatigue associated with depletion of endogenous CHO is a consideration of importance to elite Ironman triathletes, given the high estimated absolute (9626 kcal over the entire race duration) and rate (20.1 $kcal.min^{-1}$) of energy expenditure, which necessitates substantial CHO utilisation (2.05-3.49 $g.min^{-1}$) even with concomitant high rates of fat oxidation (0.6-1.2 $g.min^{-1}$). Indeed, this estimated CHO requirement (985-1675 g) exceeds likely whole-body CHO content (162), and is likely to be further exacerbated when one considers the hot environmental conditions in which Ironman triathlons are often performed (average temperature and relative humidity in Kona, Hawaii in October is ~30ºC, ~65% relative humidity [rH]

respectively), and the high-performance economy data used (31, 447, 520). The estimated CHO oxidation rates are noticeably reduced in the top amateur model (1.44-2.88 $g.min^{-1}$), underlining the considerable metabolic difference between a truly elite and highly-performing amateur Ironman triathlete, although the estimated absolute carbohydrate requirement is still very high (788-1573 g). Therefore, in the context of data linking endogenous CHO depletion to muscle fatigue (359, 361, 374–376), as well as those demonstrating improved endurance performance with CHO ingestion during competition (16, 69, 95, 101, 269, 275, 324, 370, 420, 462), this model provides further rationale that endogenous CHO availability should be considered a physiological determinant of ultra-endurance performance.

2.2.4.2 Profiling the substrate metabolism of endurance athletes

Given the potential importance of endogenous CHO availability for endurance sport (Chapter 2.2.4.1), a common practice in elite, and even sub-elite, endurance sport is profiling of exercise metabolism during an incremental step test assessment known as the "Fat_{max}" test (2). This graded exercise test measures whole-body CHO and fat oxidation rates across a range of exercise intensities in order to determine an individual athlete's capacity to make use of infinitely available fat stores for energy production during exercise, and therefore their reliance on finite endogenous CHO stores at submaximal workloads. Thus, the key parameters obtained from these assessments are the maximal rate of fat oxidation (MFO) and the intensity at which the MFO occurs (Fat_{max}). This test advances on previous protocols using four incremental submaximal workloads (386) that required multiple laboratory visits (154, 311).

The original "Fat_{max}" protocol consisted of 3-min, 35-W step increments performed after an overnight fast on a cycle ergometer until the respiratory exchange ratio reached 1.0, after which 2-min 35-W steps were employed, and this was validated against protocols involving longer stages (5 min) and prolonged exercise (>35 min) (2). Thus, the authors concluded two key theoretical limitations of step-test determination of substrate metabolism, namely shifts in substrate utilisation over time and effects of prior steps, were not influential (2), although it should be acknowledged that step durations of 6 min may be required for sedentary individuals to reach steady-state (46). The 3-min step protocol described here is indicative of those used in the literature subsequently (3–5), with conceptually identical treadmill protocols also used (6), and the starting workload and work increment magnitude is adjusted in accordance with participant training status (98, 343, 417). This relatively short protocol duration makes Fat_{max} testing a viable monitoring tool for endurance athletes concerned with substrate metabolism during competition. Lastly, the practicality of this protocol is particularly

important given attempts to predict MFO and Fat_{max} based on heart rate, power, and estimated maximum oxygen uptake ($\dot{V}O_{2max}$) have not been successful (58).

The reliability of Fat_{max} assessments has been examined. The first reliability study of the Fat_{max} protocol described above reported a coefficient of variation (CV) of 9.6% for Fat_{max} in a cohort of overnight fasted moderately-trained males with 24-h pre-trial dietary repetition (4). Interestingly, a similar study reported a CV of just 3% for Fat_{max} and 11% for MFO (99). These CVs are similar to those for MFO measured in sedentary cohorts using 4-5 pre-defined submaximal workloads based on prior assessment of maximal aerobic power (154, 311). In contrast, a 6-min step test used to determine Fat_{max} in a heterogeneous cohort of healthy males and females demonstrated wide limits of agreement and therefore considerable intra-individual variability (331). However, and critically, pre-trial diet and menstrual cycle was not controlled in this study, likely contributing to intra-individual variability given the reported influence of these variables on substrate oxidation during exercise (18, 66). Indeed, reliability of a similar treadmill protocol with 24-h dietary control conducted after an overnight fast reported CVs of 7 and 5% for MFO ($g.min^{-1}$) and treadmill velocity at MFO ($km.h^{-1}$), respectively (457). However, high CVs (>15%) have been reported with 24-h dietary control (92). The reason for this disparity in reliability is unclear, but may be related to the *effectiveness* of the pre-exercise dietary and exercise control measures (20). Failing to adequately match pre-exercise muscle glycogen content is likely to impact MFO given muscle glycogen availability is an independent regulator of substrate metabolism during exercise (179). Thus, rigorous pre-trial dietary and physical activity controls are emphasised in the experimental trials of this thesis.

As described above, the validity of the original Fat_{max} protocol was examined against prolonged exercise bouts at intensities equivalent to those in the step test, with results from the step test demonstrated to be reflective of those over longer duration (2). Interestingly, Schwindling *et al.* (443) had trained cyclists perform step Fat_{max} tests, and then 1-h constant-load tests at Fat_{max}, one workload above Fat_{max}, and one workload below Fat_{max}. No significant differences in absolute fat oxidation rates were observed between-intensities in the 1-h bouts, suggesting that results from short-duration Fat_{max} tests may *not* be reflective prolonged exercise. Therefore, Fat_{max} testing might be used to quickly and non-invasively monitor metabolic adaptations to training, rather than to elucidate the metabolic consequences of given exercise bouts, which might require prolonged, steady-state assessments. Indeed, MFO has recently been correlated with performance in Ironman triathlon ($r = 0.35$, $P < 0.01$) (141), which supports its utility in training monitoring for endurance events likely limited by CHO availability. Similarly, it is unclear whether Fat_{max} assessments can be used to derive training prescriptions,

given inconsistent observation of relationships with physiological thresholds (5, 161, 476, 483).

An issue for practitioners and researchers regarding Fat_{max} testing is the absence of normative values to contextualise individual measurements, such as those the American College of Sports Medicine provide for $\dot{V}O_{2max}$ (388). Therefore, I systematically identified all studies reporting these measures from studies with homogeneous cohorts performing assessments after an overnight fast on a cycle ergometer in order to generate normative values. Studies were subsequently partitioned into five populations: endurance-trained, lean males (2–6, 141, 368), recreationally-active, lean males (21, 40, 92, 93, 170, 271), recreationally-active, lean females (21, 40, 225), overweight/obese males (11, 12, 17, 99, 224, 270, 271, 337, 338, 343, 367, 417, 479), and overweight/obese females (37, 47, 99). “Endurance-trained” was defined by a sample mean $\dot{V}O_{2max}$ >55 $mL.kg^{-1}.min^{-1}$ and active engagement in training for endurance events. “Recreationally-active” was defined as physically active according to the individual study, not training for endurance events, and, where measured, by a sample mean $\dot{V}O_{2max}$ <55 $mL.kg^{-1}.min^{-1}$. The division between “lean” and “overweight/obese” was defined in males as a body fat percentage of 25% and/or body mass index of 25 $kg.m^{-2}$, and in females as a body fat percentage of 30% and/or body mass index of 25 $kg.m^{-2}$. Owing to often-absent definitions of physical activity status in overweight populations, those considered overweight/obese were not further defined by physical activity status. Baseline values were used for intervention studies. For synthesis, a sample size-weighted mean and SD for MFO was calculated for each population as described above for sex-mediated comparisons. Subsequently, normative percentile values were generated for each population assuming a within-population normal distribution (Table 3). This work has since been published in *Frontiers in Physiology* (316), and will be considered when interpreting the substrate oxidation rates of participants included in the present thesis.

Table 3. Normative percentile values for MFO ($g.min^{-1}$) in different subject populations during assessments performed on a cycle ergometer after an overnight fast. For example, measurement of MFO at 0.67 $g.min^{-1}$ in an endurance-trained, lean male would place them in the 80th percentile

Population	**N**	**Mean MFO ($g.min^{-1}$)**	**20th percentile**	**40th percentile**	**60th percentile**	**80th percentile**
Endurance-trained males	201	0.53 ± 0.16	0.40	0.49	0.58	0.67
Recreationally-active males	105	0.46 ± 0.14	0.34	0.42	0.49	0.58
Recreationally-active females	68	0.35 ± 0.12	0.25	0.32	0.38	0.45
Overweight/obese males	193	0.28 ± 0.14	0.16	0.24	0.31	0.39
Overweight/obese females	144	0.16 ± 0.05	0.12	0.15	0.17	0.20

Abbreviations: MFO = maximal rate of fat oxidation.

2.3 Summary

In summary, the metabolic qualities sought after in an endurance athlete are a high $\dot{V}O_{2max}$ brought about by a well-developed capacity to transport oxygen from the lungs to the working skeletal muscle tissue, a high maximal metabolic steady-state workload or physiological threshold secondary to advanced mitochondrial protein content and function, economical movement, and maintenance of endogenous CHO availability. As described above, these physiological parameters are malleable in response to exercise training, and can also be measured and monitored by exercise physiologists in elite sport settings. Consideration of the implications of different stimuli for these physiological determinants of endurance performance is therefore warranted. The acute and chronic effects of one such stimulus, environmental heat stress, will be the focus of the remainder of this thesis.

3 Literature review: How does acute and repeated exposure to heat stress impact metabolic responses to exercise?

Environmental heat stress is a potent stimulus that has seen much investigation in the exercise physiology literature. This is unsurprising given numerous elite athletic competitions take place in hot environments, and that many athletes train under environmental heat stress, either by virtue of their local climate or for specific heat acclimation (71, 403). Thus, exercise physiologists have comprehensively defined the physiological changes that occur during prolonged exercise performed under environmental heat stress (>30°C) compared to equivalent exercise in temperate conditions. These include rising core temperatures (369), greater peripheral blood flow (160, 434) and evaporative sweat losses (242, 474) for heat dissipation, progressive dehydration (195) and upward cardiovascular drift (369, 418) explained by the Starling's law of the heart (384), and an augmented stress response including greater circulating catecholamine concentrations (121). The physiological effects of exercise-heat stress are therefore substantial, as well as deleterious to prolonged exercise performance (387, 474, 480). However, it should also be acknowledged that in possessing relatively hairless, narrow-frames, an elaborate network of eccrine sweat glands, and bipedal gaits, we as humans are uniquely adapted to coping with combined-exercise heat stress (68, 501), and these adaptations were likely critical for survival in the hot, arid environment in which we evolved (56).

Alongside these well-described acute cardiovascular and thermoregulatory perturbations, a number of investigators have assessed acute metabolic responses to combined exercise-heat stress (Chapter 3.1), and there is emerging interest in the implications of performing exercise under environmental heat stress to augment metabolic adaptations to endurance training (Chapter 3.2). Given the importance of substrate metabolism for performance during prolonged endurance events, as well as the frequency with which environmental heat stress is encountered during endurance sport in both competition and training, any metabolic implications of environmental heat stress are worthy considerations for endurance athletes.

3.1 Acute effect of heat stress on substrate metabolism during endurance exercise

A number of investigations have examined metabolic alterations when performing prolonged exercise under environmental heat stress compared to temperate conditions (

Table 4). Exposure to environmental heat stress during prolonged submaximal exercise appears to augment intramuscular and whole-body endogenous CHO metabolism (118). Well-controlled studies have demonstrated enhanced net muscle glycogenolysis (121, 122, 124, 126, 242), whole-body CHO oxidation (104, 121, 122, 124, 242, 307), and hepatic glucose output (177) during prolonged exercise performed under environmental heat stress (>30°C) compared to equivalent exercise in temperate conditions, with reduced whole-body fat oxidation rates (104, 121, 122). These studies have been conducted solely in males, and so whether this effect is seen in females is not currently known.

However, a stimulatory effect of environmental heat stress on CHO metabolism during prolonged exercise has not always been observed (1, 148, 455, 514, 516). These contradictory results may be reconciled when considering that those studies failing to observe an effect of heat stress may have provided insufficient combined exercise-heat stress stimulus to effect a substantial difference in core and/or muscle temperatures between-conditions (1, 148, 455, 514), particularly when the acclimation status of the participants is considered (514), or failed to control pre-exercise muscle glycogen content (516). Indeed, pre-exercise muscle glycogen content is an independent determinant of muscle glycogenolysis during exercise (179). Interestingly, one further study failed to observe altered muscle glycogen utilisation when 30 min of cycling at 70%$\dot{V}O_{2max}$ was performed in 49 vs. 21°C, despite a greater end-exercise mean T_{re} of 0.7°C in the heat stress vs. temperate condition (517). However, this may be accounted for by the untrained status of the participants ($\dot{V}O_{2max}$ = 44.8 ± 1.3 mL.kg^{-1}.min^{-1}), who elicited near 100% CHO oxidation even in the temperate trial (respiratory exchange ratio [RER] = 0.97 ± 0.01). Indeed, when applying standard equations (246) to the presented $\dot{V}O_2$ and RER data, fat oxidation was very low in the temperate condition (~0.11 g.min^{-1}), and further suppressed in the heat stress trial (~0.02 g.min^{-1}) (517). Therefore, it is possible that environmental heat stress in this study was unable to exert a significant stimulatory effect on CHO metabolism without concomitantly increasing the energy cost of the exercise, because CHO metabolism already provided almost all energy production in the temperate trial.

Table 4. Studies comparing substrate metabolism during prolonged, constant-load endurance exercise (≥20 min) performed under environmental heat stress (≥30°C) and in temperate conditions (15-25°C)

Reference	Participants	Protocol	Metabolic effects	Thermoregulatory effects
Dolny & Lemon (104)	N = 8 healthy males ($\dot{V}O_2$max, 55 ± 8 mL.kg^{-1}.min^{-1}), 4-h postabsorptive	90-min cycle at 65%$\dot{V}O_{2max}$ in 5, 20, 30°C	RER, $\dot{V}O_2$: 30 > 20°C Estimated CHO ox: ~6.4% greater 30 vs. 20°C Blood [La]: 30 > 20°C Serum [NEFA]: 30 = 20°C Serum [Adr, Noradr]: 30 > 20°C	Mean T_{re}: ~0.24°C greater 30 vs. 20°C
Febbraio *et al.* (121)	N = 7 endurance-trained males ($\dot{V}O_2$max, 65 ± 13 mL.kg^{-1}.min^{-1}), overnight fasted	40-min cycle at 70%$\dot{V}O_{2max}$ in 20, 40°C (20% rH)	RER: 40 > 20°C, $\dot{V}O_2$: 40 < 20°C Estimated CHO ox: ~16.3% greater 40 vs. 20°C Net muscle glycogenolysis: 40 > 20°C Blood [Glu, La]: 40 > 20°C Muscle [La]: 40 > 20°C Plasma [Adr]: 40 > 20°C Plasma [Noradr]: NSD	Post-exercise T_{re}: ~1°C greater 40 vs. 20°C Post-exercise T_{mus} ~1.3°C greater 40 vs. 20°C
Febbraio *et al.* (122)	N = 12 endurance-trained males ($\dot{V}O_2$max, 65 ± 7 mL.kg^{-1}.min^{-1}), overnight fasted	40-min cycle at 70%$\dot{V}O_{2max}$ in 20, 40°C (20% rH)	RER: 40 > 20°C, $\dot{V}O_2$: 40 = 20°C Estimated CHO ox: ~17.4% greater 40 vs. 20°C Net muscle glycogenolysis: 40 > 20°C Net muscle CrP degradation: 40 > 20°C Net muscle Cr accumulation: 40 > 20°C Muscle [La]: 40 > 20°C Muscle [ATP, ADP, AMP, IMP]: NSD ([AMP] rose pre-to-post-exercise in 40, not 20°C) Muscle [NH_3]: 40 > 20°C	Post-exercise T_{re}: ~1°C greater 40 vs. 20°C Post-exercise T_{mus}: ~1.7°C greater 40 vs. 20°C

Fernández-Elías *et al.* (124)	N = 7 endurance-trained males ($\dot{V}O_2$max, 55 ± 3 mL.kg^{-1}.min^{-1}), controlled pre-trial exercise and diet	Dehydrating exercise in heat, 4-h rehydration, 40-min cycle at 75%$\dot{V}O_{2max}$ in 25, 36°C	CHO ox: 36 > 25°C Net muscle glycogenolysis: 36 > 25°C Serum [La]: 36 > 25°C	Post-exercise T_{re}: ~0.7°C greater 36 vs. 25°C
Galloway & Maughan (148)	N = 8 active males ($\dot{V}O_2$max, ~56 mL.kg^{-1}.min^{-1}), overnight fasted	Cycle TTE at 70%$\dot{V}O_{2max}$ in 4, 11, 21, 31°C	CHO ox: NSD 31 vs. 21°C Blood [Glu, La]: NSD 31 vs. 21°C Serum [Glyc, NEFA]: NSD 31 vs. 21°C	Exercise T_{re}: ~0.3-0.5°C greater 31 vs. 21°C
Hargreaves *et al.* (177)	N = 6 endurance-trained males ($\dot{V}O_2$max, ~64 mL.kg^{-1}.min^{-1}), overnight fasted	40-min cycle at 65%$\dot{V}O_{2max}$ in 20, 40°C (<50% rH)	RER: 40 > 20°C, $\dot{V}O_2$: NSD CHO ox: ~19.8% greater 40 vs. 20°C Estimated muscle glycogen ox: ~16.8% greater 40 vs. 20°C HGP: 40 > 20°C Plasma [Glu, La, Adr, Noradr, Cort, GH, Glucag]: 40 > 20°C	Post-exercise T_{re}: ~0.9°C greater 40 vs. 20°C
Hettinga *et al.* (192)	N = 10 well-trained males ($\dot{V}O_2$max, ~66 mL.kg^{-1}.min^{-1}), fasted for >2 hours	20-min cycle at 60%MAP in 15.5°C (20% rH), 35.5°C (16% rH)	RER: NSD, $\dot{V}O_2$: 35.5 > 15°C GE: 35.5 < 15°C Blood [La]: 35.5 > 15°C	Post-exercise T_{re}: ~0.3°C greater 35.5 vs. 15.5°C
Jentjens *et al.* (242)	N = 9 endurance-trained males ($\dot{V}O_2$max, 65 ± 3	90-min cycle at 55%W_{max} in 16°C (60% rH), 35°C (27% rH)	(All values 60-90 min of exercise) RER, $\dot{V}O_2$: NSD Total CHO ox: NSD	Post-exercise T_{re}: ~0.8°C greater 35 vs. 16°C

	mL.kg^{-1}.min^{-1}), overnight fasted	with ~1.5 g.min^{-1} CHO ingestion	Exogenous CHO ox: 35 < 16°C Fat ox: NSD Estimated muscle glycogen ox: 35 > 16°C Plasma [Glu, Ins, NEFA]: NSD Plasma [La]: 35 > 16°C	
Marino *et al.* (307)	N = 9 endurance-trained males ($\dot{V}O_2$max, 66 ± 4 mL.kg^{-1}.min^{-1}), acute nutrition unclear	30-min run at 70%PTRS in 15, 35°C (60% rH)	RER: 35 > 15°C, $\dot{V}O_2$: NSD CHO ox: 35 > 15°C Plasma [La]: NSD	T_{re} at 30 min: ~0.6°C greater 35 vs. 15°C
Parkin *et al.* (382)	N = 8 endurance-trained males ($\dot{V}O_2$max, 55 ± 8 mL.kg^{-1}.min^{-1}), overnight fasted	Cycle TTE at 70%$\dot{V}O_{2max}$ in 3, 20, 40°C (<50% rH)	RER, $\dot{V}O_2$: NSD Estimated rate of net muscle glycogenolysis: 40 > 20°C Plasma [Adr]: 40 > 20°C Plasma [Noradr]: NSD	T_{re} at matched time-points: ~0.5°C greater 40 vs. 20°C
Snow *et al.* (455)	N = 7 untrained males ($\dot{V}O_2$max, 51 ± 4 mL.kg^{-1}.min^{-1}), overnight fasted	40-min cycle at 70%$\dot{V}O_{2max}$ in 20, 40°C (<20% rH)	RER, $\dot{V}O_2$: NSD Plasma [NH_3]: 40 > 20°C Muscle [NH_3]: 40 > 20°C	Post-exercise T_{re}: ~0.6°C greater 40 vs. 20°C
Yaspelkis III *et al.* (514)	N = 7 endurance-trained, heat-acclimatised males ($\dot{V}O_2$max, 69 ± 1 mL.kg^{-1}.min^{-1}),	60-min cycle at ~74%$\dot{V}O_{2max}$ in 24, 34°C (~50% rH)	RER, $\dot{V}O_2$: NSD CHO ox: NSD (numerically 34 > 24°C) Net muscle glycogenolysis: NSD (numerically 34 > 24°C) Plasma [Glu, La]: 34 > 24°C Blood [Glyc, NEFA]: NSD	Post-exercise T_{re}: ~0.4°C greater 34 vs. 24°C

	overnight fasted			
Young *et al.* (517)	N = 13 untrained males ($\dot{V}O_2$max, 45 ± 5 mL.kg^{-1}.min^{-1}), 4 h-post liquid mixed meal	30-min cycle at 70%$\dot{V}O_{2max}$ in 21°C (30% rH), 49°C (20% rH)	RER: 49 > 21°C, $\dot{V}O_2$: 49 < 21°C Estimated CHO ox: ~3.8% greater 49 vs. 21°C Net muscle glycogenolysis: NSD Plasma [La]: 49 > 21°C Muscle [La]: 49 > 21°C	Post-exercise T_{re}: ~0.7°C greater 49 vs. 21°C

Abbreviations: Adr = adrenaline, CHO = carbohydrate, Cort = cortisol, Cr = creatine, CrP = phosphocreatine, GE = gross efficiency,GH = growth hormone, Glu = glucose, Glucag = glucagon, Gly = glycerol, La = lactate, Ins = insulin, HGP = hepatic glucose production, IMTG = intramuscular triglyceride, MAP = maximum aerobic power, Noradr = noradrenaline, NEFA = non-esterified fatty acids, NSD = not significantly different between-trials, ox = oxidation, PTRS = peak treadmill running speed, rH = relative humidity, TG = triglycerides, T_{re} = rectal temperature, TTE = time-to-exhaustion, $\dot{V}O_{2max}$ = maximum oxygen uptake, W_{max} = maximum power output.

Mechanistically, it appears that the environmental heat stress-induced shift toward CHO metabolism during prolonged exercise is related to increases in core temperature (123), muscle temperature (119, 460), and circulating catecholamines (120, 212, 213, 234, 495), as well as greater levels of hypohydration (178, 287, 288) (Figure 1). The evidence for these mechanisms will now be described in turn.

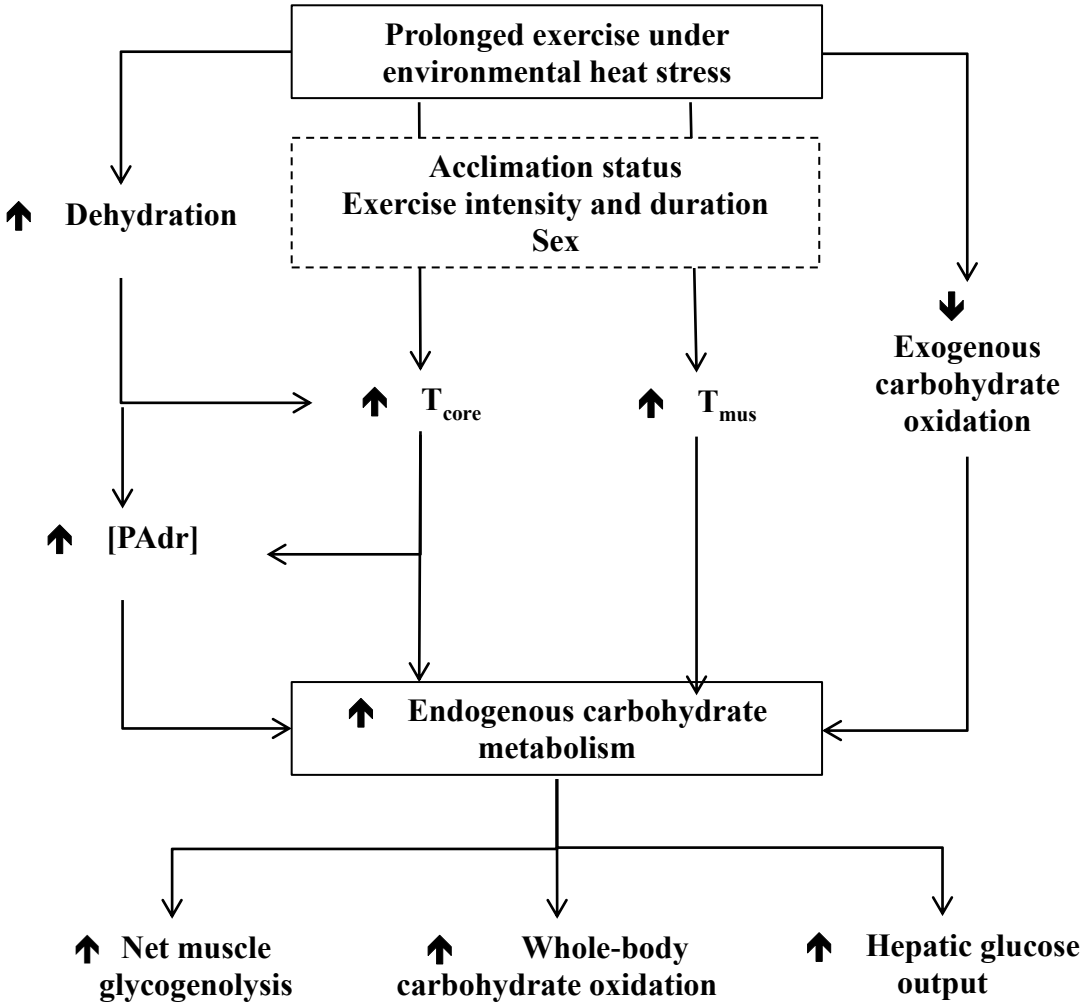


Figure 1. Acute effects of environmental heat stress (>30°C) during prolonged, submaximal exercise on whole-body and intramuscular substrate utilisation. Abbreviations: PAdr = plasma adrenaline, T_{core} = core temperature, T_{mus} = working skeletal muscle temperature.

3.1.1 Core temperature

Blunting the rise in body temperatures with external cooling reduces muscle glycogenolysis in humans (123) and dogs (267), while passive pre-heating prior to prolonged, submaximal exercise in temperate conditions augmented blood glucose and lactate concentrations after 15 min of exercise (169). Furthermore, during submaximal exercise performed under environmental heat stress, simulated rain exposure within an environmental chamber reduced oesophageal temperature and plasma lactate and adrenaline concentrations, further indicative of suppressed CHO metabolism when the rise in core body temperature is blunted (228). Interestingly, given the blunted evaporative heat loss response to given absolute exercise-heat stress, and therefore augmented rise in core temperature, in females (145, 146), it is possible the stimulation of CHO metabolism observed during prolonged exercise under environmental heat stress is exacerbated in female compared to male endurance athletes.

3.1.2 Muscle temperature

Research using limb-heating protocols demonstrated that part of the stimulatory effect of environmental heat stress on CHO metabolism during prolonged exercise can be independently attributed to elevated working skeletal muscle temperature (119, 460). Febbraio *et al.* (119) had participants perform 2 min of cycling exercise at 115%$\dot{V}O_{2max}$ in temperate conditions with and without pre-heating of the working lower limb musculature. This limb heating protocol resulted in increased pre-exercise muscle temperature of ~1.8°C compared to the control trial, and effected ~31% greater net muscle glycogenolysis without concomitant between-trial differences in rectal temperature or circulating catecholamines. Subsequently, the same laboratory adopted a pioneering study design to demonstrate this effect is robust during prolonged, submaximal exercise (460). Endurance-trained males performed 20 min of cycling exercise at 70%$\dot{V}O_{2max}$ in temperate conditions, with the upper part of both lower limbs wrapped in water-perfused cuffs. One cuff was perfused with warm water (50-55°C), while the other was perfused with cool water (0°C), such that one limb was heated and one cooled for 20 min prior to exercise. Net muscle glycogenolysis was ~76% greater in the heated vs. cooled limb, despite, by virtue of the study design, core temperature and circulating catecholamine concentrations being exactly equal. Thus, an independent effect of working skeletal muscle temperature on muscle metabolism during prolonged exercise was elucidated (460). Interestingly, the mechanism through which elevated muscle temperature augments CHO metabolism may be related to impairments in fat oxidation. A recent *ex vivo* study of rat myofibres demonstrated that a physiological temperature elevation partially uncoupled fatty acid oxidation from ADP

phosphorylation, possibly mediated by increased expression of mitochondrial uncoupling proteins (472). Theoretically, this effect would subsequently require greater reliance on CHO metabolism to support a given rate of ATP synthesis.

3.1.3 Circulating catecholamines

Several studies have demonstrated increased plasma adrenaline and/or noradrenaline concentrations during prolonged exercise under environmental heat stress compared to equivalent exercise performed in temperate conditions (104, 121, 177, 363, 382). Catecholamines have wide-ranging physiological and metabolic effects, including elevation of heart rate (252) and respiration (502), stimulation of lipolysis (153) via activation of lipolytic enzyme hormone-sensitive lipase in adipose tissue (497) and skeletal muscle (499), and activation of rate-limiting glycogenolytic enzyme glycogen phosphorylase (408). In order to elucidate the effect of circulating adrenaline on substrate metabolism during exercise, a number of laboratories have employed combined exercise-infusion protocols to artificially maintain plasma adrenaline at elevated concentrations, and demonstrated augmented net muscle glycogenolysis (120, 234), whole-body CHO oxidation rates (495), and hepatic glucose output (212, 213), although it is worth acknowledging that adrenergic blockade does not impact hepatic glucose production during exercise (214), so non-adrenergic regulation of hepatic glucose output must also be present. Particularly interesting in the present context is again a study performed by Febbraio and colleagues (120). Subsequent to their work demonstrating elevated plasma adrenaline concentrations during prolonged cycling at 70%$\dot{V}O_{2max}$ in 40 vs. 20°C (121, 177), this group had a similar cohort of endurance-trained males perform the same exercise in temperate conditions, with a saline or adrenaline infusion to elevate circulating adrenaline concentrations to those seen in their previous heat stress studies. This study therefore evaluated the metabolic impact of the elevated plasma adrenaline concentrations observed during combined exercise-heat stress independent of heat stress-induced changes in core and muscle temperature. Net muscle glycogenolysis was significantly augmented by ~35% in the adrenaline infusion trial, with evidence of increased whole-body CHO oxidation through indirect calorimetry and elevated accumulation of muscle lactate (120). Thus, an independent effect of plasma adrenaline concentration on muscle and whole-body substrate metabolism during prolonged exercise was elucidated (120).

3.1.4 Hydration status

Studies comparing substrate metabolism during prolonged exercise in temperate conditions with and without fluid ingestion to match losses have observed augmented net muscle glycogenolysis, whole-body CHO oxidation rates, and muscle and plasma

lactate accumulation in the fluid restriction trials (178, 286–288). Mechanistic research suggests permissive dehydration during exercise performed under environmental heat stress reduces cardiac output, leg blood flow, and leg oxygen consumption late in exercise (greater leg a-vO_2 difference throughout), with greater plasma catecholamine concentrations (157, 158). However, the absence of dehydration-induced alterations in delivery of glucose and non-esterified fatty acids to skeletal muscle suggests it is the hyperthermic effect of dehydration that effects these metabolic changes (158). Thus, given the greater demand for evaporative sweat loss in order to regulate core temperature during prolonged exercise performed under environmental heat stress, and the accompanying greater rate of fluid loss and likely magnitude of dehydration, it can be suggested that the dehydrating effect of environmental heat stress contributes to its observed stimulatory effects on CHO metabolism during prolonged exercise.

3.1.5 Exogenous carbohydrate oxidation

When evaluating the effect of environmental heat stress on CHO metabolism during prolonged exercise, another pertinent consideration is *exogenous* CHO utilisation. Many endurance athletes ingest CHO in large quantities during competition (62 ± 26 $g.h^{-1}$ in 53 finishers during the Ironman World Championship (390)), and published sports nutrition guidelines recommend CHO ingestion rates up to 90 $g.h^{-1}$ ingested in multiple-transportable form during events exceeding 2.5 h (62). CHO ingestion during exercise in temperate environments has been shown to suppress hepatic glucose output (245), but not during prolonged exercise in 35°C with 1 $g.min^{-1}$ glucose ingestion (15). Indeed, the capacity to oxidise exogenous CHO substrate is impaired when prolonged exercise is performed in 35 vs. 16°C (242). Despite this observed blunting of exogenous CHO oxidation with environmental heat stress, an endogenous CHO-sparing effect is still observed with 90 $g.h^{-1}$ glucose-fructose co-ingestion in a 2:1 ratio compared to water alone during 120 min cycling at 50%$\dot{V}O_{2max}$ in 32°C (241). Therefore, the endogenous CHO-sparing effect of exogenous carbohydrate ingestion during prolonged exercise is reduced when performed under environmental heat stress, thus serving to further increase the oxidation of endogenous CHO stores compared to equivalent prolonged exercise performed in temperate conditions with the same nutritional regimen.

3.1.6 Remaining questions

Therefore, the existing literature identifies a stimulatory acute effect of environmental heat stress on substrate metabolism during exercise (121, 122, 177, 242). This effect appears to be mediated by the augmented core and muscle temperature (119, 122, 460), elevated circulating catecholamines (120, 121), greater hypohydration (178), and

impaired exogenous CHO oxidation rates (242) observed when exercise is performed under environmental heat stress compared to in a temperate environment.

However, it is not possible using the literature to-date to accurately identify the combinations of exercise and heat stress in which these important metabolic changes are likely to be seen. Studies demonstrating a stimulatory effect of heat stress on CHO metabolism during prolonged exercise have been conducted at single relative exercise intensities, and the literature as a whole covers a narrow range of intensities when expressed either as a percentage of $\dot{V}O_{2max}$ (65-75%) (104, 121, 122, 124, 177, 307), aerobic power (W_{max}, 55%) (242), or aerobic speed (70%) (307). One study assessed rates of substrate oxidation during an incremental exercise test performed under environmental heat stress (34.1°C), but comparisons were made with a cold (4.6°C) rather than thermoneutral trial, preventing inferences regarding the independent effect of heat stress (147). It is possible the stimulatory effect of environmental heat stress on CHO metabolism is more pronounced as exercise intensity increases due to greater metabolic heat production in working skeletal muscle associated with higher energy expenditures. This might in turn elicit greater perturbations in aforementioned thermoregulatory variables known to augment CHO metabolism during exercise (119–121, 460).

As described above (Chapter 3.1), several studies have failed to observe an effect of environmental heat stress on CHO metabolism (1, 148, 455, 514, 516), and in these investigations it appears the heat stress stimulus was insufficient to induce meaningful changes in thermoregulatory variables to exert metabolic effects (1, 118, 148, 455, 514). This therefore suggests a minimum magnitude internal heat strain and/or external heat stress is required before stimulatory effects on CHO metabolism during exercise are observed. However, no studies have investigated the magnitude of the internal or external heat stress required for these effects, with existing studies assessing the effect of heat stress on substrate metabolism in a single heat stress condition (1, 104, 121, 122, 124, 148, 177, 242, 307, 455, 514, 516).

Lastly, studies investigating the effect of environmental heat stress on CHO metabolism during exercise have generally been relatively short in duration (<60 min) (121, 122, 124, 126, 148, 177, 192, 307, 382, 455, 514, 517). There is no data available to assess how substrate metabolism changes over time within an acute bout of exercise when performed under heat stress. Typically, when exercise is performed in a temperate environment, the rate of CHO oxidation progressively decreases and the rate of fat oxidation increases (320, 496), likely an effect of the progressive reduction in muscle glycogen content, given muscle glycogen concentration has an

autoregulatory effect on its own oxidation (516). However, when exercise is performed under heat stress thermoregulatory variables known to stimulate CHO metabolism, such as core temperature, muscle temperature, circulating adrenaline, and hypohydration, progressively increase (121, 122, 157, 158, 177). It might therefore be hypothesised that the stimulation of CHO metabolism by heat stress is exacerbated over time. Examination of the effect of exercise duration on the stimulation of CHO metabolism by heat stress is warranted, as ultra-endurance events likely limited by endogenous CHO availability are in fact defined by their long durations. For instance, the World Ironman Championship takes place under heat stress in Kaliua-Kona, Hawaii every year, and even elite male competitors require ~8 h to finish the course (312).

3.2 Endurance training and environmental heat stress: More metabolic bang for your buck?

Moving on from the acute implications of environmental heat stress for substrate metabolism during prolonged exercise, an important consideration that has received little attention in the literature is the *adaptive* metabolic consequences of training under environmental heat stress. One of the major purposes of training for endurance athletes is to increase skeletal muscle mitochondrial protein content and function (198). This is because β-oxidation of fatty acids to acetyl CoA, oxidation of acetyl CoA from fatty acid and non-fatty acid-derived sources in the citric acid cycle, and oxidative phosphorylation along the electron transport chain all take place in the mitochondria (199, 322, 512), making mitochondria the foundation of aerobic energy production.

The seminal investigation demonstrating mitochondrial biogenesis and improved mitochondrial oxidative capacity in response to exercise training was published in 1967 and conducted in Wistar rats (197). Subsequent human studies demonstrated those of endurance-trained status exhibited greater succinate dehydrogenase activity, a mitochondrial enzyme involved in the citric acid cycle and electron transport chain (156), as well as training-induced increases in succinate dehydrogenase activity in a longitudinal design (155). In the ~50 y since this pioneering research, mitochondrial adaptations to endurance training have been further elucidated (299), with increases in mitochondrial volume density (209, 339), mitochondrial oxidative capacity (166, 167), and mitochondrial enzyme content and activity (167, 436, 458) all observed. A major consequence of these exercise-induced training adaptations is glycogen-sparing at a given workload through augmented capacity to utilise fat as a metabolic substrate, and increased energy expenditure and external workloads at which a metabolic steady-state can be achieved (82, 219, 310). Given that many endurance athletes strategically perform training in artificial and/or natural hot environments (71), a largely unexplored consideration of great pertinence is how environmental heat stress impacts adaptations

to skeletal muscle mitochondria in response to endurance exercise training. Speculatively, a hypothesis for how environmental heat stress might impact mitochondrial adaptations to training can be made based on existing knowledge of the signalling cascades leading to skeletal muscle mitochondrial biogenesis.

3.2.1 Hypothesised role for heat stress in metabolic adaptation to endurance exercise training

The prevailing paradigm suggests peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α), a transcriptional co-activator (105) residing primarily in the cytosol of human skeletal muscle at rest (283), is the main regulator of skeletal muscle mitochondrial biogenesis (171, 176, 513). According to this paradigm, exercise exerts its adaptive effects by activating existing PGC-1α protein via phosphorylation and through increasing PGC-1α mRNA expression and therefore PGC-1α protein content (198). It is likely the initial phase of mitochondrial adaptation in response to an exercise stimulus is mediated by activation of existing PGC-1α, followed by an increase in PGC-1α protein synthesis (199). In this regard, exercise-induced activation of PGC-1α protein has been observed (510), and PGC-1α mRNA expression increases in response to endurance exercise (237, 394, 490) (Figure 2).

Exercise factors inducing cytosolic PGC-1α phosphorylation include activation of p38 mitogen-activated protein kinase (p38 MAPK) (265, 402), adenosine 5'-monophosphate-activated protein kinase (AMPK) (230) via rising intramuscular adenosine monophosphate (AMP) concentrations (289, 508), and calcium/calmodulin-dependent protein kinases (CaMK) (372, 511) via rising cytosolic Ca^{2+} concentrations (371, 372). It might be hypothesised that PGC-1α phosphorylation is augmented when prolonged exercise is performed under environmental heat stress via the acute stimulation of muscle glycogenolysis (392), an assertion made more compelling by the augmented responses to endurance training regimens featuring exercise performed with low-glycogen availability (216, 308, 309, 515). For instance, muscle glycogenolysis increases intramuscular osmolality *in vitro* (294). Indirectly, 30 s of sprint exercise, via significant muscle glycogenolysis (45, 114, 383), acutely decreases extracellular volume, likely as a result of an osmotic fluid shift into hyperosmotic muscle cells (325). Intramuscular hyperosmotic stress is thought to augment p38 MAPK activity (448), and thus PGC-1α phosphorylation (265, 402). Similarly, binding of glycogen to AMPK's glycogen-binding domain (215) inactivates AMPK (321), which suggests muscle glycogen content inversely regulates AMPK activity (392). Indeed, low glycogen exercise has been shown to augment AMPK activity (509), while exogenous glucose ingestion during exercise attenuates AMPK activation (8) when it spares muscle glycogen (276). It is therefore possible that reduced glycogen-AMPK

binding as a result of combined exercise-heat stress augments AMPK activity, and therefore PGC-1α phosphorylation. AMPK activation during prolonged exercise performed under environmental heat stress might be further augmented as a result of the increased interleukin-6 (IL-6) response to combined exercise-heat stress (461), an effect likely mediated through reduced splanchnic blood flow, increased gastrointestinal permeability, and endotoxin leakage (268). Importantly, there is data in mice (256) and humans (302) to suggest AMPK activation is stimulated by IL-6. Thus, the IL-6 response to prolonged exercise performed under environmental heat stress might further increase PGC-1α phosphorylation through its effects on AMPK.

Exercise-induced PGC-1α mRNA expression is regulated by binding of upstream transcription factors to the PGC-1α promoter region (125). These upstream transcription factors include cAMP response element-binding protein (CREB) (175, 190), activating transcription factor 2 (ATF2) (9, 10), myocyte enhancer factor 2 (MEF-2) (96, 175), and, compellingly in the present context, heat shock factor 1 (HSF1) (76, 301, 470). HSF1 is activated in response to cellular stresses include heat stress (285, 352, 470), and coordinates transcription of heat shock proteins (HSP) (431). Importantly, a recent study suggested HSF1 binding to the PGC-1α promoter region stimulates PGC-1α mRNA expression in skeletal muscle (301), which in turn might provide a further link between environmental heat stress and positive mitochondrial adaptations to endurance training. Another putative mechanism by which training under environmental heat stress might augment binding to the PGC-1α promoter region is through the observed increase in circulating catecholamines when exercise is performed in hot environments (104, 121, 177, 363, 382). β-adrenergic signalling appears to have implications for promotion of PGC-1α mRNA expression (13, 57, 79, 336), speculatively via interaction with CREB (392, 503). It is therefore possible that the augmented catecholamine response observed when prolonged exercise is performed under environmental heat stress might also contribute to an increase in PGC-1α mRNA expression.

In order to exert its adaptive effects, phosphorylated PGC-1α must be translocated from the cytosol to the nucleus and/or mitochondria. Human data comprehensively demonstrates nuclear PGC-1α translocation in response to acute exercise (168, 186, 283), and there is now animal (423) and human (454) data to support exercise-induced mitochondrial PGC-1α translocation. Once inside the nucleus or mitochondrion, it is proposed that PGC-1α is activated by the deacylating effect of sirtuin 1 (SIRT1) (171, 172). Interestingly, PGC-1α may be deacylated independently of SIRT1, thus suggesting possible SIRT1-independent PGC-1α activation. This hypothesis is based on SIRT1-knockout rodent data demonstrating unimpaired PGC-1α deacylation

compared to wild-type mice in response to acute exercise (391). Nuclear abundance of acetyltransferase general control of amino acid synthesis 5 (GCN5) was reduced in response to acute exercise in both rodent groups, suggesting observed PGC-1α deacylation was mediated by reduced acylation (391). Interestingly, in a further rodent study, GCN5-knockout did not augment mitochondrial adaptation to exercise training (102). The authors speculated this was mediated by compensatory actions of other acetyltransferases (102), such as P300/CBP-associated factor (PCAF) (279). Regardless, only phosphorylated, deacylated, nuclear and mitochondrial PGC-1α is ready to induce mitochondrial adaptations through co-activation of downstream transcription factors.

PGC-1α acts in concert with several other transcription factors in order to induce mitochondrial biogenesis and adaptation (105). These transcription factors include nuclear respiratory factor 1 (NRF-1) (423, 513), MEF-2 (175, 281), oestrogen-related receptor-α (ERRα) (80, 220, 439) via PGC-1- and ERR-induced regulator in muscle 1 (Perm1) (80), GA binding protein transcription factor α-subunit 60 kDa (NRF-2) (485, 513), peroxisome proliferator-activated receptor nuclear receptor-δ (PPARδ) (491, 492), and mitochondrial transcription factor A (TFAM) (423, 513). The interaction of active, nuclear PGC-1α with NRF-1, NRF-2, ERRα, and MEF2 induces transcription of nuclear genes implicated in a range of mitochondrial functions, hence synthesis of new mitochondrial protein (105). Clearly, newly-synthesised nuclear-encoded mitochondrial proteins need to be chaperoned from the nucleus to the mitochondria, a function undertaken by HSPs (518). Intuitively, HSP expression is augmented when prolonged exercise is performed under environmental heat stress (452), which might be expected to improve the efficiency of mitochondrial adaptation. Indeed, HSP72 overexpression has been shown to increase skeletal muscle mitochondrial content in rats without concomitant alteration in PGC-1α mRNA expression (188), which supports the hypothesis linking HSP function to improved mitochondrial adaptation downstream of nuclear-encoded mitochondrial protein synthesis. Lastly, exercise-induced mitochondrial TFAM translocation has been observed in rodent but not human models (454), suggesting that if PGC-1α impacts the mitochondrial genome in humans, this may not occur through direct mitochondrial PGC-1α-TFAM interactions (226).

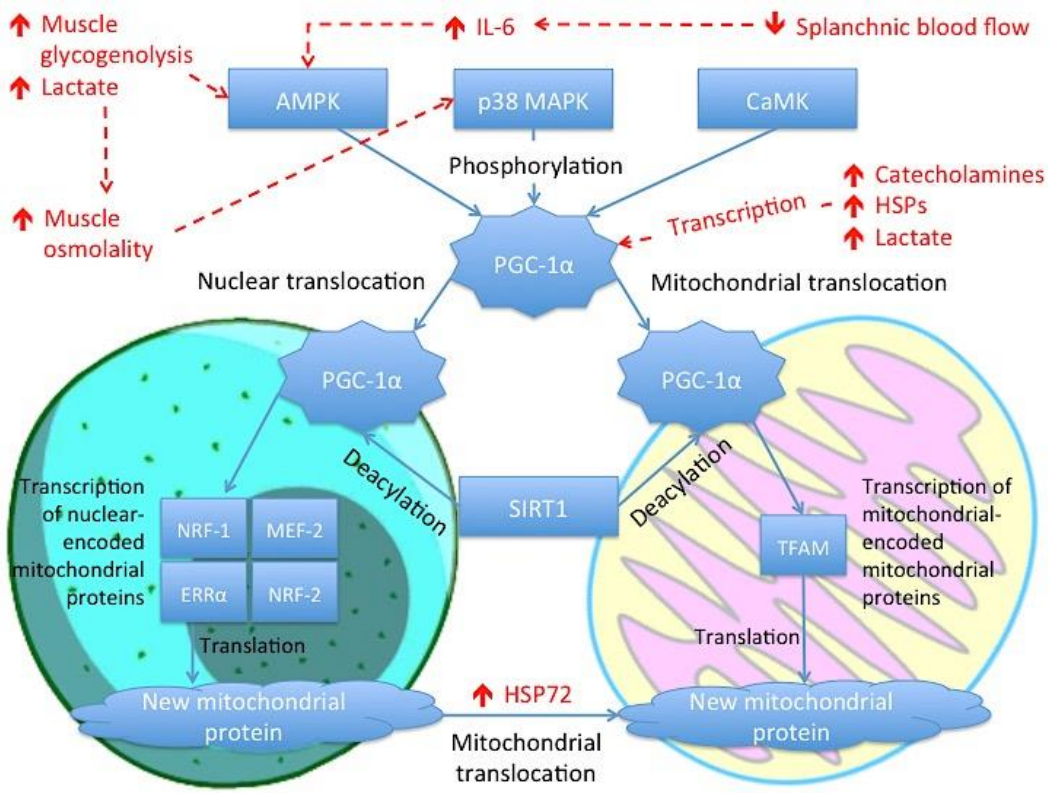


Figure 2. Schematic illustration of the possible mechanism by which episodic bouts of exercise augment skeletal muscle mitochondrial biogenesis through activation of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α). Speculative up-regulatory effects of combining exercise with environmental heat stress are shown in red.

An interesting recent study in mice demonstrated that lactate may contribute to mitochondrial adaptation to endurance training (469). Intraperitoneal administration of sodium lactate prior to endurance training sessions to successfully elevate circulating lactate concentrations augmented mitochondrial adaptation compared to administration of a phosphate-buffered saline (PBS) placebo (469). Specifically, with the same training stimulus, mice treated with lactate administration had significantly greater maximal citrate synthase, β-hydroxyacyl CoA dehydrogenase, and cytochrome c oxidase activities in the soleus muscle following the training period than mice treated with PBS, suggestive of a role of lactate in stimulating mitochondrial adaptations to endurance training, although this could not be explained through effects on AMPK, p-acetyl-CoA carboxylase (ACC), or p38 MAPK activation. Previous work demonstrated that the presence of lactate increases PGC-1α mRNA expression in cell and mouse models (181, 263), as well as a negative effect of blunting lactate accumulation through dichloroacetate administration during high-intensity exercise training on mitochondrial

biogenesis in mice (210). These findings therefore support a role for lactate in mitochondrial adaptation to exercise training (353). This literature relevant in the context of combined exercise-heat stress, given that a given exercise workload elicits higher muscle and plasma lactate concentrations when performed under environmental heat stress compared to in temperate conditions (104, 121, 122, 124, 192, 242, 514, 517), and may therefore add to the hypothesis that performing endurance training under environmental heat stress might augment mitochondrial adaptations to training. However, it should be acknowledged that this study was conducted in mice with very high lactate concentrations following sodium lactate administration (14 ± 2 $mmol.L^{-1}$), and thus the relevance of its application to humans is unknown.

Therefore, through our understanding of the physiological and metabolic responses to combined exercise-heat stress, a hypothesis can be presented for why performing repeated bouts of endurance exercise under environmental heat stress may have implications for mitochondrial adaptations to training. This hypothesis relates to speculative effects of heat stress on PGC-1α activation and mRNA expression, chaperoning of newly-synthesised nuclear-encoded mitochondrial to mitochondria via HSPs, and an effect of lactate. In the next section I will describe the limited studies that have explored this hypothesis directly.

3.2.2 Existing evidence

A limited number of studies have investigated the hypothesis linking environmental heat stress to mitochondrial adaptation. Indeed, a series of recent *in vitro* studies suggest adaptive effects of *passive* heat exposure on various mitochondrial markers (470). The first study in this regard used C2C12 myotubes and increased culture temperature to 40°C for 1 h, and observed augmented AMPK activity immediately post-heat stress, as well as elevated mRNA expression of several key genes implicated in mitochondrial biogenesis including PGC-1α at 24 h (284). When this heat exposure was repeated once per day for five days, protein content of PGC-1α and oxidative phosphorylation subunits were increased, suggesting repeated heat exposures effectively induce mitochondrial biogenesis (284). A subsequent *in vivo* study in mice demonstrated post-exercise exposure to a hot environment (30 min at 40°C five days per week for three weeks) additively enhanced exercise training-induced increases in mitochondrial enzyme activity and respiratory protein content, an effect apparently mediated by augmented acute p38 MAPK phosphorylation, and in spite of blunted AMPK phosphorylation (471).

To-date, only seven studies adopting a human, *in vivo* design have investigated the implications of environmental heat stress for mitochondrial adaptation; two in a combined exercise-heat stress model (186, 304), the remainder with passive heat exposure (173, 174, 191, 259, 519). Passive two stress models have produced mixed results. Positive responses were observed in three studies, with upregulated acute AMPK expression in response to local heat therapy (174), increased expression of electron transport complex I and V after six days of local heat therapy (174), and an attenuation of immobilisation-induced reductions in all five proteins of the electron transport chain after 10 days of limb immobilisation and local heat therapy (173). Other studies reported unchanged acute mRNA expression of numerous genes related to mitochondrial biogenesis after 3 h of passive whole-body exposure to 33°C vs. 20°C (519), no evidence of mitochondrial adaptation after eight weeks of passive local heat therapy (259), and no evidence of mitochondrial adaptation after six weeks of passive whole-body heat therapy (191). The first study of mitochondrial signalling responses to combined-exercise heat stress reported that 1-h cycling at 60%W_{max} in 33°C appeared to blunt transcription of genes related to mitochondrial biogenesis (PGC-1α, ERRα, GABPA, NRF-1, SIRT1, and vascular endothelial growth factor [VEGF]) compared to exercise performed in a temperate environment (186). Based on the available *in vitro* data, this blunting of mitochondrial biogenesis-related gene transcription was in contrast to the authors' hypotheses. Interestingly, nuclear PGC-1α translocation immediately or 3-h post-exercise was not impacted by the environmental temperature

in which exercise was performed (186). Speculatively, it remains possible that this apparent blunting of mitochondrial biogenesis-related gene transcription might be an effect of a shift in time-course of adaptive mechanisms when exercise is performed under environmental heat stress that was not detected in this study, or due to altered SIRT1 or GCN5 activity impacting the activation of downstream targets by nuclear PGC-1α (186). This time-course explanation is supported by *in vitro* data, which showed passive heat exposure upregulated mitochondrial biogenesis-related gene expression at 24 but not 2-h post-heat exposure (284). A subsequent study of 10-d low-intensity heat acclimation (90 min daily treadmill walking in 42°C, 30-50% rH) reported no evidence of mitochondrial biogenesis (304). Specifically, significant changes in expression of PGC-1α, cytochrome c oxidase IV, and electron transport chain complex I-IV (COX I-IV) in muscle biopsy samples were not significantly post- vs. pre-intervention. However, it should be acknowledged that no control group was utilised, and the exercise stimulus may have been insufficient to drive metabolic adaptations.

Interestingly, a few studies have measured adaptations to acute substrate metabolism during combined exercise-heat stress in response to periods of heat acclimation training. These studies have generally observed that performing exercise training (5-14 days) in environmental heat stress (>30°C) partially blunts the heat stress-induced stimulation of CHO metabolism during prolonged exercise (121, 261), or indirect evidence suggestive of this effect (232, 422). Importantly, these studies have only investigated the effects of heat acclimation training on substrate metabolism during prolonged exercise *under environmental heat stress*, and have never compared these changes against a temperate training control group. It is therefore not currently possible to discern if heat acclimation training exerts effects on substrate metabolism during prolonged exercise performed under environmental heat stress above that achieved through an equivalent period of temperate training, or if adaptations to substrate metabolism during prolonged exercise with heat acclimation training cross-over to prolonged exercise performed in temperate environments. The answers to these questions are likely determined by the independent effect of environmental heat stress during exercise on the adaptive mitochondrial response to training.

Several studies have measured functional metabolic and temperate performance adaptations to short periods (10-14 d) of heat acclimation (HA) training (Table 5). Lorenzo *et al.* (292) observed improved aerobic performance metrics with 10-d HA, but the training programme utilised was not reflective of real-world endurance sport, particularly for the temperate condition (90 $min.d^{-1}$ at power eliciting 50%$\dot{V}O_{2max}$), and elicited substantially greater physiological strain (as evidenced by greater heart rates)

in the heat condition. These results have been replicated recently in a study of temperate aerobic capacity responses to HA (486). In contrast, Mikkelsen *et al.* (333) observed similar improvements in 15-km time-trial performance in temperate conditions between-groups performing 5.5 weeks of training in 15 and 35-40°C. However, whilst both groups performed some interval training in temperate conditions, the heat stress training intervention (and therefore matched programme performed in temperate conditions) was not reflective of real-world endurance sport (5 x 60-min at 60%$\dot{V}O_{2max}$ each week). As such, these studies collectively did not provide, nor were they designed to provide, a fair comparison of aerobic or metabolic adaptation to endurance training programmes performed in temperate and heat stress environments. Similarly, Rendell *et al.* (407) reported improved 30-min TT performance and aerobic performance metrics such as lactate threshold following 10-d of isothermal HA, but this was not compared against a control group. The most relevant studies assessing temperate performance adaptations from training under heat stress were published in 2020. Rønnestad and colleagues (413) assigned elite male cyclists to a heat or control training group, and performed five sessions per week of 50 min at 45% of power at 4 mmol.L^{-1} blood lactate concentration in ~36°C or ~16°C for five weeks, alongside their regular training. Haemoglobin mass increased in the heat (~4.6%) but not control group (~0.5%), and power output at 4 mmol.L^{-1} blood lactate concentration increased in the heat but not control group (9 ± 12 vs. 0 ± 5%), although between-group changes were not significant (P > 0.05). Performance was assessed using a 15-min TT, which increased in both groups (7 ± 8 vs. 3 ± 5% in heat and control, respectively), with no significant between-group differences, although a small effect size favoured greater changes in the heat group (ES = 0.22). Finally, a recently published study reported similar improvements in 20-km temperate time-trial performance in groups performing three weeks of training in 32°C and 22°C, although in this study heat exposure amounted to only 4 hours.$week^{-1}$ (323).

Other studies have failed to demonstrate training-induced improvements in aerobic performance measures and/or physiology in temperate conditions, either with HA-type training protocols not reflective of real-world training (255, 355, 456), or with self-regulated continuation of ‘habitual’ training programmes (253). These studies are troublesome to interpret in the context of my research question, because without an improvement in aerobic performance in the temperate condition, we cannot be sure if the failure to see aerobic performance adaptation in the heat stress condition was due to (i) a blunting of the adaptive process induced by elevated temperature or (ii) an ineffective endurance training programme. Effective determination of whether heat stress augments or blunts aerobic metabolic adaptation to endurance training needs to be studied within a model whereby temperate endurance training induces favourable

adaptations, given the wealth of research demonstrating that this is the case in the long-term (29, 110, 200).

Table 5. Effect of endurance exercise training performed under environmental heat stress for aerobic-metabolic adaptations manifesting in temperate conditions

Reference	Participants	Protocol	Physiological and performance adaptations
Karlsen *et al.* (253)	N = 18 male cyclists ($\dot{V}O_{2max}$ = 63 ± 5 mL.kg^{-1}.min^{-1})	N = 9 two weeks self-regulated outdoor training in Denmark (<15°C) N = 9 two weeks self-regulated outdoor training in Qatar (~34°C)	43.4-km TT performance unchanged in both groups $\dot{V}O_{2max}$ and power at $\dot{V}O_{2max}$ unchanged in both groups Gross cycling efficiency unchanged in both groups
Keiser *et al.* (255)	N = 7 male cyclists ($\dot{V}O_{2max}$ = 61 ± 4 mL.kg^{-1}.min^{-1})	Randomised crossover 10 days HA (90 min.d^{-1}, 38°C) vs. control (90 min.d^{-1}, 18°C), HR fixed at 50%$\dot{V}O_{2max}$	30-min TT performance unchanged $\dot{V}O_{2max}$, power at $\dot{V}O_{2max}$ unchanged in both groups
Lorenzo *et al.* (292)	N = 20 cyclists (17 male, 3 female, $\dot{V}O_{2max}$ = ~67 mL.kg^{-1}.min^{-1})	N = 12 HA 10-d fixed power (90 min.d^{-1}, 40°C, 50%$\dot{V}O_{2max}$) N = 8 control 10-d fixed power (90 min.d^{-1}, 13°C, 50%$\dot{V}O_{2max}$)	60-min TT performance improved with HA, not control $\dot{V}O_{2max}$, LT improved with HA, not control
Mang *et al.* (304)	N = 13 active subjects (7 male, 6 female, $\dot{V}O_{2max}$ = ~51.0 ± 10.2 mL.kg^{-1}.min^{-1})	Pre/post 10-d HA (walking 90 min.d^{-1}, 42°C, 30-50% rH)	$\dot{V}O_{2max}$ increased (50.3 vs. 52.1 ml.kg^{-1}.min^{-1}) VT_1 and VT_2 unchanged
McCleave *et al.* (323)	N = 20 male cyclists ($\dot{V}O_{2max}$ = ~61 mL.kg^{-1}.min^{-1})	N = 11 three-weeks heat training (4 x 60 min interval session.week^{-1} in 32°C, 50% rH) added to regular training N = 9 three-weeks CON training (4 x 60 min interval session.week^{-1} in 22°C, 50%rH) added to regular training	20-km TT performance improved similarly in heat (-2.8% ± 1.8%) and CON (-2.0% ± 1.8%) Small increase in PV in heat (ES = -0.04 to 0.57) but not CON (-0.32 to 0.38)

Mikkelsen *et al.* (333)	N = 21 male cyclists ($\dot{V}O_{2max}$ = ~59 mL.kg^{-1}.min^{-1})	N = 12 HA 5.5 weeks (5 x 60 min sessions.week^{-1} at 60 % $\dot{V}O_{2max}$ in 35-40°C) with other training performed in ~15°C N = 9 control 5.5 weeks (5 x 60 min sessions.week^{-1} at 60 % $\dot{V}O_{2max}$ in ~15°C) with other training performed in ~15°C	15-km TT performance improved with HA and control to a similar extent (6.0 ± 1.1 vs. 5.5 ± 1.6%) $\dot{V}O_{2max}$ and power at $\dot{V}O_{2max}$ unchanged in both groups Gross cycling efficiency unchanged in both groups
Neal *et al.* (355)	N = 8 males ($\dot{V}O_{2max}$ = 57 ± 7 mL.kg^{-1}.min^{-1})	Pre/post 11-d HA at T_{re} = 38.5°C using cycling in 40°C for 90 min	LT, $\dot{V}O_{2max}$ unchanged Power at $\dot{V}O_{2max}$ increased
Rønnestad *et al.* (413)	N = 23 elite male cyclists ($\dot{V}O_{2max}$ = 76 ± 8 mL.kg^{-1}.min^{-1})	N = 11 five-week heat training (5 x 50 min at 45% power at [BLa] 4 mmol.L^{-1} in ~38°C, 65%rH) added to regular training N = 12 five week CON training (5 x 50 min at 45% power at [BLa] 4 mmol.L^{-1} in ~16°C, 25%rH) added to regular training	Hb_{mass} increased in heat (4.6%) but not CON PV increased in heat (4.8%) and CON (2.1%) $\dot{V}O_{2max}$ increased in heat (4.6 ± 5.6%) and CON (3.2 ± 3.9%) Power at [BLa] 4 mmol.L^{-1} increased in heat (9.1 ± 12.4%) but not CON 15-min TT power increased in heat (6.9 ± 8.4%) and CON (3.4 ± 5.1%)
Rendell *et al.* (407)	N = 8 males ($\dot{V}O_{2max}$ = 59 ± 8 mL.kg^{-1}.min^{-1})	Pre/post 10-d HA at T_{re} = 38.5°C using cycling in 40°C for 90 min	30-min TT performance increased LT, power at $\dot{V}O_{2max}$ increased, $\dot{V}O_{2max}$ unchanged
Sotiridis *et al.* (456)	N = 12 males ($\dot{V}O_{2max}$ = 55 ± 6 mL.kg^{-1}.min^{-1})	Pre/post 10-d HA at T_{re} = 38.5°C using cycling in 35°C for 90 min	$\dot{V}O_{2max}$, Q_{max} unchanged Power at $\dot{V}O_{2max}$ increased
Waldron *et al.* (486)	N = 22 males ($\dot{V}O_{2max}$ = ~60 mL.kg^{-1}.min^{-1})	N = 12 HA 10-d fixed power (60 min.d^{-1}, 38°C, 50%$\dot{V}O_{2max}$) N = 10 control 10-d fixed power (60 min.d^{-1}, 18°C, 50%$\dot{V}O_{2max}$)	$\dot{V}O_{2max}$ increased in both groups, greater increase in HA group 4-d post-HA

Abbreviations: HA = heat acclimation, HR = heart rate, LT = lactate threshold, Q_{max} = maximum cardiac output, TT = time trial, $\dot{V}O_{2max}$ = maximum oxygen uptake, VT_1 = first ventilatory threshold, VT_2 = second ventilatory threshold

3.2.3 Does performing endurance training under environmental heat stress carry risk?

It is therefore *possible* that conducting endurance training under environmental heat stress may augment the adaptive metabolic response to endurance training, although this has not yet been convincingly demonstrated in endurance athletes (183). However, a consideration for endurance athletes undertaking this practice is possible maladaptive effects of the increased physiological stress, which, if not managed effectively, could diminish any adaptation and even lead to maladaptation. Indeed, a recent study of a 5-d high-intensity interval training intervention in male cyclists and triathletes reported performance impairments when training was conducted in 35°C and 60% rH (406).

Prolonged exercise performed under environmental heat stress augments exercise-induced gastrointestinal hypoxia (268), plasma catecholamines (121), plasma and muscle lactate (121, 177), and plasma interleukin-6 (IL-6) (461) compared to equivalent exercise performed in temperate conditions, all of which could plausibly suppress appetite (185). For instance, circulating catecholamines have been significantly correlated with suppression of the orexigenic hormone ghrelin (449), whilst ghrelin-producing cells possess lactate-binding receptors that inhibit ghrelin release (113), a finding explaining research reporting reduced *ad libitum* energy intake after peripheral lactate administration (441). Indeed, recently published work found significant negative correlations between exercise-induced changes in plasma lactate concentration and plasma acylated ghrelin area under the curve ($r = -0.60$, $P < 0.001$) and subjective appetite ($r = -0.48$, $P = 0.006$) during a 90-min post-exercise recovery (227). Similarly, a significant positive correlation between exercise-induced changes in plasma IL-6 concentration and plasma active glucagon-like peptide-1 (GLP-1) area under the curve ($r = 0.42$, $P = 0.02$), and an accompanying negative correlation with subjective appetite ($r = -0.36$, $P = 0.04$), has been observed during a 90-min post-exercise recovery (227). GLP-1 is an anorexigenic hormone, and thus elevated GLP-1 would be expected to suppress appetite. There is tentative endocrine (117, 450), and subjective (266, 493) data to support the hypothesis in humans in response to acute prolonged exercise performed under heat stress, although this has not always been shown (117, 274). These laboratory investigations may not be the most appropriate means of discerning the real-world effects of combined exercise-heat stress on subsequent appetite and energy intake; indeed, these responses have not been measures in free-living conditions. The effects of performing prolonged exercise training under environmental heat stress on appetite and energy intake have profound implications for endurance athletes, who require large daily energy intakes to sustain body mass given the high daily energy expenditures associated with endurance

training, and therefore warrant specific investigation given the popularity of this training practice.

Similarly, prolonged exercise performed under environmental heat stress may also have implications for cardiac autonomic balance and athlete wellbeing. Exercise performed under environmental heat stress elicits greater heart rate (369, 418) and circulating catecholamines (104, 121, 177) compared to equivalent exercise performed in temperate environments, which may be indicative of greater sympathetic activation. Greater sympathetic and suppressed parasympathetic activation, which can be measured using heart rate variability (HRV) upon waking, may ultimately be predictive of negative performance consequences such as fatigue and non-functional overreaching (396). One potential means of combatting this effect is to prescribe training relative to heart rate at pre-determined individual physiological thresholds, such that training intensity is maintained according to the desired distribution. This is a common practice in endurance sport *per se* (395, 468, 477), however, the stability of heart rate at physiological thresholds between temperate and heat stress environments is not known. As such, investigating the stability of heart rate at individual lactate thresholds between environmental temperatures, as well as determining the consequences of performing well-controlled prolonged exercise training under environmental heat stress for HRV and athlete wellbeing, will provide further useful considerations for endurance athletes considering undergoing this training modality.

A last, but critical, consideration for endurance athletes considering undertaking training under environmental heat stress is the likely negative effect on absolute workloads achieved during training. As previously described, the myriad of physiological responses observed during exercise performed under heat stress reduces the individual capacity for work output, with the pace and power output at lactate threshold reduced (232, 292). Indeed, reduced power outputs have been observed during interval training sessions performed under heat stress (54). Therefore, similar to altitude training, where the hypoxic exposure is utilised as a physiological stressor to drive specific adaptations, a reduction in absolute training intensities is likely to take place with exercise training performed under heat stress that requires careful consideration (349). It is possible that this negative effect of heat stress on absolute training intensities may work against any positive direct effects of heat stress on physiological adaptations to endurance training. The balance of any outcome may therefore hinge on the duration of the intervention, with longer interventions allowing for thermoregulatory acclimation and a reduced reduction in absolute training intensities.

3.3 Remaining questions

In summary, exposure to environmental heat stress appears to exert an influence on substrate metabolism during acute endurance exercise, with a shift away from fat and towards CHO metabolism observed in many previous studies (Table 4). However, further investigation is required to identify the specific combinations of exercise-heat stress in which metabolic changes are most likely to occur; specifically, study of the regulatory effects of exercise intensity, exercise duration, and their interaction with the magnitude of the environmental heat stress is warranted. Also, repeated exposure to environmental heat stress may have implications for metabolic adaptations to endurance training (Figure 2), but this has not been fully investigated in humans. However, due to the additional physiological stress, repeated exposure to heat stress during training has the potential to compromise athlete wellbeing, and can in some instances induce maladaptation to training. Investigation of the potential adaptive effects of training under environmental heat stress, and strategies to minimise the likelihood of maladaptation, is warranted given the widespread use of heat stress training camps in endurance sport. Specifically, description of how these training camps are conducted and monitored in the real-world of elite endurance sport may give researchers and practitioners insight into the considerations made when embarking on training camps of this nature, and the tools that might be used in the field to monitor the wellbeing of athletes and minimise the risk of maladaptation to training. This information could subsequently be used in the design of rigorous research studies seeking to identify if environmental heat stress can be used as an additive stimulus during training to augment the adaptive metabolic response. Investigation of these remaining questions will be the subject of the acute (Chapter 5) and training-related (Chapter 6) experimental studies of this thesis.

4 Research methods and methodology

The experimental chapters of this thesis utilise some common methodologies. The purpose of this chapter is to provide more advanced background and principles behind these methodologies.

4.1 Research philosophy

Philosophically, the research methodology in this thesis adopts a post-positivist viewpoint utilising objectivist ontology and single-truth epistemology (282). By using a single-truth epistemology, the possibility for inter-individual variation in observed responses is still acknowledged given the complex, interactive mechanisms through which human physiology and metabolism is controlled during exercise. Therefore, differences in one or more of the physiological parameters relating to any of these mechanisms gives scope for differences in observed physiological and metabolic responses to the same stimulus. For instance, elevated plasma adrenaline concentrations have been observed in response to combined exercise-heat stress (104, 121, 177, 363, 382), and plasma adrenaline has been independently shown to augment muscle glycogenolysis and CHO oxidation during exercise (120). However, plasma adrenaline concentration might not increase by the same magnitude in each individual in response to the same exercise-heat stress stimulus, due for instance to anthropometric factors such as surface area-to-body mass ratio or sweat gland density, which would alter heat dissipation (68, 501) and therefore the magnitude of the internal heat stress. Therefore, there is possible inter-individual variation in the measured response to a given combination of exercise and environmental temperature, but this variation is grounded in a 'truth'; in this case an innate stimulatory effect of plasma adrenaline concentrations on skeletal muscle CHO metabolism. This philosophy is taken in agreement with the view of human metabolism, at the molecular level, as an objective phenomena existing outside the perception of individuals that responds in a generalisable manner. Such a view therefore encourages deductive, controlled experimentation and inferential statistics for identification of cause-and-effect, a hallmark of the natural sciences (305).

Post-positivism is adopted in this thesis ahead of traditional positivism in line with the principle that the scientific method can only expose probable truths, as investigated phenomena occur independent of observation under the influence of variables not measured or considered in the experimental design; a view reflected in Bhaskar's principle of intransitivity (38). Indeed, the continually emerging research concerning human metabolism is testament to our incomplete understanding of the field.

Therefore, description of metabolic phenomena and subsequent causal inferences based on the results of the present thesis, or indeed any study, may not perfectly explain the mechanisms under investigation in their entirety. This concession is the key point of epistemological divergence between positivistic and post-positivistic philosophies (401), and acts to guard against inappropriate certainty in identification of causative mechanisms that may incompletely explain a relationship between environmental heat stress and acute and/or chronic metabolic responses to exercise.

4.2 Participants

Due to the invasive and time-consuming nature of the data collection procedures in this thesis, recruiting a cohort of elite athletes to participate would be likely impossible, particularly given budgetary constraints. As such, well-trained amateur athletes were recruited to take part, as is commonplace in the exercise physiology literature (121, 216, 489). Given the widespread amateur participation in endurance sports such as Ironman triathlon, particularly in New Zealand, studies using this population have immediate transfer to the population of interest. Furthermore, studying this population may provide a starting point from which to answer these research questions in an elite athletic population. A logical extension of the present thesis would be to study any specific observed effects in a less-invasive, less-time consuming manner in an elite athletic population.

Female athletes were excluded from the studies presented in this thesis on the basis of observed differences in whole-body substrate metabolism during exercise between-sexes (130, 264, 483), and the design constraints associated with the need to control for the menstrual cycle. Circulating oestrogen and progesterone appear to impact substrate metabolism during exercise (97), and menstrual phase impacts resting core temperature (71). Therefore, repeat experimental trials need to occur in the ~9 d window during the follicular phase when circulating oestrogen and progesterone concentrations, and resting core temperature, are stable (373, 487), and in eumenorrheic subjects not taking hormonal contraceptive medication. Thus, it is suggested that the research questions of the present thesis should be investigated in future studies in a female-only cohort, using designs that specifically account for these important considerations.

4.3 Pre-trial controls

In order to make within- and between-subject comparisons of metabolism or performance, it is critical that measurements are made with adequate pre-trial controls to isolate the effect of the intervention of interest (e.g. environmental temperature or

training). For instance, metabolic responses to exercise are sensitive to pre-exercise feeding and hydration status, with both CHO ingestion (3, 109, 488) and hypohydration (286–288) increasing dependence on CHO metabolism to support energy expenditure during exercise. Accordingly, the experimental studies in this thesis required participants to replicate pre-trial diet, involving weighed food records and pre-trial measurement of urinary specific gravity as a low-cost indicator of hydration status (19). Similarly, as exercise can deplete endogenous CHO availability (35, 162), and endogenous CHO availability is a critical regulator of utilisation during exercise (179), pre-trial exercise was similarly replicated throughout this thesis in an attempt to replicate pre-trial muscle and liver glycogen availability.

4.4 Thermoregulatory sampling and analyses

Rectal temperature is often considered the gold standard in assessment of core temperature (111, 335). Due to discomfort and difficulty associated with rectal temperature measurement, particularly outside of the laboratory, several less-invasive models have been investigated and found to have some acceptability (111, 137, 329). However, given the laboratory-based nature of studies requiring core temperature measurement in the present thesis, rectal temperature was selected as the most appropriate and accurate technique for measurement of this variable.

This technique does have limitations that should be considered, particularly in the context of this thesis. For instance, there is likely a time-lag between changes in the internal temperatures of some organs and tissues before this is observed in the rectum (111), particularly during rapid changes in body temperature (466), and this is possibly related to the relatively small vessels that supply and drain the rectum relative to its mass (475). The potential for a time-lag should be considered when measurement of rectal temperature is made during exercise, or exercise segments, of short duration. Indeed, it is possible that in some instances true changes in internal temperatures will not be measurable at the rectum after insufficient exercise durations. However, rectal temperatures are related to metabolic heat production in the lower-body musculature, possibly via conduction (475). Thus, as the exercise modality used in this thesis is cycling, rectal temperature was considered an appropriate means of core temperature measurement. An alternative, oesophageal measurements, was deemed likely impractical with concomitant collection of expired gases, whilst the cost, and logistics (e.g. assurance that the pill is swallowed sufficiently in advance of the trial), associated with gastrointestinal measures rendered this technique unfavourable.

As described above (Chapter 3.1.2), elevated muscle temperature is one of the mechanistic causes of the augmented CHO metabolism observed during exercise

performed under environmental heat stress (119, 460). However, direct assessment of muscle temperature is a highly invasive procedure involving insertion of a needle thermistor directly into the muscle tissue, often through an incision made for a muscle biopsy (119, 460). A recent non-invasive estimate of muscle temperature that measures the temperature of insulated skin over the muscle of interest has been developed (55, 136). This method requires measurement of the skin temperature over the muscle of interest underneath a layer of neoprene, which theoretically insulates the skin such that it equalises with the temperature of the muscle below it (55, 136). With the use of corrective equations, this non-invasive method has been validated against direct measurement as an accurate assessment of working skeletal muscle temperature during exercise (136). Given its practicality and low-cost, this insulated skin technique to estimate muscle temperature during exercise was utilised in the present thesis.

Whilst performing an in-house validation of the technique would have been favourable, this was logistically challenging in the present thesis, due to the ethical requirement for a medical practitioner to insert any indwelling muscle thermometer. Given how well-described the method is in its validating research (55, 136), use of this technique in this thesis was appropriate. Similar to rectal temperature measurement, one potential weakness in this method that should be considered in this thesis is the potential for a time-lag in the equalisation of skin and muscle temperatures during periods when muscle temperature is changing rapidly, such as at the onset of exercise or during changes in exercise intensity. This should be considered if muscle temperatures are estimated early on in an exercise trial, or soon after an intensity-transition.

4.5 Indirect calorimetry

Indirect calorimetry uses the collection of expired gases for the estimation of $\dot{V}O_2$ and $\dot{V}CO_2$, and stoichiometric equations, to make inferences regarding whole-body substrate utilisation rates. Indirect calorimetry requires measurement of expiratory flow rate ($L.min^{-1}$) and expired gas content. This data can be used to estimate $\dot{V}O_2$ and $\dot{V}CO_2$ according to the Haldane transformation (182) , which assumes the absolute nitrogen content of inspired and expired air is equal. The basic stoichiometry associated with the combustion of given quantities of CHO and fat can then be used to estimate whole-body rates of CHO and fat oxidation (142, 246).

However, indirect calorimetry is certainly not without its limitations. Indeed, Rowlands (419) used physicochemical modelling to suggest decreases in whole-body and compartmental CO_2 stores can occur secondary to metabolic acidosis during high-intensity exercise, resulting in inclusion of non-metabolic $\dot{V}CO_2$ within whole-body

estimates of CHO and fat oxidation rates. Theoretically, non-metabolic $\dot{V}CO_2$ has the potential to increase RER, thereby overestimating CHO and underestimating fat oxidation rates. However, Romijn *et al.* (411) demonstrated that estimates of substrate oxidation rates calculated using indirect calorimetry during high-intensity exercise at 80-85%$\dot{V}O_{2max}$ were accurately reflected by the $^{13}C/^{12}C$ ratio expired breath method, which is independent of $\dot{V}CO_2$. These results led the authors to conclude that measurements of $\dot{V}CO_2$ during exercise provide a valid reflection of CO_2 production from metabolically active tissues. It is worth acknowledging that the subjects under investigation were endurance-trained, and indeed of similar training status to those in this thesis ($\dot{V}O_{2max}$ = 62 ± 3 mL.kg^{-1}.min^{-1}), and that plasma lactate concentrations were elevated to 4.55 ± 0.92 mmol.L^{-1} after 30 min of exercise.

Also importantly in the context of the present thesis, $\dot{V}CO_2$, as well as $\dot{V}O_2$ and therefore RER, reached steady-state after only 3-4 min of the 30-min bout exercise (411). It is similarly worth noting that this response was observed despite commencing the 30-min bout of exercise at 80-85%$\dot{V}O_{2max}$ from complete rest, thus inducing a much more dramatic change in respiratory parameters compared to a transition from lower-intensity exercise, as may be observed during an incremental step test assessment such as the Fat_{max} test (2, 316).

4.6 Mass spectrometry

When combined with indirect calorimetry, it is possible to use mass spectrometry to make inferences regarding the oxidation rate of substrate ingested during exercise (244). If CHOs ingested during exercise are naturally or artificially enriched with molecular weight 13 carbon (^{13}C), $^{13}CO_2$ is produced and excreted in the breath rather than typical $^{12}CO_2$ (244). Therefore, breath samples can be collected into evacuated tubes during exercise and analysed for ^{13}C:^{12}C ratio using mass spectrometry, thus providing insight into the proportion of CHO metabolism attributable to ingested and endogenous sources using standard equations (89). This technique can therefore be used to assess the effect of a given intervention on exogenous CHO metabolism during exercise (242, 320, 489).

There are however a number of methodological considerations when attempting to use this technique to measure the oxidation of ingested high ^{13}C CHO during exercise. Firstly, it is possible that systematic underestimation of ingested CHO oxidation takes place early in an exercise bout, as a portion of the associated $^{13}CO_2$ production is retained in the endogenous bicarbonate pool (28, 187, 244, 277, 334, 381, 451, 478). Accordingly, ingested CHO oxidation rates have only been estimated in this thesis after 60 min of exercise, at which point full turnover of the bicarbonate pool can be

expected, and therefore $^{13}CO_2$ exiting the bicarbonate pool is likely to match $^{13}CO_2$ entry to the bicarbonate pool when ingesting high ^{13}C CHO at a constant rate (398).

A second consideration relates to the potential for systematic *over*estimation of ingested CHO oxidation rates through 'glycogen dilution'. This refers to non-oxidative $^{13}CO_2$ production and expiration on the breath derived from the use of ingested high ^{13}C CHO in glycogen synthesis (138). However, it is considered unlikely that significant glycogen synthesis takes place during exercise at the intensities adopted in the present thesis when this method is used (77, 247) (80% of the power output associated with the first ventilatory threshold, Chapter 6.4), given glycogenesis consumes energy and glycogen is likely directed towards oxidation to support exercise (239, 362). The inhibition of glycogen synthesis during exercise may mechanistically related to elevated circulating adrenaline and AMPK activation, and therefore inactivation of key enzyme glycogen synthase at site 2 (362). Regardless, any minor contribution of ingested high ^{13}C CHO to glycogen synthesis during exercise would not be expected to bias between-trial comparisons. Accordingly, no correction for possible 'glycogen dilution' was made in this thesis.

A final consideration is the possibility of background shifts in expired ^{13}C during exercise associated with the greater naturally-occurring ^{13}C enrichment of CHO than fat (244). Accordingly, shifts in metabolism within an exercise bout, typically an increase in fat oxidation associated with reduced endogenous CHO availability (496), can influence the 'background' ^{13}C enrichment of expired breath unrelated to alterations in oxidation of the ingested carbohydrates. Some studies have measured these background shifts using control trials with water or specific low ^{13}C CHO ingestion, and applied correction factors to subsequent experimental trials, or prior bouts of glycogen-depleting exercise and dietary controls to reduce endogenous ^{13}C and therefore the possibility for background shifts (240, 489). In the present thesis, a high ^{13}C tracer was added to naturally-high ^{13}C carbohydrates ingested during exercise, therefore producing a much larger ^{13}C 'signal' on expired breath during exercise than in studies in which a naturally-high ^{13}C ingested CHO was used without a tracer, and participants were asked to refrain from ingestion of naturally high ^{13}C dietary sources for 48 hours in advance of experimental trials making measurement of ingested CHO oxidation. As such, any effect of background shifts in breath ^{13}C enrichment over time within the exercise bout were considered to have negligible effects. However, the possibility for background shifts in breath ^{13}C enrichment associated with shifts in substrate utilisation should be considered when examining the results.

4.7 Relative exercise intensity

When seeking to make inferences about the effect of an intervention, such as heat stress or a period of heat stress training, on metabolic variables during exercise, it is critical that the relative exercise intensity is held constant between the experimental trials. This has traditionally been achieved in a number of ways, such as using the workload that a previous incremental test showed to elicit a given percentage of an individual's $\dot{V}O_{2max}$ (122, 177, 320) or maximum heart rate (HR_{max}) (149–151), or at a given percentage of the maximum work rate an individual achieved during an incremental test (W_{max}) (192, 242, 307). However, it is my contention that these definitions of relative exercise intensity are inadequate when seeking to maintain a consistent relative workload between-individuals within a study as well as within-individuals across the study's experimental trials. Along with a colleague, I set out my views on the definition of relative exercise intensity in a short comment article in the *Journal of Applied Physiology* (429).

We proposed that relative exercise intensity is best quantified using individual measurement of physiological thresholds, namely the ventilatory and lactate thresholds (295, 296, 298, 395, 468, 477), a view that has since been echoed (42). Indeed, these parameters are used effectively in endurance sport to ensure training sessions evoke a given physiological stress and are conducted according to the desired training intensity distribution (395, 468, 477). For instance, low-intensity training sessions are prescribed below the first ventilatory or lactate threshold (LT) to ensure that the physiological response to the training session achieves the desired low physiological stress. It is this point – that exercise is below the individual's LT – that defines the exercise as low-intensity. Where LT exists as a $\%\dot{V}O_{2max}$ varies considerably between-individuals (5). Therefore, exercise at $65\%\dot{V}O_{2max}$ may be below the first ventilatory or lactate threshold in one athlete, but above it in another, and thus of different physiological stress and relative intensity. More directly, research has shown metabolic responses such as plasma lactate concentrations during acute exercise are more consistent between individuals of different training status when relative exercise intensity is expressed as a percentage of the LT compared to $\dot{V}O_{2max}$ (22), with similar results observed in a study of glycogen utilisation between subjects of similar W_{max} but different LT (88).

This is troublesome as a given intervention, such as heat stress, may plausibly have different metabolic or physiological effects at low compared to high relative exercise intensities. Therefore, when seeking to experimentally examine the effect of a given intervention on physiological function during exercise, it is critical that the relative

exercise intensity is held constant both within- and between-individuals. As such, relative exercise intensity was defined using individual measurement of physiological thresholds throughout this thesis.

4.8 Plasma sampling and analyses

Throughout this thesis, venous blood was sampled serially during the various experimental exercise protocols to provide further insights into metabolism. This occurred via pre-exercise insertion of an antecubital venous cannula, which allows repeated venous blood sampling without the need for multiple needle punctures. The patency of cannulas was maintained through frequent (~15 min) flushing with saline. This required that ~1-2 mL of 'waste' was extracted from the cannula before the sample for analysis was drawn, as saline from prior flushing must be removed from the cannula to avoid risk of sample dilution. Samples were stored on ice until the end of each experimental trial, when they were analysed for haematocrit and haemoglobin concentration (AcT 5diff, Beckman Coulter, Miami, USA) to allow for correction of sequential samples for changes in plasma volume (103). Samples were then centrifuged and plasma was extracted before storage at -80°C for further analysis. It is important to recognise that whilst assessment of plasma variables relevant to substrate metabolism can provide some insight, measurement of simple concentrations does not provide direct inferences regarding production or removal; simply the balance between production and removal over time. Accordingly, the results of plasma data in this thesis have been interpreted cautiously and in the context of previous research demonstrating the likely cause (changes in production vs. removal) of any observed fluctuation.

Specific analyses performed in this thesis include enzymatic colorimetric diagnostic assays using an automated clinical chemistry analyser (cobas Modular P800, Roche Diagnostics New Zealand Ltd, Auckland, NZ), as well as commercially-available enzyme-linked immunosorbent assays (BI-CAT® ELISA, Diagnostika GMBH, Hamburg, GER). These assays enable estimation of the concentration of a given variable within a sample via detection of light absorption; a capture antibody is used to bind the variable of interest to the analysis plate, a detection antibody labeled with a specific enzyme is next added which binds the variable of interest, and finally the substrate for that enzyme is added. The subsequent reaction evokes a colour change, the magnitude of which is proportional to the concentration of the variable of interest in the sample. The colour change is measured using a spectrophotometer and quantified using a standard curve.

4.9 Muscle sampling and analyses

As discussed by Bishop *et al.* (42), mitochondrial protein content and function has been measured using a variety of methods, which differ in terms of outputs, as well as cost and resource requirements. The most commonly adopted method for assessing mitochondrial adaptation in exercise studies is measurement of citrate synthase activity in homogenised muscle samples using a kinetic assay. Citrate synthase is an enzyme located in the mitochondrial matrix that catalyses the first step of the Krebs cycle, where acetyl coenzyme A and oxaloacetate are condensed to form citrate (504). Therefore, maximal citrate synthase activity of human muscle lysates stimulated *in vitro* correlates strongly with mitochondrial protein content as measured by transmission electron microscopy imaging (r = 0.84, $P < 0.001$) (273), which is considered the gold-standard (393). Compellingly, training volume appears to predict changes in mitochondrial protein content determined by citrate synthase activity (165). This citrate synthase activity assay is affordable and can be performed in-house, whereas the transmission electron microscopy imaging technique requires specialised equipment not available in our laboratory, hence would incur costs exceeding the budget allocated to this thesis. I learned the citrate synthase activity assay in preparation for analyses to be performed in Chapter 6.4 using lysates of C2C12 murine myotubes, and achieved an acceptable level of consistency (CV = 5.6 ± 5.1%). Thus, in the present thesis, where changes in mitochondrial protein content in response to exercise training interventions are of interest, assays for citrate synthase activity on muscle samples collected before and after training were performed.

4.10 Statistical analyses

The statistical practices used in sport and exercise science research have seen considerable attention in the recent literature, with divergent views on the most appropriate statistical approach to make inferences from collected data (72, 208, 424, 425, 500). As such, a thorough review of the literature has been performed ahead of time in order to both identify the most appropriate statistical approach as well as research design, with the temporality of the latter point suggested in a recent review of the subject (65). Accordingly, thorough consideration of the relevant statistical literature has resulted in adoption of conservative null-hypothesis testing (NHT) in the present thesis to detect significant differences between-data sets via frequentist *P*-values (128, 144, 164, 201, 506), which are, when appropriate, assessed for magnitude in raw units or using Cohen's *d* effects sizes (83, 100). The key background that encouraged adoption of this approach is detailed in the following paragraphs.

Briefly, NHT uses inferential statistical tests, selected in accordance with the study design and normality of data distribution, to discern the probability of obtaining the observed data-set if the null hypothesis is true (63). Thus, a *P*-value of 0.01 indicates the probability of obtaining the observed data-set if the null hypothesis is true is 1%. There is widespread use of an arbitrary *P*-value threshold of 0.05 for rejecting the null hypothesis; that is, *P*-values less than or equal to 0.05 are deemed significant and the null hypothesis is rejected, whereas values greater than 0.05 are deemed non-significant and the null hypothesis is accepted. The *P*-value obtained is dependent on the size of the effect, the variability in the dataset, and the sample size.

When multiple comparisons are made, such as when a group of subjects perform an experiment twice in a cross-over design, and a particular measure is made several times during each experimental trial (e.g. CHO oxidation rate at 15, 30, 45, and 60 min during exercise), it is necessary to correct for the family-wise error rate. That is, if all comparisons (e.g. CHO oxidation at 15 min in both trials, at 30 min in both trials, *etc*.) are made using basic NHT, the probability of making a type I error (incorrect rejection of the null hypothesis) increases in proportion with the number of comparisons made. In these instances, appropriate analyses of variance are applied to the dataset as a whole (128, 144), and, if a statistically significant effect of trial, time, or an interaction between trial and time is indicated, post-hoc assessments are made to locate variance. A plethora of post-hoc inferential tests are available. In the present thesis, Holm-Bonferroni stepwise correction is used (Equation 1) (201). This approach uses basic NHT (i.e. *t*-testing), and then corrects obtained *P*-values using a stepwise multiplication factor, whereby the lowest *P*-value is multiplied by the number of comparisons, and the second lowest *P*-value is multiplied by the number of comparisons minus one, and so on. This is a less conservative approach than the traditional Bonferroni adjustment (107, 108), which multiples all *t*-test *P*-values by the number of comparisons. Where a large number of data-points are collected, and therefore a large number of comparisons are made, this approach may be overly-conservative.

$$P_{cor} = P_{cal} \times (N_{tot} - N_{pri})$$

Equation 1. where P_{cor} = corrected *P* value, P_{cal} = *P* value calculated in *t*-test, N_{tot} = total number of comparisons, and N_{pri} = number of previous comparisons made. Note that this test is conducted in a stepwise manner whereby the comparison of highest significance is corrected first (in which case $N_{pri} = 0$).

The *P*-value provides no information regarding the magnitude of any statistically significant effect (85, 348). Thus, in the present thesis computation of Cohen's *d* effect

sizes (ES) are used in some cases (83, 100) and expressed ± 95% confidence intervals, which, in this case, reflects the range of ES with which the reader can be 95% confident the 'true' ES value resides (94). Cohen's *d* is a ratio of the mean difference, or effect, against the standard deviation, and is typically defined by Cohen's arbitrary criteria: 0.2-0.5, small; 0.5-0.8, moderate; >0.8, large (83). However, it is important to recognise that these criteria for small, moderate, and large effects are in fact arbitrary, and do not necessarily constitute small, moderate, or large effects in terms of their physiological or practical meaningful. Indeed, Cohen himself was reluctant to provide these arbitrary thresholds, and instead preferred that ES was interpreted by the researcher in accordance with their own specific knowledge of their field (84). Thus, whilst Cohen's criteria are used in some cases in the present thesis, their evaluation will be based on the physiological meaning of the absolute magnitude of any observed effect in raw units.

Aside from the absence of magnitude-based information, NHT has been criticised regarding its dependence on sample size. The type II error rate, or false acceptance of the null hypothesis, can be unacceptably large with NHT when the sample size is suboptimal, whereas the type I error rate can also be large with NHT when the sample size is inappropriately large (207). These concerns make *a priori* sample size estimation a key aspect of the research process. Indeed, it has been suggested that *ad hoc* sample sizing, whereby researchers begin a study without a sample size target in mind, is a form of so-called '*P*-hacking' as researchers are unlikely to continue collecting data after a significant effect has been achieved (348). This artificially inflates the likelihood of achieving statistical significance. For instance, if an effect is measured at $P = 0.05$ with N-2 subjects, a researcher using *ad hoc* sample sizing may terminate the study and report a significant effect. However, with the addition of two further subjects to make the sample size N, it is possible that P may have exceeded 0.05, but this is not measured or reported. Conversely, if $P = 0.06$ with N subjects, a researcher using *ad hoc* sample sizing may decide to collect further data in order to discern a significant effect, and see that $P = 0.05$ with N+2. *Ad hoc* sample sizing therefore inflates the likelihood of statistical significance, as this is typically the desired outcome of researchers. Accordingly, sample size estimates were made for all experimental studies in this thesis.

A recently developed, alternative, and apparently less-conservative statistical approach to NHT is magnitude-based inferences (MBI) (30). MBI is a variety of Bayesian statistics, where inferences about the probability of an effect being beneficial, trivial, or harmful are made using the confidence interval of effect statistics (207). Based on the confidence interval, and prior determination of the smallest worthwhile effect, MBI

calculates makes a probabilistic inference that the true effect is beneficial, trivial, or harmful, assigns that probability a qualitative label, as well as applying a qualitative label to the magnitude of the observed effect (30). For instance, I have previously reported that the effect of ice slurry ingestion compared to cold fluid ingestion during 40-km cycling time-trial performance under heat stress may be *likely harmful* and of *small* magnitude (315). For physiological measures, where the direction of an effect does not necessarily infer *benefit* or *harm*, these labels can be replaced with *positive* or *negative*. Therefore, a clear strength of MBI is that the reader is given both a probabilistic inference regarding the likelihood of any observed effect, as well as its magnitude. It has been suggested that use of this approach deflates the type II error rate with the small sample sizes often seen in exercise physiology research (207).

However, like the more established NHT, MBI has been criticised extensively in the sports science literature. This – the publication and debate of MBI in *sports science* rather than the statistics literature – is in itself a criticism of MBI, which some authors feel has not been suitably exposed to the more sophisticated critique that could be provided by true statistical experts (63, 424), and may instead have been debated with an “us-and-them” rhetoric that moves away from evidence-based investigation (425). Regardless, published critiques of MBI have raised concerns over several fundamental aspects. Firstly, Barker and Schofield (23) demonstrated that Batterham and Hopkins’ (30) claim that MBI is part-Bayesian but makes no prior assumptions about the true value of the outcome parameter is in fact incorrect, and that an implicit prior is used. The proponents of MBI suggest that the prior used is uniform, and thus gives equal probability to all possible parameter values (30, 207). However, this may not be true in many situations in which MBI is used, including presently, where small sample sizes ($N < 20$) are the norm and non-parametric distributions are possible (23, 63, 500). This is a valid concern given a key purported benefit of utilising MBI is the deflated type II error rate with suboptimal sample sizes (207). Indeed, a recent simulation study demonstrated MBI exhibits much larger type I error rates in small sample size datasets than conventional NHT (424).

Due to the considerable strengths and weaknesses of NHT *and* MBI, it can be difficult to determine the most appropriate statistical paradigm, particularly for scientists specialist in fields other than statistics (72). Therefore, it might be argued that it is sensible to retain use of the more established, conservative, and transparent NHT approach, and interpret outputs in the context of its flaws, until a theoretically-sound method that advances on these flaws has been developed and demonstrated through rigorous simulations studies. Therefore, NHT is adopted in the present thesis, and interpreted with acknowledgement of its flaws, particularly with small sample sizes.

Philosophically, adoption of the more conservative NHT paradigm over MBI aligns with a more cautious scientific approach, and desire to make reduce type I statistical errors, while acknowledging the greater likelihood of type II errors, within the constraints of non-specialist statistical knowledge. Interestingly, a viewpoint gathering momentum in the medical and basic science literature is that the *P*-value threshold of 0.05 may in fact *not be conservative enough*, and that tightening this threshold is necessary to reduce the number of false-positives or type I errors in science (33). Indeed, a highly controversial article published in 2005 suggested that most published findings are in fact false, a disheartening suggestion based on researcher bias, independent laboratories working on the same research questions, and small-scale studies (223). Therefore, movement towards a *less* conservative approach than NHT, *i.e.* MBI, does come with legitimate concern, particularly given the debated statistical ground on which it resides (424, 425).

A further concern with MBI, related specifically to the metabolic focus and design of the proposed thesis, is control of the family-wise error rate, or inflation of the type I error rate when multiple comparisons are made. Typical descriptions of the use of MBI relate to single effects, *e.g.* a single intervention-induced performance measure, such as time-trial performance in my previous work (315). However, the proposed thesis seeks to identify physiological differences between-trials, which, aside from acknowledged difficulties associated with assigning the smallest worthwhile change for physiological measures necessary in MBI (60), consists of multiple-comparisons of the same variable. For example, in the proposed thesis, whole-body CHO oxidation rates are compared during prolonged exercise performed under environmental heat stress and in temperate conditions. A rate will be measured and compared between-trials at a number of exercise time-points, *e.g.* 15, 30, 45, and 60 min. This elevates the risk of type I error, as the alpha value generated from each *t*-test is essentially additive. Thus, post-hoc correction through the Holm-Bonferroni stepwise approach is adopted in order to deflate this elevated type I error risk. It does not appear that MBI accounts for multiple-comparisons, and so would see a further elevated risk of type I error in the present thesis, where multiple-comparisons are made for physiological variables on many occasions.

The aforementioned concern over use of an implicit, supposedly uniform prior with MBI (30, 207), that in fact may be flawed with small or non-parametric samples (23, 63, 500), is a further concern specific to the present thesis. Given the difficulties associated with recruitment and retention in these types of studies, small sample sizes, certainly $N < 20$, are commonplace in the literature and included in these experimental studies. Indeed, *a priori* estimates in the present thesis demonstrated these sample sizes of

>20 were not even required to detect a significant effect in the main outcome measures with 80% statistical power, and so it could be considered unethical to recruit beyond this in order to match the sample size requirements to justify use of MBI.

Perhaps the most fundamental theoretical benefit of using MBI over NHT is the ability to make inferences about the magnitude of any observed effect (30, 60, 207). If statistically sound, this is a key advantage given the importance effect magnitude is likely to define its relevance in real-world, applied contexts (60). It is for this reason that Cohen's *d* effect sizes are used in the present thesis, presented in accordance with Cohen's arbitrary criteria (83), and then discussed in the context of the specific physiological and metabolic background, which is in accordance with Cohen's own wishes (84). Therefore, inferences about the magnitude of effects cautiously identified through NHT can be made.

Therefore, in the present thesis, NHT is used to detect the existence of statistical differences between various conditions, although resultant *P*-values are interpreted in the context of the aforementioned limitations. Insight into the magnitude of observed differences are sought using Cohen's *d* effect sizes, raw units, and percentage changes, where appropriate. The specific statistical approaches used are detailed in the methods sections of each individual experimental study.

5 Acute studies: In what situations might environmental heat stress be expected to impact substrate metabolism during acute endurance exercise?

5.1 Introduction

Environmental heat stress exerts stimulatory effects on CHO metabolism during endurance exercise (Chapter 3.1). When exercise is performed in a hot (>30°C) compared to temperate environment, greater net muscle glycogenolysis, (121, 122, 124, 126, 242), whole-body CHO oxidation (104, 121, 122, 124, 242, 307), and hepatic glucose output (177) has been observed (

Table 4). There is compelling data to suggest that environmental heat stress exerts these acute metabolic effects through the augmented muscle temperature (119, 122, 460), catecholamine response (120, 121), and progressive dehydration (178) observed when prolonged exercise is performed under environmental heat stress compared to in temperate conditions. As aforementioned (Chapter 2.1), this is an important consideration given endogenous CHO availability can be depleted to near-zero concentrations after exercise of sufficient length and intensity (35), and has been mechanistically associated with muscle fatigue (376).

However, these responses have not always been observed (1, 148, 455, 514, 516), possibly due to imposition of an insufficient heat stress stimulus that failed to induce the thermoregulatory perturbations required to exert a stimulatory effect on CHO metabolism during exercise (1, 148, 455, 514). Indeed, the majority of studies demonstrating a stimulatory effect of heat stress on CHO metabolism during prolonged exercise evoked quite extreme heat stress (~36-40°C), particularly when coupled with high relative exercise intensities expressed as a percentage of $\dot{V}O_{2max}$ (70%$\dot{V}O_{2max}$), and the absence of convective fan cooling to simulate outdoor exercise (121, 122, 124, 177). Thus, it would be prudent to more precisely elucidate the exercise situations under which heat stress is likely to augment CHO metabolism during endurance exercise. This information would give practitioners improved details regarding the endurance training sessions and competitions in which their athletes are likely to have augmented CHO metabolism, and thus adjust their training prescriptions and/or nutritional strategies accordingly.

Three key variables that may impact the magnitude of heat stress effects on CHO metabolism during prolonged exercise that warrant consideration by athletes and practitioners are exercise intensity, exercise duration, and environmental temperature. Indeed, the literature regarding the effects of heat stress on substrate metabolism during prolonged exercise involves studies conducted at single relative exercise intensities, and the literature as a whole covers a narrow range of intensities when expressed either as %$\dot{V}O_{2max}$ (65-75%$\dot{V}O_{2max}$) (104, 121, 122, 124, 177, 307), aerobic power (W_{max}, 55%) (242), or aerobic speed (70%) (307). It is possible that the stimulatory effect of environmental heat stress on CHO metabolism is more pronounced as exercise intensity increases due to greater metabolic heat production in working skeletal muscle associated with higher energy expenditures. This might in turn elicit greater perturbations in working skeletal muscle temperature and therefore further augment CHO metabolism during exercise (119, 460). Understanding this effect has implications for endurance athletes exercising under environmental heat stress, as the stimulatory effects on CHO metabolism might be exacerbated in training sessions or

competitions characterised by more frequent or longer transient periods of high-intensity work, such as is seen during high-mountain or time-trial stages of Grand Tour cycling races (295).

An insufficient heat stress stimulus might be used to explain the studies failing to observe a stimulatory effect of heat stress on CHO metabolism during prolonged exercise (1, 118, 148, 455, 514); however, the heat stress stimulus and thermoregulatory perturbations required to exert stimulatory effects on CHO metabolism during exercise are not known. Much like exercise intensity (104, 121, 122, 124, 177, 242, 307), studies in this field have exclusively compared a single heat stress condition against a temperate environmental temperature (

Table 4). The heat stress stimulus required to evoke metabolic changes during exercise cannot be discerned from this experimental design, nor can it be determined if observed effects are graded to the magnitude of the environmental temperature. This data would be useful in helping endurance athletes competing under environmental heat stress determine what the specific conditions under which augmented CHO metabolism might be expected during competition.

Lastly, the existing literature cannot identify how CHO metabolism changes over time within an exercise bout performed under environmental heat stress, or if this differs compared to the kinetics of substrate metabolism during endurance exercise performed in a temperate environment (

Table 4). When prolonged exercise is performed in temperate conditions, CHO metabolism progressively declines, whereas fat metabolism progressively increases, at a constant workload (496). This is likely an effect of the progressive reduction in muscle glycogen content, given muscle glycogen concentration has an autoregulatory effect on its own oxidation (179). However, when prolonged exercise is performed under environmental heat stress, thermoregulatory variables known to stimulate CHO metabolism, such as core temperature, muscle temperature, circulating adrenaline, and hypohydration, progressively increase (121, 122, 157, 158, 177). It might therefore be hypothesised that the CHO oxidation rate initially *increases* during prolonged exercise performed under environmental heat stress, before declining when these inputs are outweighed by the reduction in muscle glycogen content. Elucidating the influence of environmental heat stress on this relationship would again provide endurance athletes with more information regarding the precise situations during which environmental heat stress influences substrate metabolism during prolonged exercise.

Thus, the aim of the series of acute studies within this chapter was to investigate the following research questions:

1. Does the intensity of exercise impact the acute metabolic effect of environmental heat stress?
2. Is the acute metabolic effect of environmental heat stress on substrate metabolism during exercise graded to the environmental temperature?
3. Does the duration of exercise impact the acute metabolic effect of environmental heat stress?

5.2 Acute Study 1: Regulating effect of exercise intensity on the acute effect of heat stress on substrate oxidation rates during exercise

The purpose of Acute Study 1 was to investigate the effect of acute environmental heat stress on substrate oxidation rates across a range of exercise intensities. It was hypothesised that the stimulatory effect of environmental heat stress on CHO oxidation rates during exercise would be exacerbated by increasing exercise intensity, and that this would be explained by perturbations in estimated muscle temperature. Acute Study 1 is published in the *European Journal of Sport Science* (318).

5.2.1 Methods

5.2.1.1 Participants

Nine endurance-trained, competitive male cyclists and triathletes participated in this study (Table 6). The Auckland University of Technology Ethics Committee approved experimental procedures, and all participants provided written informed consent.

Table 6. Participant characteristics for Acute Study 1

Age (y)	37 ± 9
Height (cm)	184 ± 6
Mass (kg)	81 ± 5
$\dot{V}O_{2max}$ (L.min^{-1})	4.58 ± 0.37
Maximum fat oxidation rate (g.min^{-1})	0.65 ± 0.15
Power at $\dot{V}O_{2max}$ (W)	396 ± 28

5.2.1.2 Study design

Acute Study 1 utilised a randomised, counterbalanced, cross-over study design conducted during the non-summer months in Auckland, New Zealand. Incremental exercise tests (IET) were performed in 18 and 35°C, with two subsequent laboratory visits involving prolonged cycling in 18 and 35°C (all at 60% rH).

5.2.1.3 Experimental procedures

Participants arrived for the first IET of Acute Study 1 at ~8:00 am after an overnight fast, having kept a weighed diet record for 48-h and refrained from alcohol and vigorous exercise for 24 h. After providing written informed consent, participant

height and mass was measured and a mid-stream urine sample was used to confirm euhydration (urinary specific gravity <1.026) (19). Participants were then fitted with an antecubital venous cannula, from which a 5-mL venous blood sample was drawn. Participants then self-inserted a rectal thermometer (Monatherm Thermistor, 400 Series, Mallinckrodt Medical, St Louis, MO) ~10 cm beyond the anal sphincter for continuous determination of rectal temperature (T_{re}). A skin temperature thermistor was taped over the *vastus lateralis* ~15 cm above the patella and covered by a 6-mm-thick insulative neoprene layer for continuous estimation of muscle temperature (T_{mus}) (136). Heart rate was recorded continuously (RS800, Polar Electro Oy, Kempele, Finland).

Following baseline measurements, participants entered an environmental chamber set to 60% rH and either 18°C (IET_{temp}) or 35°C (IET_{heat}) and rested passively for 20 min. Thereafter, cycling commenced on a laboratory ergometer (Excalibur Sport, Lode, Groningen, NET) at 95 W, with the workload increasing by 35 W every 3 min. Convective air flow (~3.2 $m.s^{-1}$) was provided by an industrial fan (FS-75, FWL, Auckland, NZ). Continuous measurements of thermoregulatory variables (1 HZ; Grant Instruments, Shepreth, UK) and expired gases using indirect calorimetry (TrueOne2400, ParvoMedics, Sandy, UT, US) were obtained. A 5-mL venous blood sample was obtained after each 3-min stage. The test continued until the respiratory exchange ratio reached 1; at this point venous blood sampling ceased and workload increased by 35 $W.min^{-1}$ until volitional exhaustion. Participants returned to the laboratory at the same time of day, having adhered to the same pre-trial controls and replicated their 48-h weighed diet record, after a minimum of four days for repetition of this protocol in the remaining environmental condition. The two IETs were conducted in randomised, counterbalanced order. The two IETs were conducted in randomised, counterbalanced order. The first ventilatory threshold (VT_1) was identified as the work rate at which the ventilatory equivalent for oxygen ($\dot{V}E.\dot{V}O_2^{-1}$) began to increase in the absence of changes in the ventilatory equivalent for carbon dioxide ($\dot{V}E.\dot{V}CO_2^{-1}$), the second ventilatory threshold (VT_2) was identified as the first work rate at which $\dot{V}E.\dot{V}O_2^{-1}$ and $\dot{V}E.\dot{V}CO_2^{-1}$ increased alongside a reduction in $PetCO_2$ (296).

A minimum of four days following the second IET, participants reported to the laboratory for the first prolonged exercise test (PROL) at ~8:00 am after an overnight fast, with the same pre-trial instructions and procedures as for the IETs and having again replicated their 48-h weighed diet record. An identical 20-min passive rest

period in the environmental chamber set to 60% rH and either 18°C ($PROL_{temp}$) or 35°C ($PROL_{heat}$) followed. Cycling then commenced for 60 min at the absolute workload eliciting VT_1 in IET_{heat}. Participants consumed water *ad libitum* during PROL and convective air flow (~3.2 $m.s^{-1}$) was provided by an industrial fan (FS-75, FWL, Auckland, NZ). Thermoregulatory variables were recorded continuously, and expired gases were collected at 4-min intervals every 10 min. At exercise cessation, participants towelled down and body mass was measured to determine the degree of fluid loss, accounting for the mass of fluid consumed. Participants returned to the laboratory at the same time of day, having adhered to the same pre-trial controls and replicated their 48-h weighed diet record, after a minimum of four days for repetition of this protocol in the remaining environmental condition. The two PROL were conducted in randomised, counterbalanced order.

5.2.1.4 Gas analysis

Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) values were averaged for 30 s prior to each time-point in the IETs, and 3 min prior to each measurement time-point in PROL. Whole-body energy expenditure (EE), CHO, and fat oxidation rates were subsequently calculated according to standard equations (Equation 2) (246):

$$\text{Energy expenditure } (kcal.min^{-1}) = (0.55 \times \dot{V}CO_2) + (4.471 \times \dot{V}O_2)$$

$$\text{CHO oxidation } (g.min^{-1}) = (4.21 \times \dot{V}CO_2) - (2.962 \times \dot{V}O_2)$$

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

Equation 2. Estimates of whole-body rates of energy expenditure, CHO oxidation, and fat oxidation, where $\dot{V}O_2$ and $\dot{V}CO_2$ are in $L.min^{-1}$.

5.2.1.5 Thermoregulatory analysis

Thermoregulatory measurements were averaged over the 30 s preceding each time-point. T_{mus} was estimated using the insulated skin temperature over the *vastus lateralis* in line with recent work (Equation 3) (136):

Estimated *vastus lateralis* T_{mus} at rest = (0.597 x T_{ins}) – (0.439 x $T_{ins}Lag_2$) + (0.554 x $T_{ins}Lag_3$) – (0.709 x $T_{ins}Lag_4$) + 14.767

Estimated *vastus lateralis* T_{mus} during exercise = (T_{ins} x 0.599) – [(0.311 x $T_{ins}Lag_4$) + 15.63]

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

5.2.1.6 Plasma analysis

Venous blood samples were collected into 6-mL ethylenediaminetetraacetic acid tubes and stored on ice until trial completion. A small sample of whole blood was mixed manually using a vortex and analysed for haematocrit and haemoglobin concentration (AcT 5diff, Beckman Coulter, Miami, USA) to allow correction of plasma volume relative to baseline (103). Plasma was then isolated from the remaining whole blood by centrifugation at 1500 g in 4°C for 10 min, and stored at -80°C prior to subsequent analyses. Plasma glucose (sensitivity, 0.11 mmol.L^{-1}) and lactate (sensitivity, 0.22 mmol.L^{-1}) concentrations were determined through specific enzymatic colorimetric diagnostic assays (CV, 1.0 and 0.4%, respectively) on a Roche Diagnostics automated clinical chemistry analyser (cobas Modular P800, Roche Diagnostics New Zealand Ltd, Auckland, NZ).

5.2.1.7 Statistical analysis

Sample data is expressed as mean ± standard deviation. Observed thermoregulatory, substrate oxidation, and plasma variables were compared at matched absolute workloads; the absolute workload prior to that eliciting the first ventilatory threshold under environmental heat stress (LOW, 184 ± 18 W), the first ventilatory threshold in temperate conditions (MOD, 219 ± 40 W), the second ventilatory threshold under environmental heat stress (VIG, 247 ± 25 W), and the second ventilatory threshold in temperate conditions (HIGH, 266 ± 37 W). Also, individual power vs. whole-body CHO and fat oxidation rate curves were modelled using quadratic functions to produce estimates of substrate oxidation rates at matched 'physiological' loads; specifically, the first (VT_1) and second (VT_2) ventilatory thresholds, and maximal fat oxidation rates (SPSS Statistics, v25, SPSS Inc., Chicago, IL). Two-way repeated measures analyses of variance were performed (SPSS Statistics, v25, SPSS Inc., Chicago, IL), with variance was located post-hoc using Holm-Bonferroni stepwise correction of one-tailed repeated measures *t*-tests given the prior evidence demonstrating an effect of heat stress (121, 122) in line with

recent recommendations (424). Cohen's *d* effect sizes (ES) are expressed ± 95% confidence limits. Significance was inferred when $P \leq 0.05$.

5.2.2 Results

5.2.2.1 Thermoregulatory data at matched absolute workloads

During IET, a significant main effect of trial for T_{re} was observed ($P = 0.05$), although there was no significant trial x exercise intensity interaction ($P = 0.63$). A significant x exercise intensity interaction was observed for estimated T_{mus} ($P < 0.001$). The heat stress-induced increase in estimated T_{mus} was significantly lower at HIGH compared to LOW (1.95 ± 0.84 vs. 2.29 ± 0.73°C, $P = 0.04$), but not MOD (2.13 ± 0.79°C, $P = 0.07$), intensity, but MOD was significantly lower than LOW ($P = 0.04$, Figure 3). During PROL, a main effect of trial was observed for T_{re} ($P = 0.02$), and this reached significance at 40, 50, and 60 min ($P < 0.03$). A main effect of trial was observed for estimated T_{mus} ($P < 0.0001$), and this reached significance at all time-points throughout exercise ($P < 0.002$).

5.2.2.2 Substrate oxidation data at matched absolute workloads

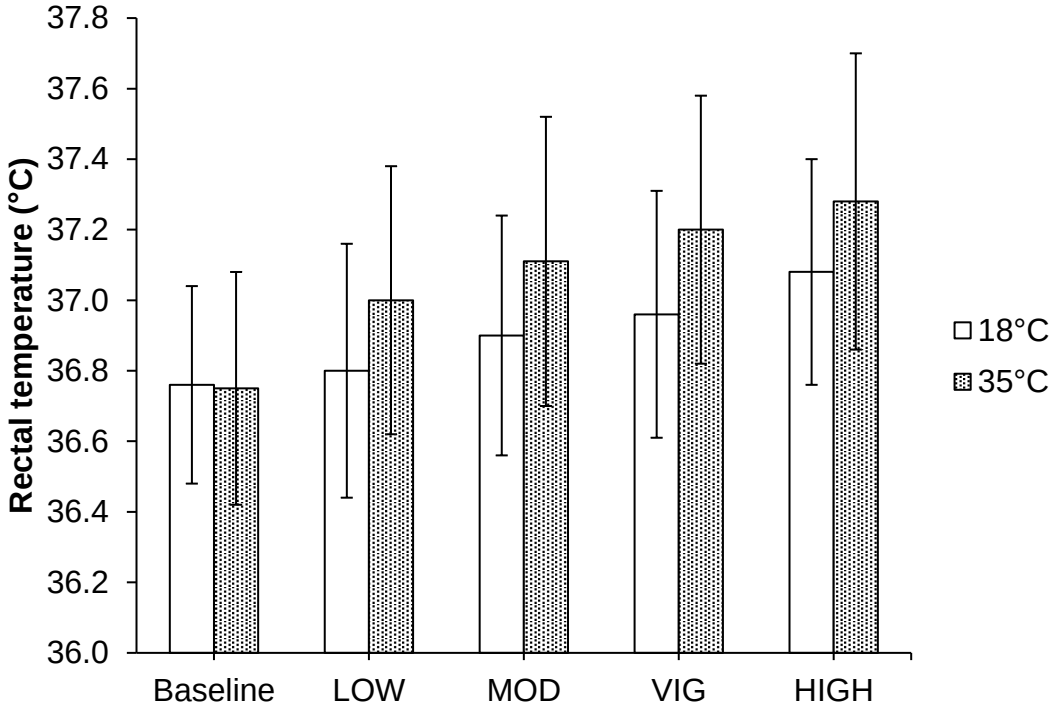
During IET, no significant main effect of trial ($P = 0.15$) or trial x exercise intensity interaction ($P = 0.88$) was observed for EE. At matched absolute workloads, a significant main effect of temperature ($P = 0.05$), and trial x exercise intensity interaction ($P = 0.0004$) was observed for CHO oxidation rate (Table 7). No significant main effect of temperature was observed for fat oxidation rate ($P = 0.20$); however, a trial x exercise intensity interaction was observed ($P = 0.001$, Table 7). Maximal fat oxidation rate was unaffected by environmental temperature (0.68 ± 0.15 vs. 0.72 ± 0.19 $g.min^{-1}$ in 18 and 35°C, respectively, $P = 0.22$).

Table 7. Whole-body CHO and fat oxidation rates at matched absolute workloads in incremental tests performed in 18 and 35°C (60% rH)

	CHO oxidation rate (g.min^{-1})				**Fat oxidation rate (g.min^{-1})**			
	LOW	**MOD**	**VIG**	**HIGH**	**LOW**	**MOD**	**VIG**	**HIGH**
18° C	1.67 ± 0.42	2.17 ± 0.70	2.79 ± 0.52	3.33 ± 0.71	0.59 ± 0.20	0.59 ± 0.20	0.50 ± 0.20	0.40 ± 0.19
35° C	1.62 ± 0.46	2.38 ± 0.80	3.12 ± 0.56*	3.89 ± 1.05*	0.66 ± 0.24	0.55 ± 0.24	0.40 ± 0.25	0.21 ± 0.31*
Δ	-0.05 ± 0.26^{bcd}	0.21 ± 0.39^{ad}	0.33 ± 0.39^{a}	0.56 ± 0.52^{ab}	0.07 ± 0.12^{bcd}	-0.04 ± 0.13^{ad}	-0.10 ± 0.14^{a}	-0.19 ± 0.21^{ab}
ES	-0.10 ± 0.43	0.27 ± 0.38	0.58 ± 0.53	0.72 ± 0.51	0.33 ± 0.43	-0.18 ± 0.43	-0.43 ± 0.48	-0.87 ± 0.74

'*' indicates significantly different vs. 18°C. '^{a}' indicates significantly different vs. LOW, '^{b}' indicates significantly different vs. MOD, '^{c}' indicates significantly different vs. VIG, '^{d}' indicates significantly different vs. HIGH. Significance was inferred when adjusted $P \leq 0.05$. Effect sizes (ES) are expressed ± 95% confidence limits.

(a)



(b)

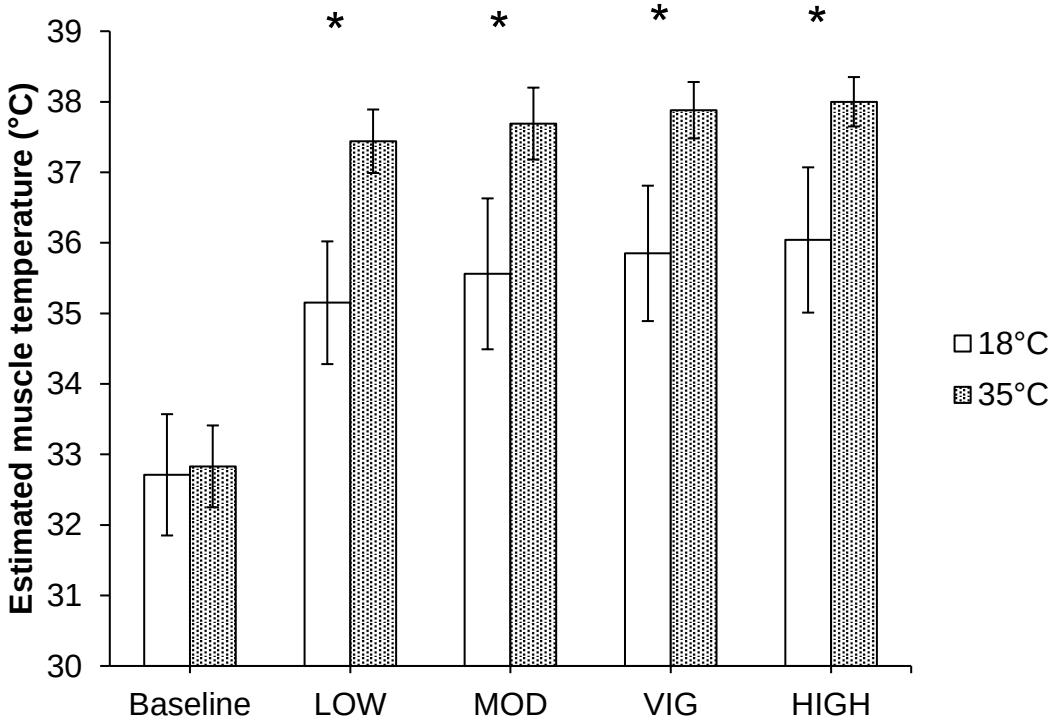
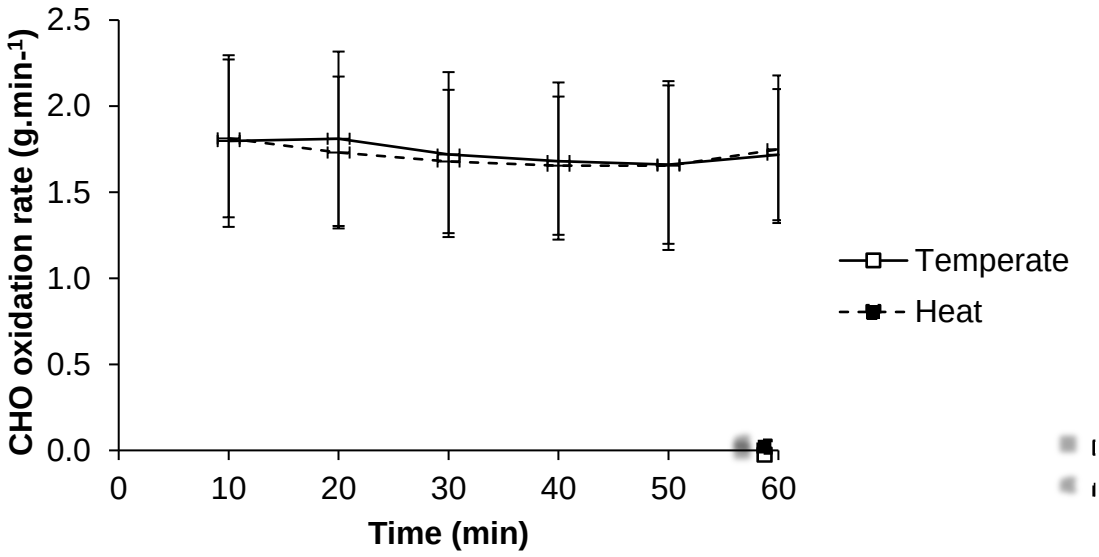


Figure 3. (a) Rectal and (b) estimated muscle temperature during incremental exercise tests performed in 18 and 35°C at baseline, and at LOW, MOD, VIG, and HIGH intensities. '*' indicates significantly different between-temperatures ($P \leq 0.05$).

During PROL (N = 9), mean CHO (1.72 ± 0.43 vs. 1.74 ± 0.46 g.min^{-1} in $PROL_{heat}$ and $PROL_{temp}$, respectively, P = 0.88) and fat (0.60 ± 0.20 vs. 0.62 ± 0.21 g.min^{-1} $PROL_{heat}$ and $PROL_{temp}$, respectively, P = 0.71) oxidation rates were not significantly different between-trials (Figure 4). No significant main effect of trial or time-point was observed for substrate oxidation rates during PROL (P < 0.05). The mean CHO oxidation rate during $PROL_{heat}$ was significantly correlated with the CHO oxidation rate during IET_{heat} (r = 0.76, P = 0.02). However, 95% limits of agreement between mean CHO oxidation rate during $PROL_{heat}$ and CHO oxidation rate during IET_{heat} were wide (-0.50-0.70 g.min^{-1}).

(a)



(b)

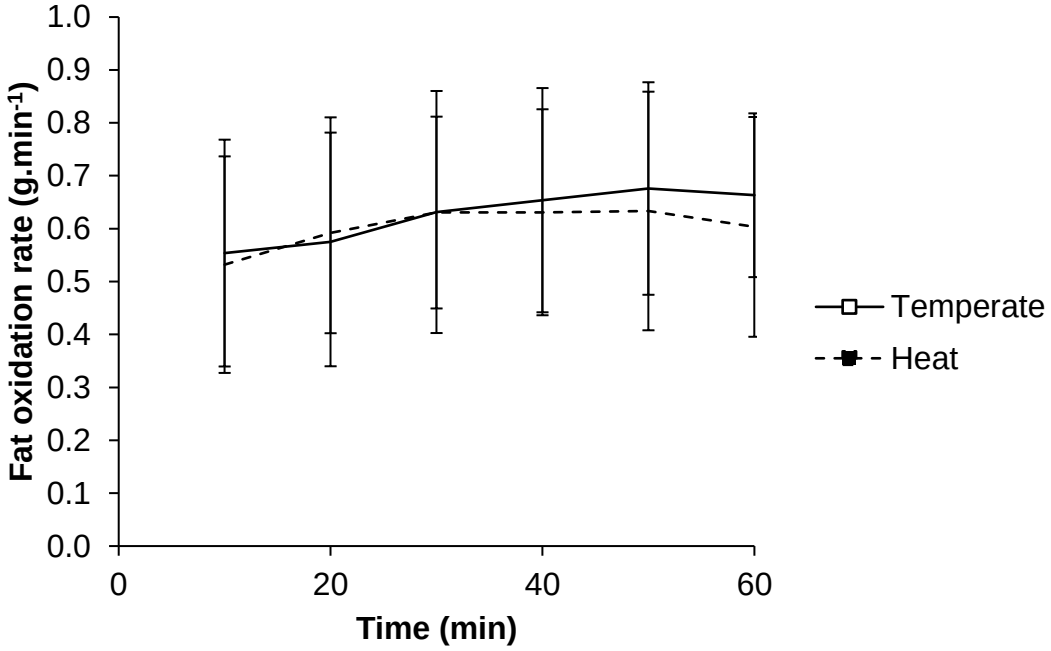


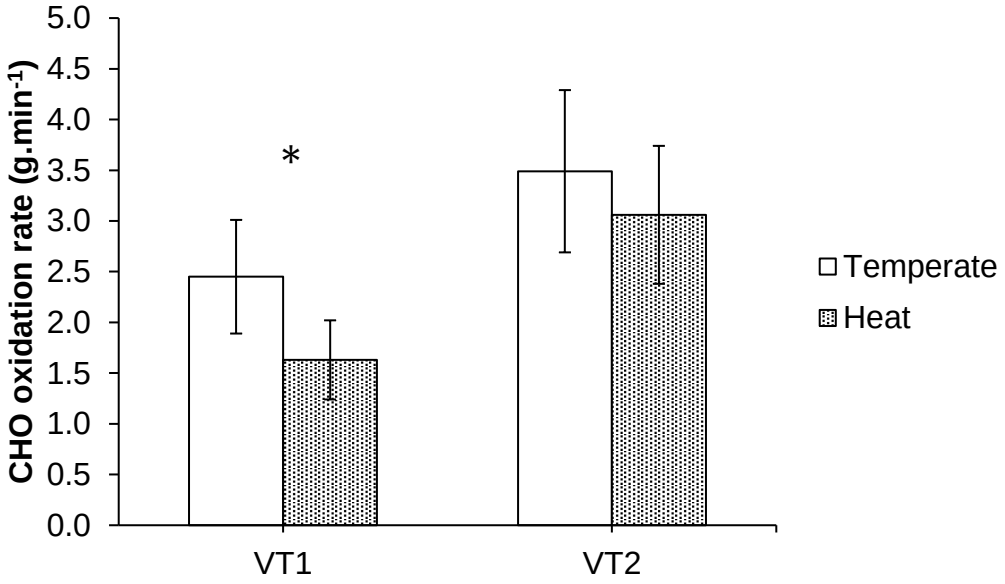
Figure 4. Whole-body (a) carbohydrate and (b) fat oxidation rates during 60-min low intensity exercise in 18 and 35°C.

5.2.2.3 Substrate oxidation data at matched physiological workloads

Individual models for CHO (18°C, r^2 = 0.98 ± 0.01, SEE = 0.24 ± 0.09; 35°C, r^2 = 0.99 ± 0.00, SEE = 0.18 ± 0.05) and fat (18°C, r^2 = 0.86 ± 0.11, SEE = 0.07 ± 0.03; 35°C, r^2 = 0.93 ± 0.05, SEE = 0.07 ± 0.02) oxidation rates during IET showed good agreement with observed data. At matched physiological workloads, significant effects of temperature were observed for CHO (*P* = 0.006) but not fat (*P* = 0.13) oxidation rates (

Figure 5). Specifically, at VT_1, modelled CHO oxidation rate was significantly lower in 35°C (1.63 ± 0.39 $g.min^{-1}$ at 184 ± 19 W) than in 18°C (2.45 ± 0.56 $g.min^{-1}$ at 225 ± 35 W, *P* = 0.01). Modelled CHO oxidation rates were not significantly different at VT_2 (3.06 ± 0.68 vs. 3.49 ± 0.80 $g.min^{-1}$ at 241 ± 35 vs. 265 ± 36 W in 35°C and 18°C, respectively, *P* = 0.06).

(a)



(b)

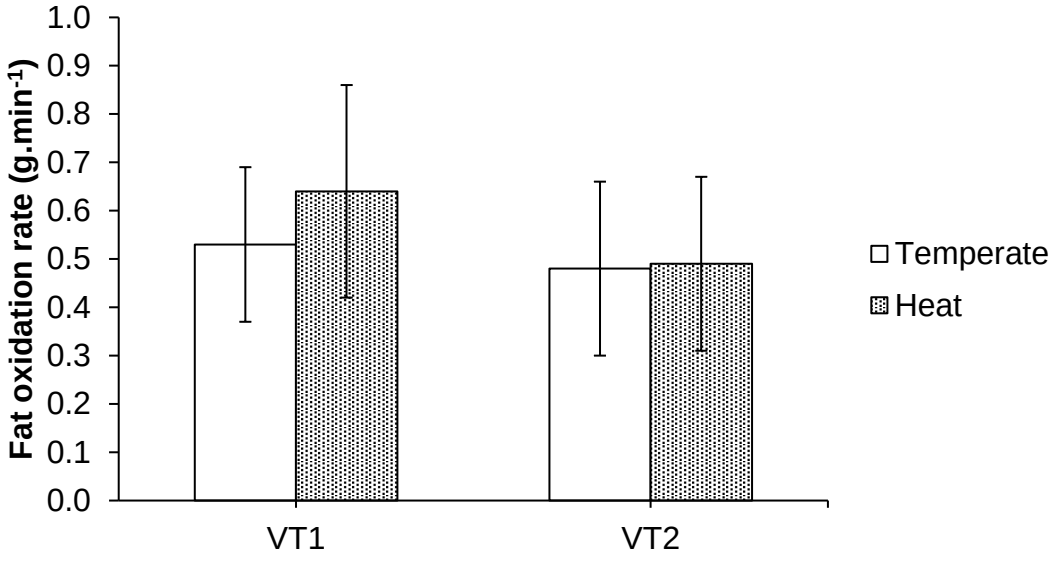


Figure 5. Modelled whole-body (a) CHO and (b) fat oxidation rates at matched physiological workloads during incremental exercise tests performed in 18 and 35°C. VT_1 occurred at 184 ± 19 W in 35°C and 225 ± 35 W in 18°C, whereas VT_2 occurred at 241 ± 35 W in 35°C and 265 ± 36 W in 18°C. '*' indicates significantly different between-environments ($P \leq 0.05$).

5.2.2.4 Plasma data at matched absolute workloads

During IET, no significant main effect of trial was observed for plasma glucose concentration (P = 0.43). A significant trial x exercise intensity interaction was observed for plasma lactate concentration (P = 0.006). The heat stress-induced increase in plasma lactate concentration was significantly greater at HIGH compared to LOW (1.21 ± 1.01 vs. 0.05 ± 0.32 mmol.L^{-1}, P = 0.04) and MOD (0.44 ± 0.51 mmol.L^{-1}, P = 0.05) intensities, and at MOD compared to LOW (P = 0.05). During PROL, no significant main effect of trial was observed for plasma glucose concentration (P = 0.10). A significant main effect of trial was observed for plasma lactate concentration (P = 0.03), but this was not significant at any specific time-point (P > 0.12).

5.2.3 Discussion

The primary aim of the present investigation was to determine if the heat stress-induced changes to CHO oxidation rates are impacted by exercise intensity. The data collected during IETs of this study provide some suggestion exercise intensity regulates the effect of heat stress on CHO oxidation rates during exercise, whereby greater effects at given absolute workloads are seen at higher relative exercise intensities (Table 7). However, when workloads were matched between-environments for physiological stress, i.e. according to environment-specific ventilatory thresholds, CHO oxidation rates may actually have been lowered by moderate environmental heat stress (Figure 5). Collectively, these data may have implications for determining the specific endurance events in which heat stress-induced changes in CHO metabolism are a relevant consideration, but needs to be confirmed in longer duration exercise and examined at different magnitudes of environmental heat stress.

Between completion of this study and submission of this thesis, a similar study was conducted elsewhere, appearing to show similar results (421). In this work, incremental cycling tests were performed in ~18 and 36°C, and main effects of environment were observed for CHO and fat oxidation rates, such that shifts towards CHO and away from fat oxidation were observed (421). Whilst environment x intensity interactions were not statistically significant for CHO (P = 0.16) or fat (P = 0.07) oxidation rates, significant between-environment differences for CHO oxidation rates did not emerge until the fourth incremental workload, which suggested an absence of differences in CHO oxidation rates at lower exercise intensities. Also, metabolic variables were compared between-environments in this study at workloads matched for percentages of temperate $\dot{V}O_{2max}$, which provide suitable comparisons within-participants, but may produce markedly different relative intensities between-participants (5, 22, 88). This is potentially influential when assessing the intensity-dependence of a given physiological response, such as the stimulatory effects of heat stress on CHO metabolism during exercise.

In the present study, during IET, the heat stress-induced increase in estimated T_{mus}, and tendency towards elevated T_{re}, may explain the observed heat stress-induced stimulation of CHO metabolism at given *absolute* workloads, but cannot explain any exercise intensity-mediated regulation of this effect. Indeed, between-trial differences in estimated T_{mus} and T_{re} were actually less pronounced at the higher absolute workloads, and were present at LOW where no between-trial difference in CHO oxidation rates was observed. It is acknowledged that the short-duration IET employed in the present investigation provides limited time for between-intensity thermoregulatory adjustments to occur, although this limitation would be expected to diminish the observed exercise

intensity-mediated heat stress-induced stimulation of CHO metabolism during exercise in the present study. As such, it is suggested that other physiological factors mediated this effect. For instance, exercise intensity may regulate either the release of catecholamines during exercise performed under heat stress, or the metabolic manifestation of elevated catecholamine concentrations, given adrenaline can exert stimulatory effects on lipolysis (153, 497, 499) as well as glycogenolysis (120, 212, 213, 408). Alternatively, or additionally, an interactive effect between exercise intensity and environmental temperature on regional blood flow during exercise is also possible. Any impairment to skeletal muscle blood flow during exercise-heat stress may only be matched by increases in plasma substrate concentrations and oxygen extraction that maintain substrate and oxygen delivery to skeletal muscle at lower exercise intensities (91, 159), resulting in greater reliance on oxidation of intramuscular substrate such as glycogen, and anaerobic ATP generation through conversion of glycolysis-derived pyruvate to lactate at higher intensities. However, circulating catecholamine concentrations and regional blood flow were not measured in the present investigation, and therefore mechanistic research into the apparent exercise intensity-mediated regulation of the effect of heat stress on CHO metabolism during prolonged exercise is warranted.

Previous research demonstrating a pronounced stimulation of CHO metabolism with heat stress has typically utilised a more extreme environmental temperature than the present investigation (40 vs. 35°C), not provided convective fan cooling to simulate outdoor cycling, which has been shown to significantly alter core and skin temperature responses to exercise heat-stress (432), and/or prescribed workloads likely of greater relative intensity (65-75%$\dot{V}O_{2max}$) (121, 122, 124, 177). Indeed, the LOW intensity workload utilised in the present study, where 35°C heat combined with convective fan cooling did not impact CHO oxidation rates, elicited only 56 ± 6%$\dot{V}O_{2max}$. Therefore, the addition of the presently collected data to the existing literature suggests the effect of moderate environmental heat stress on CHO metabolism is exercise intensity-dependent.

From a practical perspective, the results of the present investigation suggest heat stress effects on CHO metabolism during exercise may only be of relevance during endurance competitions characterised by large volumes of high-intensity *absolute* workloads, such as high-mountain or time-trial stages of Grand Tour cycling races (295). Data from the IET and PROL phases of the present investigation suggest that when cycling at low absolute workloads, such as in easy, flat stages of Grand Tour cycling races when riding within the peloton (295), CHO oxidation rates are largely unaffected by environmental heat stress. Indeed, when comparisons were instead

made between-environments at matched physiological stress – environment-specific VT_1 and VT_2 – CHO oxidation rates may even be lowered by environmental heat stress (

Figure 5). This finding has strong implications for endurance athletes training under environmental heat stress, given a likely consequence is a reduction in absolute power output during training (54) in line with the elevated physiological stress associated with given absolute workloads (231, 232, 293).

Secondly, the present data suggests whole-body fat oxidation rates are likely not meaningfully affected by moderate environmental heat stress, at least until the relative exercise intensity is high (Table 7). This is supported by the aforementioned since-published study reporting significant reductions in fat oxidation rates at 60 and 70%$\dot{V}O_{2max}$ in ~36 vs. 18°C, but not at 40 or 50%$\dot{V}O_{2max}$ or for maximal observed fat oxidation rate (0.55 ± 0.2 vs. 0.48 ± 0.2 g.min^{-1}, in ~18 and ~36°C respectively, *P* = 0.052) (421). Given that improving the capacity for fat oxidation during exercise is a training objective for many endurance athletes concerned with preserving finite endogenous CHO availability, and the possibility – in line with the training specificity principle – that use of fatty acid metabolism during training may help augment adaptations to this pathway (216), this study may partially alleviate concerns regarding use of environmental heat stress during training and rates of fatty acid metabolism. Indeed, maximal fat oxidation rates during the incremental exercise tests and whole-body fat oxidation rates during PROL were not significantly different between-environments in the present investigation, and fat oxidation rates at matched physiological workloads between-environments (VT_1 and VT_2) were again not significantly different (

Figure 5).

Thirdly, the present data suggests whilst IET performed under heat stress might not accurately predict absolute CHO oxidation rates during prolonged exercise, as evidenced by wide 95% limits of agreement between measured and predicted values based on IET_{heat}, these assessments could be used to identify those athletes who will exhibit high CHO oxidation rates during prolonged exercise under heat stress, as evidenced by the correlation between measured CHO oxidation rates during $PROL_{heat}$ and that predicted by IET_{heat} (r = 0.76, *P* = 0.02). Indeed, considerable inter-individual variation in CHO oxidation rates was observed during the heat stress IET, as well as heat stress-induced changes in CHO oxidation rates at moderate (-0.26-1.03 g.min^{-1}), VIG (-0.10-1.07 g.min^{-1}), and HIGH (-0.18-1.28 g.min^{-1}) intensities. Given the practicality of IET in elite sport settings (316), this indicates that these assessments

might be used by practitioners to determine athletes for whom heat stress-induced changes in CHO metabolism are more likely to be influential.

As described above, the short-duration IET employed in the present investigation provides limited time for between-intensity thermoregulatory adjustments to occur. Accordingly, the results of the present investigation need to be replicated during longer duration exercise before firm conclusions can be drawn regarding any exercise intensity-mediated regulation of heat stress effects on substrate oxidation rates during exercise. Similarly, it cannot be discerned from the present investigation if these effects are present at other magnitudes of environmental heat stress, given this study only compared 18 with 35°C heat. It is possible that any exercise intensity 'threshold' for heat stress to exert stimulatory effects on CHO metabolism during exercise is impacted by the magnitude of the heat stress, given the magnitude of thermoregulatory perturbation would be expected to be greater in more extreme temperatures. For instance, the MOD intensity exercise in the present study may be insufficient to elicit heat stress-induced stimulation of CHO metabolism during exercise performed at 35°C, but these effects may be observed at 40°C. These are logical future research directions that are investigated in Acute Study 2.

In summary, exercise intensity appeared to regulate the stimulation of CHO metabolism when incremental exercise was performed under environmental heat stress in the present study, with greater effects at given absolute workloads seen at higher relative intensities. When the workload was matched for physiological stress between-environments, CHO oxidation rates appeared to be *lower* under moderate environmental heat stress. These data may have implications for endurance athletes seeking to determine the specific events in which heat stress-induced stimulation of CHO metabolism is a relevant consideration. Further investigation of this effect during prolonged exercise at different magnitudes of heat stress is warranted, along with its mechanisms.

5.3 Acute Study 2: Regulating effect of environmental temperature on the acute effect of heat stress on substrate oxidation rates during exercise

Building on the main findings of Acute Study 1, which suggested that the effect of environmental heat stress on substrate oxidation rates during exercise is at least partially regulated by exercise intensity, the purpose of Acute Study 2 was firstly to establish if this effect is observed during longer duration, constant-load exercise, and secondly to determine if any exercise intensity 'threshold' for heat stress to exert stimulatory effects on CHO metabolism during exercise is impacted by the specific magnitude of the heat stress. Acute Study 2 is published alongside Acute Study 1 in the *European Journal of Sport Science* (318).

5.3.1 Methods

5.3.1.1 Participants

Eleven endurance-trained, competitive male cyclists and triathletes participated in this study (Table 8). The Auckland University of Technology Ethics Committee approved experimental procedures, and all participants provided written informed consent.

Table 8. Participant characteristics for Acute Study 2

Age (y)	39 ± 9
Height (cm)	184 ± 7
Mass (kg)	81 ± 8
$\dot{V}O_{2max}$ (L.min^{-1})	4.58 ± 0.40
Maximum fat oxidation rate (g.min^{-1})	0.76 ± 0.17
Power at $\dot{V}O_{2max}$ (W)	418 ± 40

5.3.1.2 Study design

Acute Study 2 featured an initial IET performed on a cycle ergometer in a temperate environment (18°C, 60% rH) to determine the work rate at the first (VT_1) and second (VT_2) ventilatory thresholds. In randomised, counterbalanced order using a Latin square design, participants performed 20 min of cycling exercise at VT_1 (MOD, 220 ± 19 W) immediately followed by 5 min at VT_2 (HIGH, 277 ± 19 W) in 18, 28, 34, and 40°C (60% rH).

5.3.1.3 Experimental procedures

Participants initially arrived after a >4-h fast and performed a maximal, incremental cycling test in 18°C and 60% rH. Cycling commenced at 95 W with continuous

collection of expired gases using indirect calorimetry (TrueOne2400, ParvoMedics, Sandy, UT, US) and heart rate (RS800, Polar Electro Oy, Kempele, Finland). The workload increased by 35 W every 3-min until the respiratory exchange ratio reached 1.0, after which step duration was shortened to 1 min. Again, VT_1 was defined as the work rate eliciting the first rise in the ventilatory equivalent for oxygen ($\dot{V}E.\dot{V}O_2^{-1}$), and VT_2 was identified as the work rate eliciting the first rise in the ventilatory equivalent for carbon dioxide ($\dot{V}E.\dot{V}CO_2^{-1}$) in a pro rata manner when this fell within a stage (296). Participants were provided with portable weighing scales and instructions on how to complete a 48-h weighed diet record in advance of the subsequent experimental trials.

Participants arrived for the first experimental trial at ~7:00 having fasted overnight and completed a 48-h weighed diet record and physical activity diary. On arrival, participants were asked to provide a mid-stream urine sample to confirm euhydration (urinary specific gravity <1.026) (19). Participants were weighed and then fitted with an antecubital venous cannula, from which a 5-mL venous blood sample was drawn. Participants self-inserted a rectal thermometer (Monatherm Thermistor, 400 Series, Mallinckrodt Medical, St Louis, MO) ~10 cm beyond the anal sphincter for continuous determination of rectal temperature (T_{re}), and a skin temperature thermistor was taped over the *vastus lateralis* ~15 cm above the patella and covered with a 6-mm insulative neoprene layer for estimation of muscle temperature (T_{mus}) in line with recent work (136). Heart rate was recorded continuously (RS800, Polar Electro Oy, Kempele, Finland).

Participants then entered an environmental chamber, set to 60% rH and either 18, 28, 34, or 40°C, where they sat upright on a chair for 20 min. Cycling then commenced for 20 min at the absolute workload deemed to elicit VT_1 during the incremental exercise assessment (MOD, 220 ± 19 W). Thermoregulatory variables and heart rate were recorded continuously, a 5-mL venous blood sample was obtained after 15 min, and expired gases were collected after 16 min for 4 min (TrueOne2400, ParvoMedics, Sandy, UT, US). The workload then increased to that deemed to elicit VT_2 (HIGH, 277 ± 19 W) for 5 min, during which expired gases, thermoregulatory variables, and heart rate were recorded continuously, and a 5-mL venous blood sample was obtained. These exercise intensities were adopted based on the results of Acute Study 1 where differences in the metabolic effects of heat stress were observed between MOD and HIGH, and the durations were selected to provide adequate time for metabolic steady-state and between-trial thermoregulatory perturbations to occur, whilst minimising the opportunity for thermoregulatory heat acclimation within the study. At exercise cessation, participants were removed from the environmental chamber, the antecubital

venous cannula, skin temperature thermistor, and rectal thermometer was removed. Participants towelled down and body mass was measured to determine fluid loss.

5.3.1.4 Gas analysis

Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) values were averaged for 30 s prior to each time-point in the IET, and 3 min prior to each measurement time-point in the experimental trials. Whole-body energy expenditure (EE), CHO, and fat oxidation rates were subsequently calculated according to the standard equations above (Equation 2) (246).

5.3.1.5 Thermoregulatory analysis

Thermoregulatory measurements were averaged over the 30 s preceding each time-point. T_{mus} was estimated using the insulated skin temperature over the *vastus lateralis* in line with recent work (136) using the equation above (Equation 3).

5.3.1.6 Plasma analysis

Venous blood samples were collected into 6-mL ethylenediaminetetraacetic acid tubes and stored on ice until trial completion. A small sample of whole blood was mixed manually using a vortex and analysed for haematocrit and haemoglobin concentration (AcT 5diff, Beckman Coulter, Miami, USA) to allow correction of plasma volume relative to baseline (103). Plasma was then isolated from the remaining whole blood by centrifugation at 1500 g in 4°C for 10 min, and stored at -80°C prior to subsequent analyses. Plasma glucose (sensitivity, 0.11 $mmol.L^{-1}$) and lactate (sensitivity, 0.22 $mmol.L^{-1}$) concentrations were determined through specific enzymatic colorimetric diagnostic assays (CV, 1.0 and 0.4%, respectively) on a Roche Diagnostics automated clinical chemistry analyser (cobas Modular P800, Roche Diagnostics New Zealand Ltd, Auckland, NZ). Plasma adrenaline concentration was determined through an enzyme-linked immunosorbent assay (sensitivity, 0.027 $nmol.L^{-1}$) performed in duplicate (CV, 7.1%) according to manufacturer's instructions (BI-CAT® ELISA, Diagnostika GMBH, Hamburg, GER).

5.3.1.7 Statistical analysis

Sample data is expressed as mean ± standard deviation. Absolute values as well as heat stress-induced changes (28, 34, and 40°C vs. 18°C) were used for comparison. Two-way repeated measures analyses of variance were performed (SPSS Statistics, v25, SPSS Inc., Chicago, IL), with variance was located post-hoc using Holm-Bonferroni stepwise correction of one-tailed repeated measures *t*-tests given the prior evidence demonstrating an effect of heat stress (121, 122) in line with recent recommendations (424). Associations between heat stress-induced changes in selected physiological variables and heat stress-induced changes in CHO oxidation

rate were assessed using Pearson correlation coefficients. Cohen's *d* effect sizes (ES) are expressed ± 95% confidence limits. Significance was inferred when $P \leq 0.05$.

5.3.2 Results

5.3.2.1 Mechanistic variables

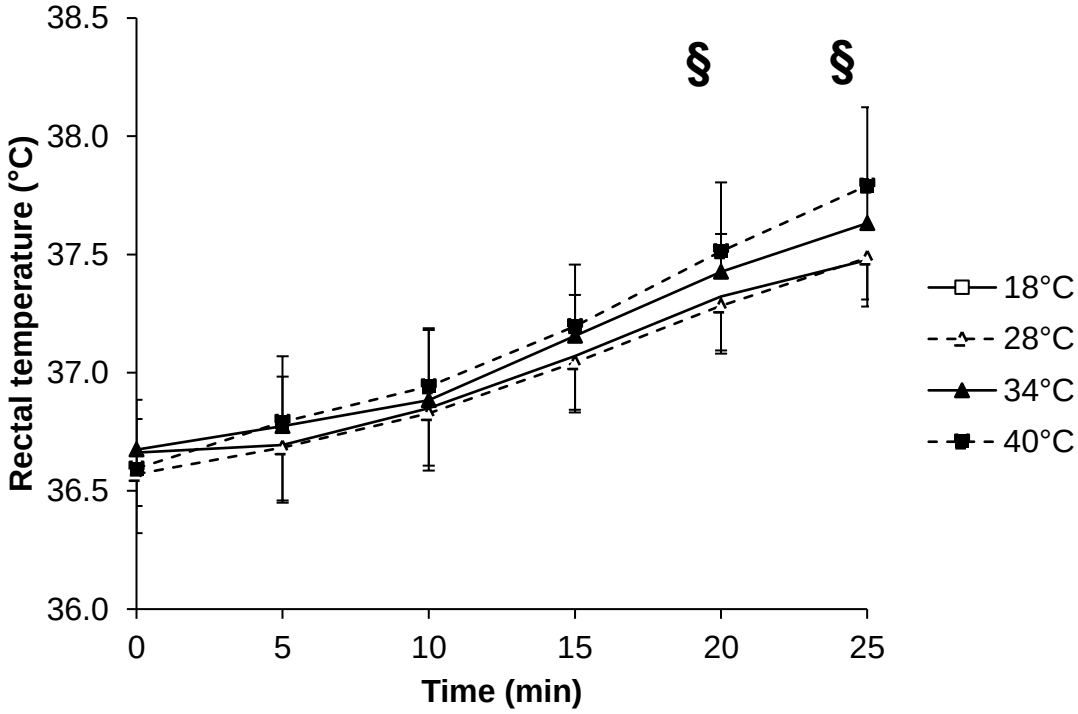
Significant trial x exercise intensity interactions were observed for T_{re}, estimated T_{mus}, and plasma adrenaline concentration ($P = 0.03$, $P < 0.0001$, and $P = 0.004$, respectively, Figure 6, Table 9). The heat stress-induced increases in rectal temperature (0.33 ± 0.28 vs. 0.16 ± 0.28°C, $P = 0.02$) and plasma adrenaline concentration (1.95 ± 1.28 vs. 0.48 ± 0.33 nmol.L^{-1}, $P = 0.01$) were significantly greater during HIGH vs. MOD exercise at 40°C, but these interactions were not significant elsewhere.

Table 9. Rectal temperature (T_{re}, °C), estimated muscle temperature (T_{mus}, °C), and plasma adrenaline concentration ([Adr], nmol.L^{-1}) during cycling exercise at moderate (220 ± 19 W) and high (277 ± 19 W) relative exercise intensities in 18, 28, 34, and 40°C in Acute Study 2

	T_{re} (°C)		**Estimated T_{mus} (°C)**		**Plasma [Adr] (nmol.L^{-1})**		**Heart rate (b.min^{-1})**	
	MOD	**HIGH**	**MOD**	**HIGH**	**MOD**	**HIGH**	**MOD**	**HIGH**
18°C	37.3 ± 0.2	37.5 ± 0.2	36.4 ± 0.8^{bcd}	36.8 ± 0.7^{bcd}	0.22 ± 0.13^{cd}	0.56 ± 0.41^{d}	140 ± 11^{bcd}	154 ± 6^{bcd}
28°C	37.3 ± 0.2^{d}	37.5 ± 0.2^{d}	37.7 ± 0.3^{acd}	37.8 ± 0.3^{acd}	0.34 ± 0.30	0.67 ± 0.38^{d}	146 ± 11^{acd}	158 ± 7^{acd}
34°C	37.4 ± 0.2	37.7 ± 0.2	38.1 ± 0.3^{abd}	38.3 ± 0.3^{abd}	0.46 ± 0.19^{a}	0.80 ± 0.50^{d}	155 ± 10^{abd}	166 ± 7^{abd}
40°C	37.5 ± 0.3^{b}	37.8 ± 0.3^{b}	38.8 ± 0.3^{abc}	39.0 ± 0.3^{abc}	0.72 ± 0.40^{a}	2.12 ± 1.55^{abc}	167 ± 7^{abc}	177 ± 6^{abc}

$P \leq 0.05$ vs. 18°C^{a}, 28°C^{b}, 34°C^{c}, and 40°C^{d} after Holm-Bonferroni correction.

(a)


38.5
38.0
37.5
37.0
36.5
36.0
Rectal temperature (°C)
0
5
10
15
20
25
Time (min)
§
§
18°C
28°C
34°C
40°C

(b)

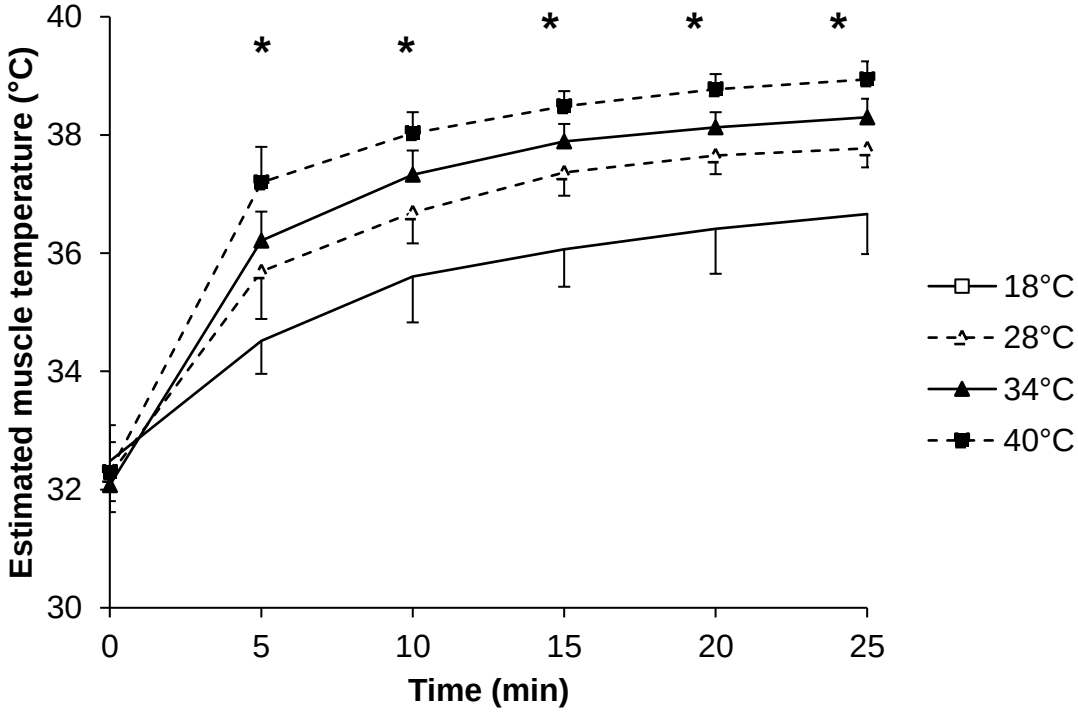

40
38
36
34
32
30
Estimated muscle temperature (°C)
0
5
10
15
20
25
Time (min)
*
*
*
*
*
18°C
28°C
34°C
40°C

Figure 6. (a) Rectal temperature (T_{re}) and (b) estimated muscle temperature (T_{mus}) responses to MOD (220 ± 19 W, 0-20 min) and HIGH (277 ± 19 W, 20-25 min) relative exercise intensities in 18, 28, 34, and 40°C in Acute Study 2. '§' denotes 40°C significantly different to 28°C ($P \leq 0.05$), '*' denotes main effect of environmental temperature whereby all conditions are significantly different to each other ($P \leq 0.05$).

5.3.2.2 Metabolic variables

A significant temperature x exercise intensity interaction was observed for CHO oxidation rate (P = 0.001). At MOD, only 40°C heat significantly elevated CHO oxidation rate compared to 18°C, whereas at HIGH this effect was evident in 34 and 40°C (Table 10). The heat stress-induced increase in CHO oxidation rate was significantly greater at HIGH compared to MOD in 40°C (0.75 ± 0.31 vs. 0.38 ± 0.38 $g.min^{-1}$, P < 0.0001, ES = 1.05 ± 0.17), and this effect approached significance in 34°C (0.32 ± 0.40 vs. 0.16 ± 0.34 $g.min^{-1}$, P = 0.08, ES = 0.71 ± 0.59, Figure 7). A significant trial x exercise intensity interaction was observed for fat oxidation rate (P < 0.0001). Fat oxidation rate was not significantly impacted by temperature at MOD, whereas 40°C heat significantly decreased fat oxidation rate compared to 18°C at HIGH (Table 10). No significant main effect of trial (P = 0.202), or trial x exercise intensity interaction (P = 0.267) was observed for EE.

Table 10. Carbohydrate (CHO) and fat oxidation rates during cycling exercise at moderate (220 ± 19 W) and high (277 ± 19 W) relative exercise intensities in 18, 28, 34, and 40°C in Acute Study 2

	CHO oxidation rate (g.min^{-1})		**Fat oxidation rate (g.min^{-1})**	
	MOD	**HIGH**	**MOD**	**HIGH**
18°C	2.25 ± 0.65^{d}	3.38 ± 0.40^{cd}	0.64 ± 0.29	0.49 ± 0.26^{d}
28°C	2.29 ± 0.74^{d}	3.56 ± 0.60	0.63 ± 0.29	0.41 ± 0.28
34°C	2.41 ± 0.79	3.74 ± 0.74^{a}	0.64 ± 0.31	0.37 ± 0.34
40°C	2.64 ± 0.77^{ab}	4.13 ± 0.47^{a}	0.50 ± 0.33	0.19 ± 0.24^{a}

P ≤ 0.05 vs. 18°C^{a}, 28°C^{b}, 34°C^{c}, and 40°C^{d} after Holm-Bonferroni correction.

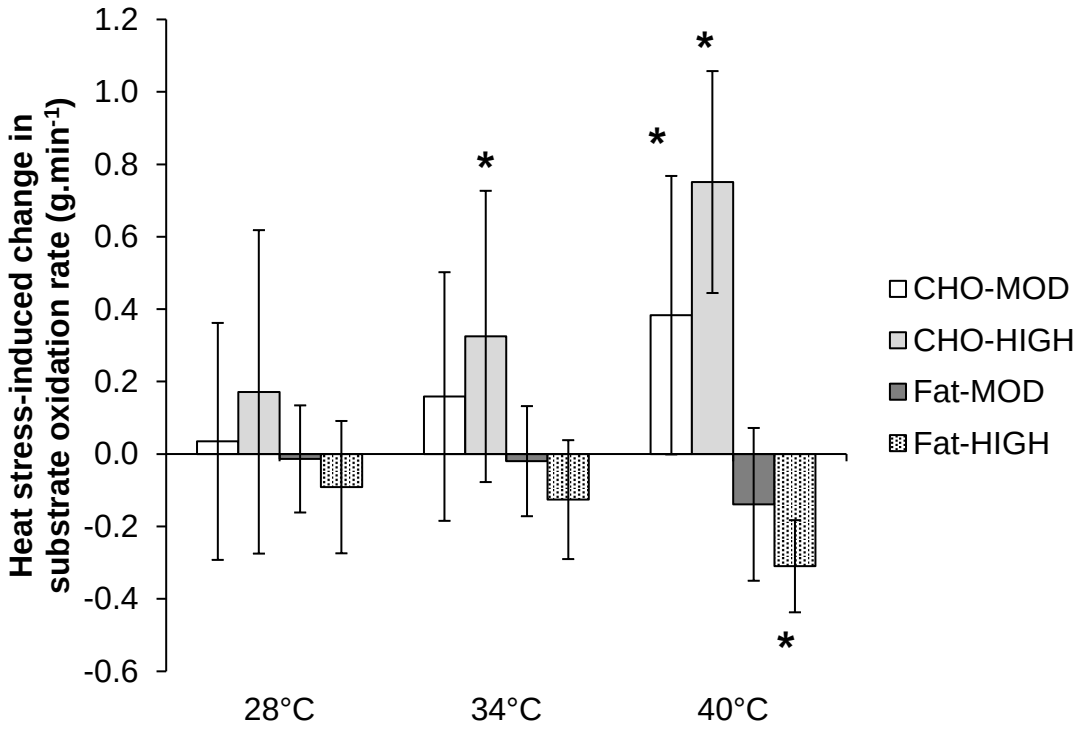


Figure 7. Heat stress-induced change in carbohydrate (CHO) and fat oxidation rates (vs. 18°C) at MOD (220 ± 19 W) and HIGH (277 ± 19 W) relative exercise intensities in 28, 34, and 40°C in Acute Study 2. '*' denotes a significant change vs. 18°C ($P \leq 0.05$).

There was no significant main effect of trial (P = 0.18), exercise intensity (P = 0.255), or trial x exercise intensity interaction (P = 0.09) for plasma glucose concentration. A significant trial x exercise intensity interaction was observed for plasma lactate concentration (P = 0.03). Specifically, the heat stress-induced increase in plasma lactate concentration was significantly greater at HIGH compared to MOD in 40°C (1.86 ± 0.92 vs. 1.17 ± 1.28 mmol.L^{-1}, P = 0.004), but these interactions were not significant elsewhere.

At MOD, the heat stress-induced increase in CHO oxidation rate tended to be related to the heat stress-induced increases in estimated T_{mus} (r = 0.33, P = 0.06) and plasma adrenaline concentration (r = 0.35, P = 0.07). At HIGH, the heat stress-induced increase in CHO oxidation rate tended to be related to the heat stress-induced increase in T_{re} (r = 0.40, P = 0.06), and was significantly correlated with the heat stress-induced increase in estimated T_{mus} (r = 0.60, P = 0.001) and plasma adrenaline concentration (r = 0.57, P = 0.01).

5.3.3 Discussion

The purpose of the present investigation was to investigate if the apparent exercise intensity-mediated regulation of heat stress effects on substrate oxidation during exercise observed in Acute Study 1 is observed during longer duration, constant-load exercise, and to determine if any exercise intensity 'threshold' for heat stress to exert stimulatory effects on CHO metabolism during exercise is impacted by the magnitude of the heat stress. The primary findings of the present investigation were that (i) evidence of exercise intensity-mediated regulation of heat stress effects on substrate oxidation during exercise was observed during longer duration, constant-load exercise was observed and (ii) the exercise intensity required for heat stress to stimulate CHO oxidation rates is lower at high environmental temperatures and, therefore, the external heat stress required to stimulate CHO oxidation rates during exercise is lower at higher relative exercise intensities. This data, in addition to Acute Study 1, therefore provides indication as to the specific combinations of exercise intensity and environmental temperature at which augmented CHO metabolism is likely to occur. Specifically, under moderate environmental heat stress (34-35°C, 60% rH), heat stress-induced changes in CHO oxidation rates are unlikely to occur unless the relative exercise intensity is high (81 ± 8%$\dot{V}O_{2max}$), whereas under more extreme environmental heat stress (40°C, 60% rH), these changes occur at more modest relative intensities (69 ± 8 % $\dot{V}O_{2max}$).

Studies investigating the acute metabolic effect of high environmental temperature during exercise have typically not provided convective fan cooling to simulate outdoor cycling (104, 121, 122, 124, 126, 177), and have been conducted at a single relative

intensity defined as a percentage of $\dot{V}O_{2max}$ or W_{max} rather than individual physiological thresholds (104, 121, 122, 124, 177, 242, 307). Whilst these studies have provided insight into the effect of heat stress on substrate metabolism during exercise (118), these designs have limited application to real-world endurance sport or capacity to identify the specific events under which CHO metabolism is likely to be stimulated by environmental heat stress. The addition of the present studies to the existing literature suggests that the effect of heat stress on whole-body CHO oxidation rates during exercise is regulated by relative exercise intensity. Heat stress-induced increases in CHO oxidation rates were greater at HIGH vs. MOD intensity during IETs performed in 35°C, as well as during MOD and HIGH intensity exercise in 34 and 40°C. Indeed, during exercise in 34°C, heat stress did not induce a significant increase in CHO oxidation rate at MOD, whereas a significant increase was observed at HIGH (Table 10). Therefore, it appears that events characterised by more frequent or longer transient periods of high-intensity work, such as is seen during high-mountain or time-trial stages of Grand Tour cycling races (295), are likely to evoke heat stress-induced stimulations of CHO metabolism at lower environmental temperatures than events characterised by lower intensity exercise, such as flat stages within the peloton (295). However, it should be acknowledged that the present investigations were performed in the fasted state, and so repetition of these results in the fed state in which athletes perform endurance competitions is required before firm recommendations can be made for real-world athletes.

Increases in T_{mus} with heat stress in the present investigations likely contributed to observed heat stress-induced stimulation of CHO oxidation rates *per se* (119, 122, 460), but not exercise intensity-mediated regulation of this effect, as larger changes in estimated T_{mus} were not seen at HIGH vs. MOD during IETs in Acute Study 1 or constant-load exercise in Acute Study 2 (Table 9), although it should be acknowledged that muscle temperature was estimated using a previously validated non-invasive technique and not directly (136). However, the present data does suggest it is possible that exercise intensity-mediated regulation of heat stress-induced changes in CHO oxidation rates could be at least partially attributed to exercise intensity-dependent effects of heat stress on plasma adrenaline concentrations, which are known to be influential in heat stress-induced stimulation of CHO metabolism (120, 121). Indeed, the heat stress-induced increase in plasma adrenaline concentration was significantly greater at HIGH vs. MOD in 40°C (1.95 ± 1.28 vs. 0.48 ± 0.33 $nmol.L^{-1}$, $P = 0.01$), where heat stress-induced changes in CHO oxidation rates were also significantly greater at HIGH vs. MOD (0.75 ± 0.31 vs. 0.38 ± 0.38 $g.min^{-1}$, $P < 0.0001$, Figure 7).

Other physiological factors could plausibly explain the metabolic effects observed in the present studies, such as exercise intensity-dependent effects on regional blood flow responses to exercise-heat stress. It is possible that at low intensities any reduction in skeletal muscle blood flow (159, 358) is matched by concomitant elevations in plasma substrate concentrations (158) and oxygen extraction (157, 442), whereas during high intensity exercise decreases in skeletal muscle blood flow might exceed the capacity to proportionally increase plasma substrate concentrations and oxygen extraction (159), thus reducing substrate and oxygen delivery. Theoretically, this could increase dependence on intramuscular substrate metabolism, such as muscle glycogenolysis and utilisation of the pyruvate-lactate pathway, and contribute to the greater heat stress-induced increases in CHO oxidation in the present studies at higher intensities. This hypothesis might be supported by the significant interaction between environmental temperature and exercise intensity for plasma lactate concentration in Acute Study 2. However, direct investigation of this hypothesis using blood flow measures would be required to make firm conclusions.

Acute Study 2 also supports the hypothesis that a minimum magnitude external heat stress is required before heat stress elicits stimulatory effects on CHO metabolism during exercise *per se*. Indeed, 28°C heat was insufficient to influence CHO oxidation at MOD or HIGH (Table 10), despite causing a significant increase in estimated T_{mus} at both intensities (Table 9). Weak linear relationships were evident between the heat stress-induced increase in estimated T_{mus} and plasma adrenaline concentration and the increase in CHO oxidation rate at MOD, with stronger relationships observed at HIGH, which suggests a grading of heat stress-induced elevations in CHO oxidation rates during exercise to the magnitude of the perturbation in these parameters. However, both of these variables were significantly elevated in this study at time-points in which augmented CHO oxidation rates were not seen in the cohort as a whole (Table 9). Therefore, this data suggests that a minimum increase in external and/or internal heat stress is required before an augmented CHO metabolism is observed, which, again, might itself be influenced by exercise intensity (Table 10).

It is possible that the apparently absent effect of moderate environmental heat stress on CHO oxidation rates during MOD *per se* observed during the present study (Table 10) was an artefact of the still relatively short exercise duration (20 min). A longer exercise duration may give opportunity for larger heat stress-induced perturbations in physiological variables relevant to substrate metabolism, such as core and muscle temperatures (119, 122, 460) or plasma catecholamines (120, 121). Thus, interrogation of the effect of moderate environmental heat stress on substrate oxidation rates during

moderate-intensity exercise of longer duration, along with the time-course of any differences during an exercise bout, is still warranted.

In summary, the results of the present investigations indicate that the stimulatory effect of heat stress on CHO oxidation during exercise is regulated by relative exercise intensity, possibly attributable to intensity-dependent effects of heat stress on circulating adrenaline concentrations, and align with the hypothesis that the exercise intensity required for heat stress to stimulate CHO oxidation rates is lower at higher environmental temperatures and, therefore, the external heat stress required to stimulate CHO oxidation rates during exercise is lower at higher relative exercise intensities. This data is valuable when seeking to elucidate if a given endurance event performed under environmental heat stress is likely to influence CHO oxidation rates, although caution should be used when generalising the present data to other exercise protocols.

5.4 Acute Study 3: Regulating effect of exercise duration on the acute effect of heat stress on substrate oxidation rates during exercise

Building on Acute Study 1 and 2, which appeared to show that moderate environmental heat stress did not exert stimulatory effects on CHO oxidation rates during short-duration exercise at moderate-intensities, the purpose of Acute Study 3 was therefore to investigate if moderate environmental heat stress elicits stimulatory effects on CHO oxidation rates during moderate-intensity exercise when exercise duration is more prolonged, and therefore if the absence of effects seen in Acute Study 1 and 2 can plausibly be explained by an insufficient exercise duration. This study is therefore intended to provide further indication as to the specific combinations of exercise and environmental temperature at which augmented CHO metabolism is likely to occur.

5.4.1 Methods

5.4.1.1 Participants

Eight endurance-trained, competitive male cyclists and triathletes participated in this study (Table 11). The Auckland University of Technology Ethics Committee approved experimental procedures, and all participants provided written informed consent.

Table 11. Participant characteristics for Acute Study 3

Age (y)	33 ± 8
Height (cm)	179 ± 7
Mass (kg)	76 ± 10
$\dot{V}O_{2max}$ (L.min^{-1})	4.20 ± 0.56
Maximum fat oxidation rate (g.min^{-1})	0.59 ± 0.14
Power at $\dot{V}O_{2max}$ (W)	384 ± 45

5.4.1.2 Study design

Acute Study 3 featured an initial IET performed on a cycle ergometer in temperate conditions (18°C, 60% rH) to determine the work rate at the first ventilatory threshold (VT_1). In randomised, counterbalanced order, participants performed 72 min of constant-load cycling in 18 and 36°C (60% rH). This involved three repetitions of 8 min at 50% of the power at VT_1 and 16 min at 100% of the power at VT_1.

5.4.1.3 Experimental procedures

Participants initially arrived after a >4-h fast and performed a maximal, incremental cycling test in 18°C and 60% rH. Cycling commenced at 95 W with continuous

collection of expired gases using indirect calorimetry (TrueOne2400, ParvoMedics, Sandy, UT, US) and heart rate (RS800, Polar Electro Oy, Kempele, Finland). The workload increased by 35 W every 3-min until the respiratory exchange ratio reached 1.0, after which step duration was shortened to 1 min. Again, VT_1 was defined as the work rate eliciting the first rise in the ventilatory equivalent for oxygen ($\dot{V}E.\dot{V}O_2^{-1}$) in a pro rata manner when this fell within a stage (296). Participants were provided with portable weighing scales and instructions on how to complete a 48-h weighed diet record in advance of the subsequent experimental trials.

Participants arrived for the first experimental trial at ~7:00 having fasted overnight and completed a 48-h weighed diet record and physical activity diary. On arrival, participants were asked to provide a mid-stream urine sample to confirm euhydration (urinary specific gravity <1.026) (19). Participants were weighed and then self-inserted a rectal thermometer (Monatherm Thermistor, 400 Series, Mallinckrodt Medical, St Louis, MO) ~10 cm beyond the anal sphincter for continuous determination of rectal temperature (T_{re}), and a skin temperature thermistor was taped over the *vastus lateralis* ~15 cm above the patella and covered with a 6-mm insulative neoprene layer for estimation of muscle temperature (T_{mus}) in line with recent work (136). Heart rate was recorded continuously (RS800, Polar Electro Oy, Kempele, Finland).

Participants then entered an environmental chamber, set to 60% rH and either 18 or 36°C, where they sat upright on a chair for 20 min. Cycling then commenced for 8 min at 50% of the absolute workload deemed to elicit VT_1 during the incremental exercise assessment (LOW), followed by 16 min at the absolute workload deemed to elicit VT_1 during the incremental exercise assessment (MOD). This sequence was repeated three times. Thermoregulatory variables and heart rate were recorded continuously and expired gases were collected for 4 min every 8 min (TrueOne2400, ParvoMedics, Sandy, UT, US).

This specific exercise protocol was adopted based on my observations in Acute Study 1 and 2. I was surprised by the absence of a statistically significant effect of moderate environmental heat stress (35 and 34°C) on CHO oxidation rates during exercise at moderate intensity, specifically the absolute power eliciting VT_1 during a preliminary assessment in 18°C (Table 7, Table 10). Accordingly, I wanted to extend the duration of this moderate-intensity exercise under moderate environmental heat stress, to see if these surprising observations could be explained by insufficient exercise duration and therefore time for thermoregulatory disparities to appear between-conditions. The environmental temperature was selected to be at the upper-end of the arbitrarily defined moderate environmental heat stresses commonly encountered during

endurance sport, and similar to those adopted in Acute Study 1 (35°C) and 2 (34°C). The specifics of the protocol were settled based on pilot testing, and sought to be consistently tolerable in unacclimated individuals. The interspersed periods of lower intensity exercise were intend to extend the total exposure and time spent at moderate intensity, without resulting in an acute reduction in thermoregulatory variables such as T_{re}.

At exercise cessation, participants were removed from the environmental chamber, and the skin temperature thermistor and rectal thermometer was removed. Participants towelled down and body mass was measured to determine fluid loss.

5.4.1.4 Gas analysis

Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) values were averaged for 30 s prior to each time-point in the IET, and 3 min prior to each measurement time-point in the experimental trials. Whole-body energy expenditure (EE), CHO, and fat oxidation rates were subsequently calculated according to the standard equations above (Equation 2) (246).

5.4.1.5 Thermoregulatory analysis

Thermoregulatory measurements were averaged over the 30 s preceding each time-point. T_{mus} was estimated using the insulated skin temperature over the *vastus lateralis* in line with recent work (136) using the equation above (Equation 3).

5.4.1.6 Statistical analysis

Sample data is expressed as mean ± standard deviation. Two-way repeated measures analyses of variance were performed (SPSS Statistics, v25, SPSS Inc., Chicago, IL), with variance was located post-hoc using Holm-Bonferroni stepwise correction of one-tailed repeated measures *t*-tests given the prior evidence demonstrating an effect of heat stress (121, 122) in line with recent recommendations (424).

5.4.2 Results

5.4.2.1 Thermoregulatory data

Significant main effects of trial and time-point, and trial x time-point interactions were observed for T_{re} and estimated T_{mus} ($P < 0.05$, Table 12). Holm-Bonferroni corrected *t*-tests were unable to locate between-trial variance in T_{re} post-hoc ($P > 0.05$). In both temperatures, T_{re} continued to rise from the end of repetition one, with T_{re} after repetition two greater than after repetition one, and T_{re} after repetition three greater than after repetition two (in all cases, corrected $P < 0.05$). The exercise-induced increase in T_{re} was only greater in 36 vs. 18°C after repetition three, and with an uncorrected *P* value (1.83 ± 0.60 vs. 1.49 ± 0.37°C, $P = 0.04$).

Estimated T_{mus} was greater in 36 vs. 18°C throughout exercise ($P < 0.05$). Estimated T_{mus} did not continue to rise after repetition one in either trial, with estimated T_{mus} after repetitions one, two, and three not significantly different (in all cases, $P > 0.05$). The exercise-induced increase in estimated T_{mus} was greater in 36 vs. 18°C after repetition one (6.02 ± 0.43 vs. 4.83 ± 1.01°C, $P = 0.02$), two (6.03 ± 0.39 vs. 5.04 ± 1.28°C, $P = 0.02$), and three (5.96 ± 0.57 vs. 5.00 ± 1.14°C, $P = 0.02$).

5.4.2.2 Metabolic data

At LOW intensity, a significant main effect of time-point was observed for whole-body CHO oxidation rate ($P = 0.03$), alongside a significant trial and time-point interaction ($P = 0.03$). Specifically, whole-body CHO oxidation rate at LOW in 18°C significantly decreased from repetition one to repetition two and three ($P = 0.02$), but not from repetition two to repetition three ($P = 0.49$). Within-trial effects were not observed in 36°C (in all cases, $P > 0.22$). There was no significant main effect of trial on whole-body fat oxidation rate at LOW ($P = 0.24$), but a within-trial effect was observed ($P = 0.02$). Specifically, whole-body fat oxidation rate at LOW in 18°C was significantly greater in repetition three vs. repetition one ($P = 0.03$). Within-trial effects were not observed in 36°C (in all cases, $P > 0.26$). No significant within- or between-trial effects on EE at LOW were observed ($P > 0.21$).

No significant between- or within-trial effects were observed for whole-body CHO ($P > 0.34$) or fat ($P > 0.32$) oxidation rates at MOD (

Table 12. Rectal temperature (T_{re}) and estimated muscle temperature (T_{mus}) during three repetitions of cycling exercise (8 min at low intensity, 16 min at moderate intensity) performed in 18 and 36°C in Acute Study 3 (N = 8). '*' indicates significantly different between-trials ($P \leq 0.05$)

		Rep 1 Low	**Rep 1 Moderate**	**Rep 2 Low**	**Rep 2 Moderat**
T_{re}	18°C	36.92 ± 0.35	37.28 ± 0.35	37.50 ± 0.33	37.84 ± 0
(°C)	36°C	36.76 ± 0.24	37.31 ± 0.32	37.64 ± 0.37	38.08 ± 0
Estimated T_{mus}	18°C	34.36 ± 0.62	36.42 ± 0.74	36.51 ± 0.79	36.62 ± 0
(°C)	36°C	36.60 ± 0.49*	38.12 ± 0.17*	38.01 ± 0.19*	38.13 ± 0

Table 13). No significant main effects of trial were observed for EE at MOD (P = 0.25), although there was an effect for repetition number (P = 0.001). Specifically, EE significantly increased at MOD in 36°C from repetition one to two (P = 0.05) and three (P = 0.001), but not between repetition two and three (P = 0.70).

Table 12. Rectal temperature (T_{re}) and estimated muscle temperature (T_{mus}) during three repetitions of cycling exercise (8 min at low intensity, 16 min at moderate intensity) performed in 18 and 36°C in Acute Study 3 (N = 8). '*' indicates significantly different between-trials ($P \leq 0.05$)

		Rep 1 Low	**Rep 1 Moderate**	**Rep 2 Low**	**Rep 2 Moderate**	**Rep 3 Low**	**Rep 3 Moderate**
T_{re}	18°C	36.92 ± 0.35	37.28 ± 0.35	37.50 ± 0.33	37.84 ± 0.38	37.82 ± 0.43	37.93 ± 0.47
(°C)	36°C	36.76 ± 0.24	37.31 ± 0.32	37.64 ± 0.37	38.08 ± 0.40	38.22 ± 0.47	38.42 ± 0.54
Estimated T_{mus}	18°C	34.36 ± 0.62	36.42 ± 0.74	36.51 ± 0.79	36.62 ± 0.96	36.44 ± 1.06	36.59 ± 0.81
(°C)	36°C	36.60 ± 0.49*	38.12 ± 0.17*	38.01 ± 0.19*	38.13 ± 0.30*	38.05 ± 0.24*	38.06 ± 0.33*

Table 13. Whole-body substrate oxidation rates during three repetitions of cycling exercise (8 min at low intensity, 16 min at moderate intensity) performed in 18 and 36°C in Acute Study 3 (N = 8)

		Rep 1 Low	**Rep 1 Moderate**	**Rep 2 Low**	**Rep 2 Moderate**	**Rep 3 Low**	**Rep 3 Moderate**
CHO oxidation	18°C	1.06 ± 0.31	2.05 ± 0.48	0.85 ± 0.20	2.00 ± 0.45	0.82 ± 0.15	2.00 ± 0.45
($g.min^{-1}$)	36°C	0.81 ± 0.19	2.00 ± 0.50	0.74 ± 0.20	2.12 ± 0.77	0.79 ± 0.25	2.16 ± 0.88
Fat oxidation	18°C	0.37 ± 0.15	0.46 ± 0.19	0.45 ± 0.14	0.51 ± 0.16	0.48 ± 0.12	0.52 ± 0.17
($g.min^{-1}$)	36°C	0.46 ± 0.11	0.51 ± 0.20	0.49 ± 0.12	0.49 ± 0.22	0.49 ± 0.13	0.48 ± 0.26
EE	18°C	7.9 ± 0.6	12.8 ± 1.9	7.8 ± 0.9	13.1 ± 1.9	8.0 ± 0.9	13.2 ± 1.9
($kcal.min^{-1}$)	36°C	7.8 ± 1.0	13.0 ± 2.2	7.7 ± 0.9	13.4 ± 2.4	8.0 ± 0.9	13.5 ± 2.1

5.4.3 Discussion

The purpose of Acute Study 3 was to assess if the absence of heat-stress induced alterations to whole-body CHO oxidation rates during moderate-intensity exercise in Acute Study 1 and 2 could be explained by insufficient exercise duration. The main finding of Acute Study 3 was that, even with extended exercise duration, 36°C heat stress did not significantly increase whole-body CHO oxidation rates during moderate-intensity exercise. Collectively, these findings provide insight into the combinations of exercise intensity and environmental temperature under which heat stress-induced changes in whole-body CHO oxidation rates are likely to be observed.

Specifically, three repetitions of 16 min at the workload eliciting VT_1 in temperate conditions separated by 8 min of lower intensity recovery elicited similar CHO oxidation rates in 18 and 36°C (60% rH), despite significant main effects of trial for T_{re} and estimated T_{mus}. This is interesting given core temperature and muscle temperature have previously been shown to contribute to the stimulatory effects on CHO metabolism during exercise (119, 123) observed when exercise is performed under environmental heat stress (121, 122, 177). These results therefore provide further support for the observations in Acute Study 1 and 2, which showed intensity-dependent regulation of the effects of environmental heat stress during exercise, and suggest that insufficient exercise duration was not the cause of the absence of heat stress effects on substrate oxidation rates at moderate intensities in these studies. Specifically, these studies collectively suggest that greater stimulatory effects of environmental heat stress on CHO oxidation rates are observed at higher workloads, and that during moderate heat stress appears unlikely to stimulate whole-body CHO oxidation rates during moderate-intensity exercise unless the environmental temperature is quite extreme (~40°C) (

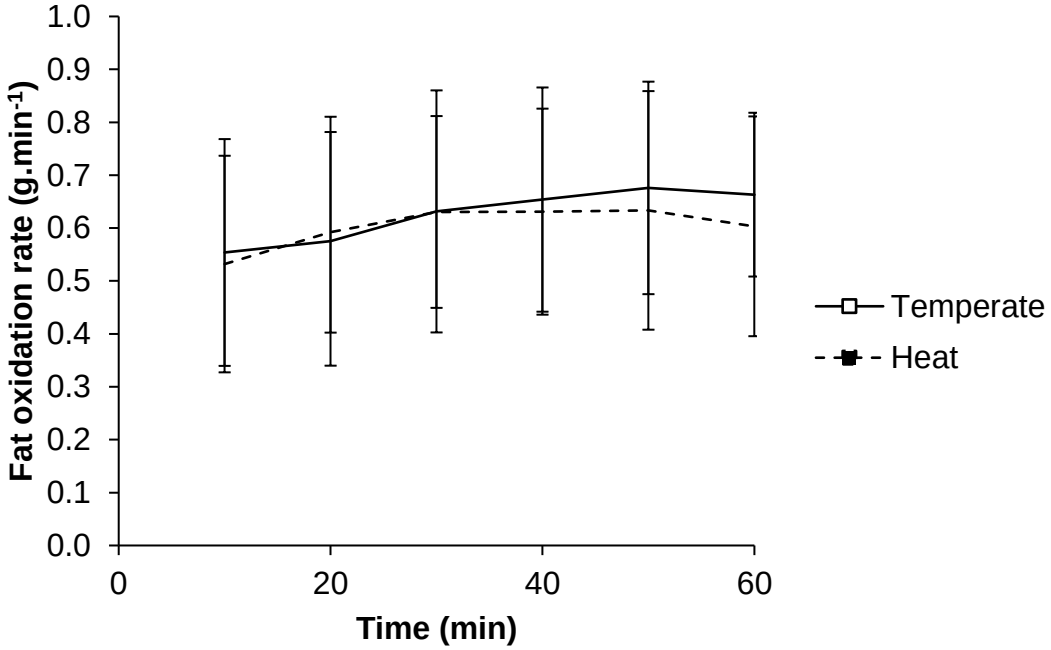


Figure 4, Figure 7, Table 7, Table 10,

Table 12. Rectal temperature (T_{re}) and estimated muscle temperature (T_{mus}) during three repetitions of cycling exercise (8 min at low intensity, 16 min at moderate intensity) performed in 18 and 36°C in Acute Study 3 (N = 8). '*' indicates significantly different between-trials ($P \leq 0.05$)

		Rep 1 Low	**Rep 1 Moderate**	**Rep 2 Low**	**Rep 2 Moderat**
T_{re}	18°C	36.92 ± 0.35	37.28 ± 0.35	37.50 ± 0.33	37.84 ± 0
(°C)	36°C	36.76 ± 0.24	37.31 ± 0.32	37.64 ± 0.37	38.08 ± 0
Estimated T_{mus}	18°C	34.36 ± 0.62	36.42 ± 0.74	36.51 ± 0.79	36.62 ± 0
(°C)	36°C	36.60 ± 0.49*	38.12 ± 0.17*	38.01 ± 0.19*	38.13 ± 0

Table 13).

In Acute Study 2, temperature x intensity interactions for plasma adrenaline concentrations were linked to the exercise intensity-mediated effects of heat stress on substrate oxidation rates, given circulating catecholamines are part of the mechanism by which heat stress-induced stimulation of CHO metabolism occurs (120, 121). It is possible that the combined exercise-heat stress in Acute Study 3 was not sufficient to elicit a rise in plasma adrenaline concentrations of large enough magnitude to stimulate CHO oxidation. However, as plasma adrenaline concentrations were not measured in Acute Study 3, this remains speculative. Regardless, the results presented here support the observations of Acute Study 1 and 2, and suggest that even with a longer exercise duration (72 min), moderate environmental heat stress (36°C and 60% rH) does not stimulate CHO oxidation rates during moderate-intensity exercise performed at the absolute workload eliciting VT_1 in temperate conditions. From a practical perspective, this further substantiates the suggestion that practitioners need not consider the effects of moderate environmental heat stress on substrate metabolism during moderate-intensity exercise, even if the exercise duration is prolonged (~72 min), at least when convective cooling is high.

In summary, moderate environmental heat stress (36°C and 60% rH) did not exert stimulatory effects on CHO oxidation rates during moderate-intensity exercise of prolonged duration (72 min). These data therefore substantiate the results of Acute Study 1 and 2, and suggest that the effects of heat stress on substrate oxidation rates during exercise are intensity-dependent.

5.5 Summary of acute studies

The primary aims of the studies presented in this chapter were to investigate the effects of exercise intensity, environmental temperature, and exercise duration on the acute metabolic effects of environmental heat stress on whole-body substrate oxidation rates. The main findings were that:

1. The effects of heat stress on substrate oxidation rates during cycling appear to be mediated by exercise intensity and environmental temperature, with greater effects seen at higher relative exercise intensities and higher environmental temperatures, and the exercise intensity 'threshold' for heat stress-induced changes in substrate oxidation rates lower in hotter environments (Table 7, Table 10, Table 13, Figure 5, Figure 7).
2. Heat stress-induced changes in substrate oxidation rates at moderate exercise intensities that are sustainable under moderate environmental heat stress were not substantial (Table 7, Table 10, Table 13, Figure 3, Figure 5, Figure 7), and perhaps less meaningful than indicated by previous research in this field (118, 121, 122). Interestingly, the lower absolute workloads at individual ventilatory thresholds under moderate environmental heat stress compared to temperate conditions may actually be supported by lower CHO but similar fat oxidation rates (Figure 5).
3. Mechanistically, the apparent regulatory effect of exercise intensity on the magnitude of heat stress-induced changes in substrate oxidation rates is not clear from the present data. Thermoregulatory perturbations under heat stress in these studies (T_{re}, estimated T_{mus}) were generally quite modest, and could not easily explain why larger effects would be observed at higher workloads (Figure 6, Table 9). However, and as discussed previously, the present data does suggest it is possible that exercise intensity-mediated regulation of heat stress-induced changes in CHO oxidation rates could be at least partially attributed to exercise intensity-dependent effects of heat stress on plasma adrenaline concentrations, which are influential in heat stress-induced stimulation of CHO metabolism (120, 121).

These findings have implications for understanding the specific combinations of exercise intensity and environmental temperature under which heat stress-induced changes in substrate oxidation rates during exercise are more likely to occur. These findings collectively suggest that athletes concerned with endogenous CHO availability as a limiting factor in competition might be less concerned about heat stress-induced stimulations in whole-body CHO oxidation rates than previously thought (118, 121,

122), unless their event takes place under more extreme environmental heat stress or involves significant periods of high-intensity work (Table 7, Table 10, Table 13, Figure 5, Figure 7), a finding supported recently (421). Indeed, as absolute workloads are generally decreased when transitioning from a temperate to hot environment, it is quite possible that the CHO oxidation rate at a given physiological intensity – i.e. VT_1 or VT_2 – may actually decline with the addition of environmental heat stress (Figure 5). However, it should be acknowledged that the studies presented in this thesis did not isolate the origin of oxidised CHO, i.e. skeletal muscle vs. hepatic glycogen, and so inferences regarding the effects of heat stress on muscle glycogen utilisation and therefore availability during endurance competitions cannot be made. Similarly, caution should be exercised when generalising these results to exercise circumstances in which convective cooling would be lower than in this thesis (~3.2 $m.s^{-1}$). This includes endurance disciplines such as running, where the lower absolute speeds elicit lower convective cooling, or indoor-cycling without meaningful fan speed. Under these circumstances, given work-rates would be expected to elicit greater thermoregulatory changes, which might in turn act to exacerbate acute substrate oxidation changes.

These studies also suggest that endurance athletes training to achieve the highest rates of fat oxidation during training need not consider the heat stress when making decisions related to training intensity. Training sessions designed to achieve the highest rates of fat oxidation will necessarily be of low-to-moderate intensity (2, 3, 316), such that, according to the data presented in this thesis, whole-body fat oxidation rates will be largely unaffected by moderate environmental heat stress (Table 7, Table 10, Table 13, Figure 5, Figure 7).

In the context of the present thesis, the data presented in this chapter has implications for the subsequent chapter, which is focused on metabolic adaptations to training performed under moderate environmental heat stress. Assuming some level of specificity of adaptation, the results of previous work would suggest that endurance training performed under heat stress would preferentially garner adaptations related to CHO rather than fat metabolism (104, 121, 122, 124, 126, 177, 242, 307). However, the data presented here suggests this may be less of a concern than previously thought when training is performed under moderate environmental heat stress (Table 7, Table 10, Table 13, Figure 5, Figure 7), particularly when one considers the likely reduction in external workloads performed during training in these conditions (54, 106, 232, 292, 369, 418). It should be acknowledged that the studies conducted in this thesis were designed to isolate the effects of exercise intensity, environmental temperature, and exercise duration, and so did not involve exercise protocols that specifically match those performed by endurance athletes during training. One could

speculate using the present data that a ‘basic endurance’ training session prescribed to maintain an athlete’s heart rate below their individual aerobic threshold (or moderate-heavy intensity transition) is unlikely to evoke greater CHO metabolism or blunted fat metabolism when performed under moderate environmental heat stress compared to a temperate environment. Indeed, CHO oxidation may even be lowered (Figure 5) secondary to a reduction in external workload when training under moderate environmental heat stress (232, 292); however, the data to directly assess this is not present. Therefore, in the context of the new level of understanding provided by the acute studies of this thesis, further study of the acute effect of environmental heat stress on whole-body substrate oxidation rates during endurance exercise protocols more closely matching those performed by endurance athletes during real-world heat stress training camps – using matched absolute and physiological workloads - is warranted.

6 Training studies: Does environmental heat stress impact the adaptive metabolic response to endurance exercise training?

6.1 Introduction

A fundamental goal of endurance training is to induce physiological adaptations that improve aerobic determinants of endurance performance such as maximum oxygen uptake ($\dot{V}O_{2max}$), physiological thresholds, and exercise economy (198). Furthermore, endurance athletes competing in stochastic events such as road cycling (295) would likely benefit from adequate metabolic flexibility in order to effectively utilise fat as an energy substrate at given submaximal workloads, alongside well-developed, rapid CHO metabolism to facilitate short periods of intense effort (183). These adaptations are at least partly mediated via effects on skeletal muscle mitochondrial content and function, both of which have been shown to adapt in response to endurance training (166, 167, 209, 339, 436, 458). Mitochondria are the cellular organelles in which β-oxidation of fatty acids to acetyl CoA, oxidation of fatty acid or non-fatty acid-derived acetyl CoA in the citric acid cycle, and oxidative phosphorylation along the electron transport chain all occur (199, 322, 512). Thus, a key focus of endurance training is to improve mitochondrial content and function in order to favourably impact metabolic determinants of endurance performance.

Endurance athletes commonly perform specific blocks of training in a hot environment for purposes of thermoregulatory adaptation (71, 403) and/or to provide an extra physiological training stressor in a 'heat stress training camp', presumably to evoke adaptations related to aerobic metabolism such as mitochondrial biogenesis (183). However, despite their widespread use, the adaptive consequences of heat stress training camps in endurance sport remain largely unexplored from a metabolic perspective, with no human study as yet interrogating the longitudinal implications of performing endurance training under environmental heat stress for mitochondrial protein content or function (183).

The only *in vivo* investigation of heat stress effects on metabolic adaptations to endurance training was an acute study, which demonstrated blunted transcription of genes related to mitochondrial biogenesis immediately and 3 h post-exercise performed in 33°C compared to exercise performed in a temperate environment (186). This was in contrast to the authors' hypothesis, which was based on *in vitro* and animal studies reporting positive effects of passive heat exposure for acute signalling and chronic mitochondrial changes (284, 385, 470, 471), and the basic principle that

greater disturbance from homeostasis leads to greater training adaptation (127, 183). It is possible this unexpected blunting reflected a shift in the time-course of adaptive signalling when exercise is performed under environmental heat stress (186), an explanation supported by an *in vitro* passive heat stress study in which the augmented mitochondrial biogenesis-related gene expression was observed at 24 h and not 2 h post-heat exposure (284). Given the incomplete understanding of the acute intramuscular signalling cascade(s) required for mitochondrial adaptation to training (226) (Figure 2), particularly when performed under heat stress (186), an appropriate means of determining the effect of environmental heat stress for mitochondrial adaptations to prolonged exercise is therefore to measure changes in mitochondrial protein content and function in response to a heat stress training intervention. Elucidating this effect is of paramount importance to endurance athletes considering engaging in an organised period of heat stress training, as well as for those athletes who live and train in hot environments, given effecting positive mitochondrial adaptations is a key goal of endurance training.

Several studies have investigated the effect of endurance training performed under heat stress for functional adaptations relevant to performance in temperate conditions, with mixed results (253, 255, 292, 333, 486). For example, Rønnestad and colleagues reported positive haematological adaptations with training performed under heat stress, along with performance responses that appeared to favour the heat stress training group, though these were not significant (413). The training intervention in this study involved 50 min cycling at 45% of the power at 4 $mmol.L^{-1}$ blood lactate concentration (measured in temperate conditions), with training performed in ~15.5°C or 37.5-38.5°C, 4-5 times per week for five weeks, on top of regular temperate training. Though workloads in the study-specific training sessions were adjusted to maintain “light” to “hard” perceived exertion, it is likely the study-specific relative training stress was greater in the heat stress training group. Indeed, time spent in power-based training zones, defined in temperate conditions, was the same between-groups, indicating greater training stress in the heat stress group, given the decreased external workloads observed at individual physiological thresholds in hot environments (232, 292). Accordingly, there has so far been no systematic evaluation of the adaptive metabolic or temperate performance effects of exposure to environmental heat stress during endurance training.

Further considerations for endurance athletes undertaking heat stress training camps are possible detraining effects through reduced mechanical power outputs, and negative effects on athlete wellbeing. Elevated environmental temperature (>30°C) reduces the absolute power output achieved for a given heart rate (369, 418), and

reduces the power output at individual physiological thresholds (232, 292), although it is not currently known if the heart rate at the individual physiological thresholds is consistent between temperate and heat stress environments. As such, the power output during specific training sessions is likely to be reduced during heat stress training camps, at least initially (54, 106). Heat stress training camps may therefore induce a reduced mechanical load compared to training performed in a temperate environment with equivalent cardiovascular demand, plausibly detraining an athlete in a manner analogous to what might be seen with high altitude training (280, 349). Similarly, because of the elevated heart rate (369, 418) and elevated plasma catecholamines (104, 121, 177) observed when exercise is performed under environmental heat stress, likely indicative of greater sympathetic activation, heat stress training camps have the potential to disturb cardiac autonomic balance when not managed effectively. Suppressed parasympathetic activation, which can be measured using heart rate variability (HRV) upon waking, may ultimately be predictive of negative performance consequences such as fatigue and non-functional overreaching (396). Indeed, a recent study reported maladaptation to short-term, high-intensity heat acclimation training, plausibly due to failure to appropriately manage training stress (406).

Despite these apparent risks, endurance athletes do commonly engage in heat stress training camps. However, a detailed description of how these camps are conducted from a practical perspective is not available. Such a description, particularly with regard to training prescription and monitoring, would provide insight into how the risks presented by training under heat stress are managed in the real-world. This information could be taken into consideration in the design of a controlled study assessing the effectiveness of a simulated heat stress training camp for metabolic adaptations and measures of temperate exercise performance.

Thus, the aim of the series of training-related studies within this chapter was to investigate the following research questions:

1. How are heat stress training camps performed by elite endurance athletes in the real-world?
2. Are heart rates at individual physiological thresholds consistent between temperate and heat stress environments?
3. Does environmental heat stress exert an additive effect on the adaptive metabolic response to endurance training in humans?

6.2 Training Study 1: A descriptive case study of the use of heat stress training camps in elite endurance athletes

The purpose of Training Study 1 was therefore to describe how a real-world heat stress training camp undertaken by two highly-trained Ironman triathletes was monitored in terms of training prescription, training load and volume, and athlete wellbeing. It was intended that the descriptive case study design would give insight into real-world practices regarding heat stress training camps in elite endurance athletes for use in the design of the final study of this thesis. This case study has since been published in the *International Journal of Sports Physiology and Performance* (314).

6.2.1 Methods

6.2.1.1 Participants

Two male Ironman triathletes participated in this case study. Ironman 1 is a multiple-time World Champion triathlete with >20 victories in half- and full-distance Ironman events, and several <8 h full-distance Ironman finishes (mass, 73.2 kg; cycling $\dot{V}O_{2max}$, 70.4 $mL.kg^{-1}.min^{-1}$; power at $\dot{V}O_{2max}$, 420 W). Ironman 2 has personal best times of 8:24 and 3:53 for full- and half-Ironman events (mass, 78.2 kg; cycling $\dot{V}O_{2max}$, 65.4 $mL.kg^{-1}.min^{-1}$; power at $\dot{V}O_{2max}$, 445 W). All procedures conformed to the Declaration of Helsinki and were approved by the Auckland University of Technology Ethics Committee.

6.2.1.2 Case study overview

The Ironman triathletes were followed prior to and during a three-week heat stress training camp in Kailua-Kona, Hawaii. The intention of the training camp was to augment metabolic adaptations to training in advance of a race performed in a temperate environment ~2 weeks following the camp. All training sessions during the heat stress camp where performed outdoors, and further passive heat exposure was deliberately minimised by residing indoors as much as possible. The average ambient temperature during training sessions was recorded using bicycle power meters, and reached 30 ± 3°C during the heat stress training camp, having been 18 ± 2°C during the pre-training camp period in Auckland, New Zealand. Three laboratory exercise assessments were made: one at baseline four weeks prior to departure for the training camp (Baseline), one in the week prior to departure for the camp (Pre-Camp), and one three days after returning to New Zealand (Post-Camp). The Baseline assessment was made after a ~2 week break from training following Ironman New Zealand.

6.2.1.3 Laboratory assessments

Participants arrived for climate-controlled laboratory assessments (~18°C and ~65% rH) at ~6:30 following an overnight fast, and having measured resting heart rate

variability upon waking (HRV4Training, Amsterdam, NET). On arrival, participants provided a mid-stream urine sample to confirm euhydration (urinary specific gravity <1.026) (19), before height and nude body mass was determined. The sum of eight skinfold assessment was performed (triceps brachii, biceps brachii, subscapular, iliac crest, supraspinale, abdominal, anterior thigh, posterior shank), and a capillary blood sample was obtained at rest and analysed for lactate concentration (Lactate Pro 2, Arkray, Tokyo, Japan).

Participants commenced cycling on personal road bicycles connected to indoor trainers (Revolution Wind Trainer, LeMond Fitness Inc., Poway, USA) and crank-based power meters (Quarq, SRAM, Chicago, USA) at 95 W with continuous collection of expired gases using indirect calorimetry (TrueOne2400, ParvoMedics, Sandy, UT, US), as well as convective fan cooling (~3.2 $m.s^{-1}$). Workload increased by 35 W every 3 min until the respiratory exchange ratio reached 1.0. Capillary blood samples were obtained in the last 30 s of each stage and analysed for lactate concentration (Lactate Pro 2, Arkray, Tokyo, Japan). Ventilatory thresholds (VT_1 and VT_2) were estimated (296). The workload eliciting a 4 $mmol.L^{-1}$ blood lactate concentration (OBLA) was estimated using available software (356).

Participants then rested passively for 10 min before commencing cycling at 260 W for 60 min (approximate Ironman power output) with *ad libitum* water ingestion. Expired gases were collected for 4-min every 10-min and analysed for non-protein whole-body rates of fat and CHO oxidation using standard equations (246). Heart rate was recorded continuously (M430, Polar Electro Inc., NY, USA; Forerunner® 920XT, Edge® 520 Plus, Garmin, Schaffhausen, SWI).

6.2.1.4 Athlete monitoring

Well-being was assessed via five-point Likert scales of fatigue, sleep quality, muscle soreness, stress, and mood, and these scores were summed to measure global well-being (327). Scales were completed over three-day periods in advance of the baseline assessments and whilst in Kona. The participants recorded waking heart rate variability daily (HRV; HRV4Training, Amsterdam, NET) and detailed training logs including heart rate, pace, and power output (M430, Polar Electro Inc., NY, USA; Forerunner® 920XT, Edge® 520 Plus, Garmin, Schaffhausen, SWI). Participants also habitually follow a high-volume, largely low-intensity training programme with the aid of target powers, paces, and/or heart rates derived from laboratory testing. During the heat stress training camp, the athletes initially used heart rate thresholds to guide training intensities, before using power outputs later in the training camp. Accordingly, training intensity distribution was quantified as time in training zones based on heart rates

observed at the first (LT_1) and second (LT_2) lactate thresholds measured during previous laboratory assessment in temperate conditions. Subsequently, training intensity distribution was expressed as the percentage of training time spent in Zone 1 ($<LT_1$), Zone 2 (LT_1-LT_2), and Zone 3 ($>LT_2$). Training load is expressed as training stress score (TSS, TrainingPeaks™ Boulder, CO, USA) calculated as follows (Equation 4).

TSS = (time [s] x session intensity x IF®)/(threshold unit x 3600) x 100

Equation 4. where "session intensity" = the average normalised power (cycling), heart rate (running) and pace (swimming), "IF®" = intensity factor, measured as session intensity/threshold power/pace, heart rate, "threshold unit" = the respective individualised upper threshold for Ironman 1 and 2. Due to differing modalities, these units (pace, power and HR) gave the most accurate representation of session intensity.

6.2.1.5 Statistical analyses

Weekly training volume and load is expressed as mean ± standard deviation (SD). Likelihood and magnitude of individual changes in measurements were estimated using a custom-made spreadsheet (206). Typical error (TE) for training load and intensity distribution in each physiological zone was 3%, taken from TE data for submaximal heart rate measures (59), and the smallest important change (SIC) was calculated as 0.3 multiplied by the individually calculated coefficient of variation (CV) for training distribution in each zone in each Ironman over five weeks preceding Ironman New Zealand and this case study. TE for perceived wellbeing was the SD of each Ironman's score during the baseline period, and a SIC of 1.5 arbitrary units (AU) was used. SIC for weekly HRV (mean log-transformed square root of the mean sum of the squared differences between R–R intervals [Ln rMSSD]), and for the pre-camp and training camp periods, was determined as 0.3 multiplied by the individual CV over a week prior to the case study, and TE was defined as the previously observed value for photoplethysmography against electrocardiography (6.9%) (397). Sum of eight skinfold thickness CV (3.8%) and TE (1.6 mm) were derived from repeat profiles performed by the anthropometrist of this study. SIC for workloads eliciting VT_1 (~1.1%), VT_2 (~0.6%), and OBLA (~1.1%) were quantified as 0.3 multiplied by the associated CV measured in a cohort of well-trained cyclists, and TE of these measurements (4.7, 2.7, and 5.3%, respectively) was calculated using the same data (380). SIC and TE for mean submaximal heart rate was 1 and 3%, respectively (59). The SIC (0.2 multiplied by the between-subject SD) and TE for CHO (~0.09 $g.min^{-1}$ and 0.25 $g.min^{-1}$, respectively) and fat (~0.02 $g.min^{-1}$ and ~0.11 $g.min^{-1}$) oxidation rates during 60 min steady-state exercise was derived from previous data (320). SIC for gross cycling efficiency (~1.3%,

expressed as a percentage of measured value for gross efficiency) was quantified as 0.3 multiplied by the associated CV in a cohort of trained cyclists, and TE was calculated using the individual values from trial 1 and 2 of the same study (~0.6%, expressed as a raw value for gross efficiency) (347). Quantitative chances of change were assessed as follows: >75%, *very likely*; 25-75%, *possible*; <25%, *very unlikely*. Change scores that were *possible* increases (25-75%) and *possible* decreases (25-75%) were deemed *unclear* (206).

6.2.2 Results

Physiological data from the exercise assessments is shown in Table 14. Notably, pre-to-post camp increases in VT_1 (~8-10%) and OBLA (~4%) were *very likely* and *possible,* respectively. Training volume and load is shown in Table 15. For Ironman 1, the change in percentage of total training time spent in Zone 1, 2, and 3 in the pre-camp and camp periods was *unclear*, *very likely* increased, and *very likely* decreased, respectively (77/15/8 vs. 78/18/4%). In Ironman 2, these changes were *very likely* increase, *very likely* decrease, and *very likely* decrease, respectively (76/19/5 vs. 88/11/1%). Perceived wellbeing and skinfold data are shown in Table 16. HRV was largely maintained throughout the case study in both athletes. In Ironman 1, changes in weekly mean HRV expressed as Ln rMSSD were *unclear*. In Ironman 2, week-to-week changes were *unclear*, except for a *possible* increase during the last week of the heat stress training camp. When the pre-camp period was compared with the heat stress training camp as a whole, Ironman 1 exhibited a *possible* increase in HRV, whereas Ironman 2 showed an *unclear* change (Table 16).

Table 14. Key physiological determinants of performance in Ironman 1 (IM1) and Ironman 2 (IM2) during each assessment across the case study. CHO = carbohydrate. Athletes were instructed to cycle at a comfortable, consistent cadence, which was almost identical across trials

Variable	ID	Baseline	Pre-Camp	Inference (Pre-Camp vs. Baseline)	Post-Camp	Inference (Post-Camp vs. Pre-Camp)
VT_1 (W)	IM1	208	216	*Possible* increase	233	*Very likely* increase
	IM2	247	270	*Very likely* increase	298	*Very likely* increase
VT_2 (W)	IM1	253	251	*Unclear*	250	*Unclear*
	IM2	265	306	*Very likely* increase	321	*Very likely* increase
OBLA (W)*	IM1	343	344	*Unclear*	359	*Possible* increase
	IM2	332	376	*Very likely* increase	392	*Possible* increase
CHO oxidation^ ($g.min^{-1}$)	IM1	2.58	2.23	*Very likely* decrease	2.26	*Unclear*
	IM2	2.06	1.53	*Very likely* decrease	1.38	*Unclear*
Fat oxidation^ ($g.min^{-1}$)	IM1	0.68	0.80	*Possible* increase	0.85	*Unclear*
	IM2	0.97	1.19	*Very likely* increase	1.17	*Unclear*
Gross efficiency^ (%)	IM1	21.8	22.0	*Unclear*	21.3	*Possible* decrease
	IM2	21.4	21.0	*Possible* decrease	21.9	*Possible* increase
Heart rate^ ($b.min^{-1}$)	IM1	135	124	*Very likely* decrease	123	*Unclear*
	IM2	151	144	*Very likely* decrease	143	*Unclear*

^At a steady-state power output of 260 W.

*Ironman 2 adopts a low-CHO, high-fat diet (as evidenced by their high rates of fat oxidation), and has always had very low blood lactate concentrations during the incremental tests. It is therefore possible that the observed 4 $mmol.L^{-1}$ OBLA values may overestimate their true physiological threshold. This parameter was chosen due to its unambiguity and for ease of objective comparison over time, and its low associated SIC (~1.1%) (380).

Table 15. Descriptive training data for Ironman 1 and Ironman 2 across the case study. Training intensity distribution is expressed as the percentage of total training time spent in Zone 1 (<LT_1), Zone 2 (LT_1-LT_2), and Zone 3 (>LT_2)

	Ironman 1		**Ironman 2**	
	Volume ($h.week^{-1}$)	**Load ($TSS.week^{-1}$)**	**Volume ($h.week^{-1}$)**	**Load ($TSS.week^{-1}$)**
Pre-Camp 18 ± 2°C	25.1 ± 2.2	1349 ± 144	21.5 ± 0.9	1059 ± 53
Swim	5.5 ± 0.6	324 ± 60	5.7 ± 0.5	207 ± 20
Bike	12.0 ± 1.7	546 ± 94	10.6 ± 0.7	605 ± 39
Run	6.6 ± 0.6	441 ± 38	4.3 ± 0.5	227 ± 20
Camp 30 ± 3°C	32.7 ± 0.4	1714 ± 51	25.0 ± 6.7	1048 ± 116
Swim	5.6 ± 0.3	330 ± 14	5.9 ± 0.3	304 ± 13
Bike	18.8 ± 0.7	792 ± 6	16.0 ± 6.1	630 ± 59
Run*	6.5 ± 0.2	499 ± 33	2.0 ± 2.4	99 ± 140
Change	*Very likely* increase	*Very likely* increase	*Very likely* increase	*Trivial*
Swim	*Unclear*	*Unclear*	*Likely* increase	*Very likely* increase
Bike	*Very likely* increase	*Very likely* increase	*Very likely* increase	*Very likely* increase
Run	*Unclear*	*Unclear*	*Very likely* decrease	*Very likely* decrease

*Ironman 2's run volume during the heat stress training camp was impacted by a minor injury that impaired running but not swimming or cycling capabilities.

Table 16. Waking heart rate variability (Ln rMSSD), global perceived wellbeing (sum of five-point Likert scales of fatigue, sleep quality, muscle soreness, stress, and mood, AU) and sum of eight skinfolds (mm) in Ironman 1 and Ironman 2 across the case study

		Ironman 1	**Ironman 2**
Waking heart rate variability (Ln rMSSD)	Pre-Camp	4.73 ± 0.20	5.21 ± 0.21
	Camp	5.04 ± 0.19	5.06 ± 0.25
	Change	*Possible* increase	*Unclear*
Perceived wellbeing (5-25, AU)	Pre-Camp	14.3 ± 0.6	15.3 ± 3.2
	Camp	14.0 ± 2.6	15.0 ± 3.6
	Change	*Very likely* trivial	*Unclear*
Sum of eight skinfolds (mm)	Baseline	40.8	38.0
	Pre-Camp	33.0	34.6
	Change	*Very likely* decrease	*Very likely* decrease
	Post-Camp	28.1	34.6
	Change	*Very likely* decrease	*Unclear*

6.2.3 Discussion

The present case study describes the careful monitoring of a three-week heat stress training camp undertaken by two highly-trained Ironman triathletes. The physiological profiles observed before and after the camp (Table 14), along with HRV and subjective wellbeing scores (Table 16), are indicative of a successful heat stress training camp. Indeed, during the heat stress training camp, training volume was *very likely* increased and load was maintained or *very likely* increased, largely through *very likely* increases in cycling training volume and load (Table 15), without compromising athlete wellbeing. The *possible* increase in resting HRV observed in Ironman 1 during the heat stress training camp is unlikely to reflect an impairment in cardiac-autonomic balance, given it remained within their normal range and the absence of changes in perceived wellbeing. Moreover, *very likely* and *possible* increases in VT_1 (~8-10%) and OBLA (~4%), respectively, were observed, which should be considered desirable outcomes indicative of a successful camp. These descriptive case study results therefore provide support for the use of the simple strategies adopted by the athletes, namely daily measurement of resting HRV, athlete wellbeing scales, and careful prescription of training using prior knowledge of physiological heart rate thresholds whilst measuring daily HRV and wellbeing scores, when undertaking a period of endurance training under moderate environmental heat stress.

Given the athletes in this case study used knowledge of their heart rate thresholds to programme and regulate training intensity during the heat stress camp, the next study, prior to the final intervention study of this thesis, will seek to explore if heart rate thresholds measured in temperate conditions are reflective of those measured under moderate environmental heat stress. In this case study, it appears this strategy was successful, given resting HRV and subjective wellbeing was apparently unaffected by the heat stress training camp (Table 16). It is in fact possible that, if heart rate thresholds were to rise when transitioning to moderate environmental heat stress; that is, heart rates at given power outputs increasing more than the decrease in power outputs at given physiological thresholds, that the strategy used in the present case study actually 'under-trained' the athletes whilst on the heat stress training camp. Understanding the agreement between heart rate thresholds measured in temperate conditions and under moderate environmental heat stress will therefore provide useful information for athletes embarking on periods of endurance training under moderate environmental heat stress, as well as researchers seeking to design intervention studies that successfully match training interventions between-conditions.

6.3 Training Study 2: Investigating the stability of heart rate at individual physiological thresholds between temperate and heat stress environments

The case study above (Chapter 6.2) describes the successful conduct of a real-world heat stress training camp undertaken by two highly-trained Ironman triathletes (314). Several strategies were implemented to minimise the risk of maladaptation to exercise-heat stress, which has been observed previously (406). These included daily monitoring of HRV upon waking, self-report athlete wellbeing scales, and careful training programming for control of intensity and load using individual physiological heart rate thresholds.

However, it should be acknowledged that the individual physiological thresholds used for training programming by the Ironman triathletes described in this case study were derived from laboratory assessments performed in temperate conditions, and not under the heat stress they faced during the heat stress training camp (314). Whilst it has been previously identified that power/pace at individual physiological thresholds is decreased by heat stress (232, 292), and that the heart rate elicited by a given power/pace increases with heat stress (369, 418), it has so far not been identified in the literature whether heart rate at individual physiological thresholds is constant between temperate and heat stress environments. Identifying the effect of environmental temperature on heart rate at physiological thresholds is of importance for endurance athletes undertaking training in a hot environment when training is programmed using threshold heart rate measurements, which are typically undertaken in thermoneutral laboratory conditions. This will ensure that training intensity and load is adequately controlled for the facilitation of optimal training outcomes, and aid in the design of research seeking to 'match' training load between training interventions performed in temperate and heat stress environments. Simplistically, identifying this effect will determine if endurance athletes need to perform a physiological threshold assessment in a simulated hot environment prior to departure for a heat stress training camp if seeking to programme training using heart rate measurements.

Therefore, in order to advance on the strategies utilised by the Ironman triathletes to minimise the risk of maladaptation and/or non-functional overreaching to heat stress training described in Training Study 1, Training Study 2 will seek to explore the stability of heart rates measured at individual physiological thresholds between temperate and heat stress environments. This information will be used to elucidate if heart rates at physiological thresholds, and therefore heart rate-based training zones, derived from assessments performed in temperate conditions can be accurately used for exercise

prescription in a heat stress training camp. Training Study 2 has recently been published in the *International Journal of Sports Physiology and Performance* (319).

6.3.1 Methods

6.3.1.1 Participants

Sixteen regularly-competitive, endurance-trained males took part in this study ($\dot{V}O_{2max}$, 58.2 ± 6.9 $mL.kg^{-1}.min^{-1}$; height, 183 ± 8 cm; mass, 81 ± 10 kg). The Auckland University of Technology Ethics Committee approved experimental procedures, and all participants provided written informed consent.

6.3.1.2 Experimental protocol

Data was collected in the non-summer months in Auckland, New Zealand. Participants, in random order and a minimum of four days apart, completed incremental exercise tests (IET) in 18 and 35°C after an overnight fast and having kept a diet record for 24-h and refrained from vigorous exercise for 24-h. A mid-stream urine sample was collected prior to IET to confirm euhydration (urinary specific gravity <1.026) (19). A blood sample was collected at baseline, either from an antecubital venous cannula (N = 8) or as finger capillary blood (N = 8). Sampling procedures were identical between-trials within each individual. Participants were fitted with a heart rate monitor, and heart rate was recorded continuously (RS800, Polar Electro Oy, Kempele, Finland).

Participants then entered an environmental chamber set to 18°C and 60% rH or 35°C and 60% rH, and rested passively for 20 min. Cycling then commenced on a laboratory ergometer (Excalibur Sport, Lode, Groningen, NET) at 95 W with workload increasing by 35 W every 3 min. Convective air flow (~3.2 $m.s^{-1}$) was provided by an industrial fan (80 cm diameter, 100 cm in front of the handlebars, FS-75, FWL, Auckland, NZ). Expired gases were collected continuously using indirect calorimetry (TrueOne2400, ParvoMedics, Sandy, UT, USA). A 5-mL venous blood sample or finger capillary blood sample was collected after each 3-min stage, and heart rate was averaged for the last 30 s of each stage. The test continued until the respiratory exchange ratio reached 1 (venous sampling) or if a blood lactate concentration of >4 $mmol.L^{-1}$ was confirmed (capillary sampling); at this point blood sampling ceased and workload increased by 35 $W.min^{-1}$ until volitional exhaustion. Participants returned to the laboratory at the same time of day, having adhered to the same pre-trial controls and replicated their 24-h diet record, after a minimum of four days for repetition in the remaining environmental condition.

6.3.1.3 Threshold analysis

Venous blood samples were collected into 6-mL ethylenediaminetetraacetic acid tubes and stored on ice until trial completion. Plasma was then isolated from the remaining whole blood by centrifugation at 1500 g in 4°C for 10 min, and stored at -80°C prior to subsequent analyses. Plasma lactate concentrations were determined through specific enzymatic colorimetric diagnostic assays on a Roche Diagnostics automated clinical chemistry analyser (cobas Modular P800, Roche Diagnostics New Zealand Ltd, Auckland, NZ). Capillary blood was analysed instantaneously using an automated analyser (Lactate Pro 2, Arkray, Tokyo, Japan).

Power and heart rate at lactate thresholds were subsequently computed using publically-available software (356). Lactate thresholds were defined as fixed blood lactate concentrations (FBLC) of 2, 3, and 4 mmol.L^{-1} (356). These absolute blood lactate threshold definitions were selected ahead of curve-fitting methods due to the lower associated coefficient of variation (380). The first ventilatory threshold (VT_1) was identified as the work rate at which the ventilatory equivalent for oxygen ($\dot{V}E.\dot{V}O_2^{-1}$) began to increase in the absence of changes in the ventilatory equivalent for carbon dioxide ($\dot{V}E.\dot{V}CO_2^{-1}$), and the second ventilatory threshold (VT_2) was identified as the first work rate at which $\dot{V}E.\dot{V}O_2^{-1}$ and $\dot{V}E.\dot{V}CO_2^{-1}$ increased, alongside a reduction in end tidal PCO_2.

6.3.1.4 Statistical analysis

Data are presented as mean ± standard deviation unless otherwise stated. Systematic variance in power and heart rate at physiological thresholds was identified between-environments using two-tailed paired t-tests and Cohen's *d* effect sizes expressed ± 95% confidence limits, and bias was calculated as the heat stress value subtract the temperate value (Microsoft Excel v14.7). Coefficient of variation and Pearson's correlation coefficients for measured heart rate values (modelled using average of the last 30 s of each stage) were computed to identify the consistency of these variables between-environments (SPSS Statistics v25).

6.3.2 Results

For all blood lactate concentrations, calculated power was decreased by heat stress (FBLC-2 mmol.L^{-1}, 258 ± 47 vs. 218 ± 41 W, ES = -0.82 ± 0.23, -16 ± 8%; FBLC-3 mmol.L^{-1}, 286 ± 46 vs. 249 ± 40 W, ES = -0.76 ± 0.26, -13 ± 8%; FBLC-4 mmol.L^{-1}, 305 ± 47 vs. 273 ± 39 W, ES = -0.65 ± 0.28, -10 ± 7%; all $P < 0.05$). Power at VT_1 (223 ± 33 vs. 183 ± 25 W, ES = -1.15 ± 0.38, -17 ± 9%) and VT_2 (275 ± 35 vs. 240 ± 30 W, ES = -0.96 ± 0.35, -12 ± 8%) was significantly decreased by heat stress (Figure 8, $P < 0.001$). Systematic variance in heart rate at any blood lactate concentration or

ventilatory threshold was not detected between-environments (P > 0.05), and mean bias was low (Table 17).

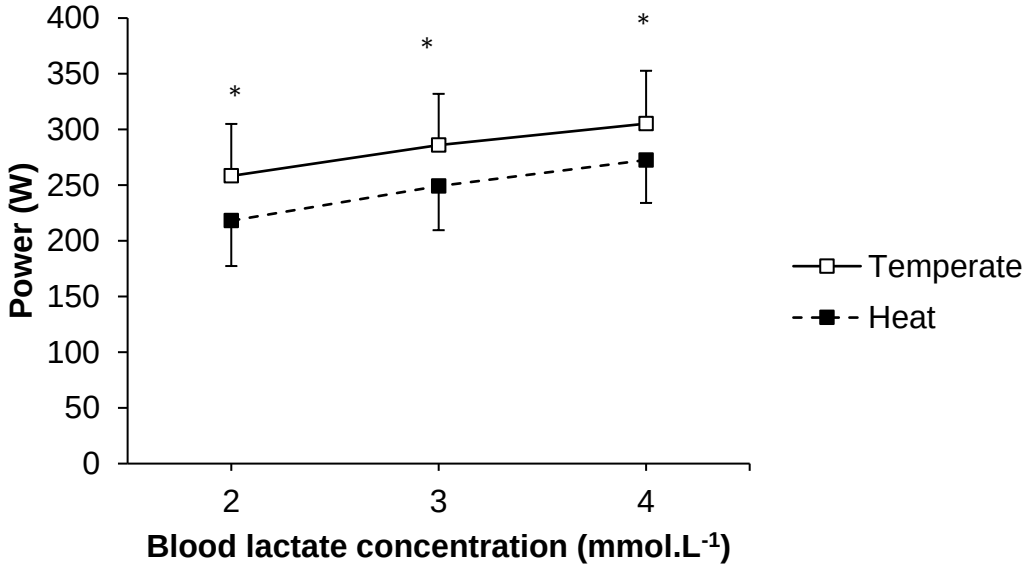
Given the absence of systematic variance in heart rate at absolute blood lactate concentrations or ventilatory thresholds between-environments, within-individual consistency of heart rate at these points was investigated (Figure 9). The coefficient of variance for measures of heart rate at fixed blood lactate concentrations and ventilatory thresholds (1.4-2.9%) between-environments were low (Table 17). Similarly, significant strong positive correlations between heart rate at fixed blood lactate concentrations and ventilatory thresholds in 18 and 35°C were observed (r = 0.92-0.95, P < 0.05, Table 17).

Table 17. Consistency of heart rate at fixed blood lactate concentrations and ventilatory thresholds during incremental cycling assessments performed in 18 and 35°C

	FBLC-2 mmol.L^{-1}	**FBLC-3 mmol.L^{-1}**	**FBLC-4 mmol.L^{-1}**	**VT_1**	**VT_2**
Mean bias (b.min^{-1})	-3 ± 5	-1 ± 4	1 ± 3	-1 ± 5	0 ± 5
CV (%)	2.9	1.7	1.4	2.7	2.3
CV (b.min^{-1})	4.2	2.6	2.2	3.6	3.5
r	0.92	0.95	0.95	0.94	0.92

Abbreviations: FBLC = fixed blood lactate concentration, CV = coefficient of variance, r = Pearson's correlation coefficient.

(a)



(b)

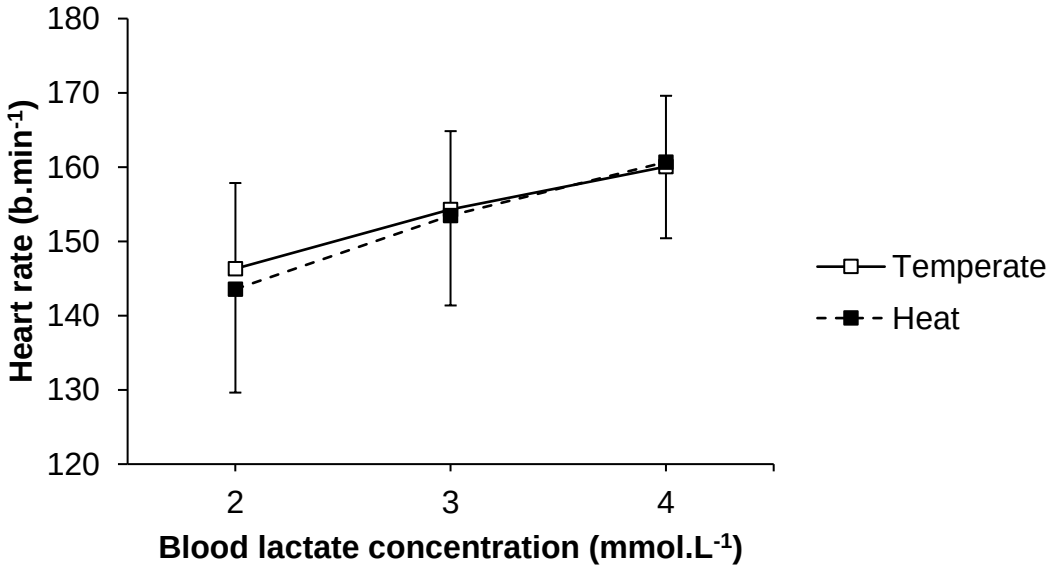


Figure 8. (a) Power output (b) and heart rate measured at 2, 3, and mmol.L^{-1} blood lactate concentrations during incremental exercise assessments performed in 18 and 35°C. '*' denotes $P < 0.05$ between-environments.

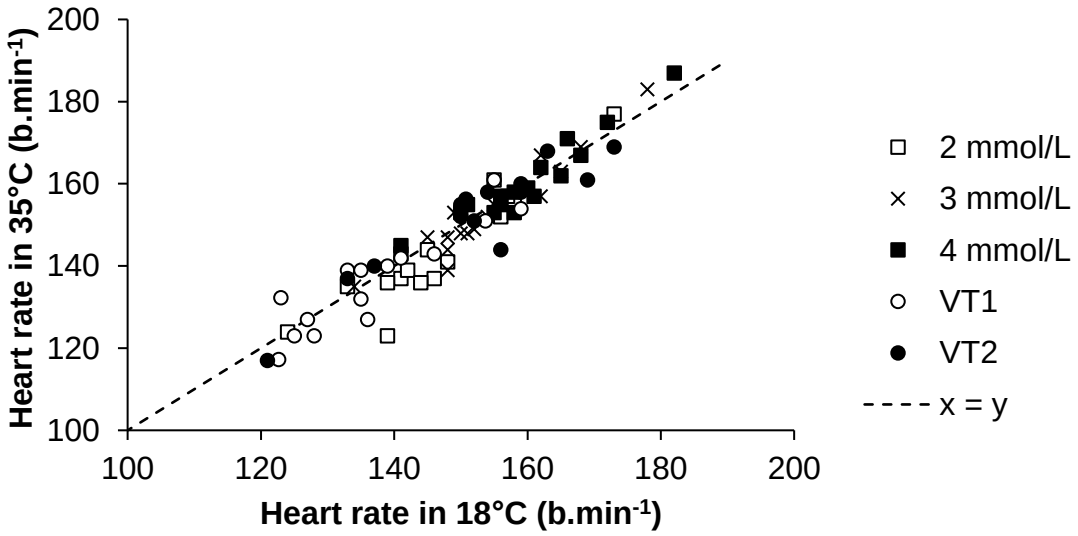


Figure 9. Consistency of heart rates measured at 2, 3, and mmol.L^{-1} blood lactate concentrations, and the first (VT1) and second (VT2) ventilatory thresholds, during incremental exercise assessments performed in 18 and 35°C.

6.3.3 Interim discussion

The purpose of Training Study 2 was to determine if laboratory-based measurements of heart rate at individual physiological thresholds made in temperate environments are reflective of those measured under moderate environmental heat stress. The primary finding was that heart rates at various blood lactate concentrations and ventilatory thresholds were similar in temperate conditions and under moderate environmental heat stress in endurance-trained males (Figure 9). These observations have implications for endurance athletes embarking on heat stress training camps, or undertaking one-off bouts of endurance exercise training under moderate environmental heat stress.

Specifically, heart rates at FBLC-2, 3, and 4 mmol.L^{-1} lactate thresholds and ventilatory thresholds were not systematically different between 18 and 35°C (Table 17), despite significant reductions in power output of ~10-17% in with observations reported elsewhere (232, 292). Moreover, the variability of heart rates measured at these thresholds between-environments was low, all demonstrating a CV similar to the ~3% reported for sub-maximal heart rate (1.4-2.9%) (59). Given the strong, positive correlations between these heart rate thresholds in 18 and 35°C ($r = 0.92$-0.95, $P < 0.05$, Table 17), the data presented here indicates heart rates measured at physiological thresholds in temperate environments are reflective of those measured under moderate environmental heat stress. Further research is required to determine if these observations can be extended to elite and female populations.

Following a period of heat stress training, it would be expected that the power achieved at specific physiological thresholds would increase, and the heart rate at given workloads would decrease (121). However, whether these adaptations occur proportionally is not known and requires further consideration. Investigation of how heart rates associated with physiological thresholds change during a period of heat stress training would give insight into whether pre-training measurements in temperate conditions can be used throughout an entire heat stress training camp. Whilst the present data suggests using heart rate thresholds derived from temperate laboratory assessments to define training intensity during the early stages of a heat stress training camp may provide an effective means of avoiding chronic high training intensities, and may provide an appropriate means of regulating training intensity during sporadic sessions performed under heat stress (e.g. during summer months), the implications of this approach later in a heat stress training camp are not known. Understanding the time-course of these adaptations would give endurance athletes insight into when power-based prescriptions might be effectively used to regulate exercise intensity during training sessions in a heat stress training camp.

The data presented here has implications for the design of studies aimed at assessing the effect of moderate environmental heat stress on metabolic adaptation, and, specifically, the need to match relative training intensity (127) and volume (165) between temperate and hot training interventions to isolate the effect of moderate environmental heat stress. Specifically, matching absolute workloads between-interventions may serve to under-train those in a temperate training group, given the effect that moderate environmental heat stress has on the external workloads at given physiological thresholds (232, 292) (Table 17). This is a challenge that has so-far not been adequately addressed in the literature (292, 333, 413). The data collected in the present investigation suggests that matching training programming using individual heart rate thresholds, measured in a temperate environment, between temperate and heat stress training interventions may be an appropriate means of isolating the effect of moderate environmental heat stress on metabolic training adaptation. Moreover, this would be an ecologically-valid simulated heat stress training camp study design, given training intensity was largely regulated according to temperate-based threshold heart rates in the real-world heat stress training camp described in Training Study 1. Accordingly, this methodology is employed in Training Study 3.

In summary, heart rate thresholds measured in temperate environments were similar to those measured under moderate environmental heat stress. Therefore, training programming for endurance athletes beginning a period of training under environmental heat stress may be based on temperate measurements of heart rate at individual physiological thresholds. Specifically, the data presented here supports programming training according to temperate-assessed heart rate threshold when seeking to manage training stress, and in research when attempting to match training interventions between temperate and moderate environmental heat stress conditions.

6.4 Training Study 3: Implications of prolonged exercise performed under environmental heat stress for the adaptive response to endurance training: A randomised controlled trial

The purpose of Training Study 3 was therefore to establish if performing endurance training under moderate environmental heat stress augments the adaptive metabolic response to training in terms of (i) mitochondrial adaptations, (ii) functional physiological responses to exercise performed in temperate conditions, and (iii) endurance exercise performance in temperate conditions. As described in detail throughout this thesis (Chapters 3.2, 6.1), it was hypothesised that exposure to moderate environmental heat stress during endurance training would increase the beneficial adaptations observed in response to training to a greater extent than matched training performed in temperate conditions.

6.4.1 Methods

6.4.1.1 Participants

Seventeen endurance-trained males took part in Training Study 3 (age, 34 ± 7 y; height, 181 ± 8 cm; mass, 80.5 ± 9.6 kg; sum of eight skinfolds, 71 ± 29 mm; recent training volume, 8 ± 2 h.week^{-1}; $\dot{V}O_{2max}$, 4.3 ± 0.7 L.min^{-1}). Three participants were in-progress when the first nationwide lock-down for COVID-19 in New Zealand was announced, hence some post-testing data in three participants (N = 2 in the heat group, N = 1 in the temperate group) was not successfully collected. The subsequent regional lock-down prevented collection of a larger sample size. The actual sample size for outcome measures is indicated in the relevant section. This study was conducted during a maintenance phase of training in all participants. The Auckland University of Technology Ethics Committee approved all procedures, and all participants provided written informed consent.

6.4.1.2 Study design

Training Study 3 adopted a randomised controlled trial design, and was conducted in the non-summer months in Auckland, New Zealand (Figure 10). The initial laboratory visit involved an incremental, maximal exercise test performed on a cycle ergometer in temperate conditions (18°C, 60% rH). This assessment was used to ascertain a physiological profile, including heart rates at individual physiological thresholds in order to individualise subsequent training interventions. Following the incremental test, participants completed a 30-min familiarisation time-trial (TT) ahead of subsequent experimental TTs. An overnight-fasted, resting muscle microbiopsy was then performed during a separate visit ~48 h following the incremental test and familiarisation TT. Participants returned to the laboratory, ~48 h later, to complete the

pre-intervention experimental TT on a cycle ergometer in temperate conditions (18°C, 60% rH). Participants subsequently commenced a three-week training programme, having been randomly allocated to either a temperate (TEMP, 18°C, 60% rH) or moderate environmental heat stress (HEAT, 33°C, 60% rH) intervention group, during which serial measures of waking HRV and subjective wellbeing were made. Following the training programme, the incremental exercise test, resting muscle microbiopsy, and experimental TT were repeated.

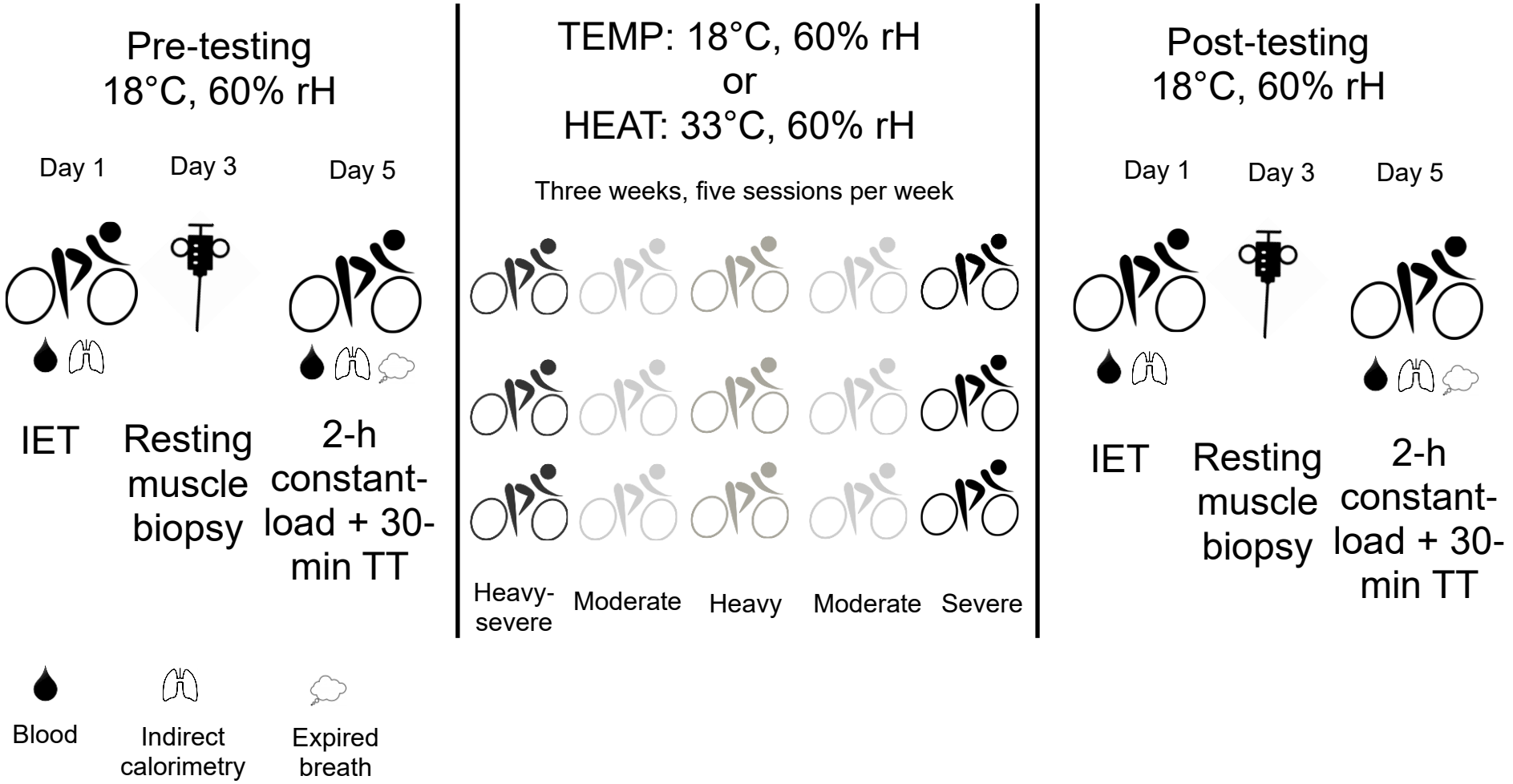


Figure 10. Schematic illustration of the design of Training Study 3.

6.4.1.3 Incremental exercise tests

Participants arrived for the incremental exercise test at ~7:00 having fasted overnight and refrained from alcohol and vigorous exercise for 24 h. Height and body mass were then recorded prior to the incremental cycling test. Cycling began at 95 W and the workload increased by 35 W every 3 min (Excalibur Sport, Lode, Groningen, NET), with continuous collection of expired gases using indirect calorimetry (TrueOne2400, ParvoMedics, Sandy, UT, US) and heart rate (RS800, Polar Electro Oy, Kempele, Finland). Capillary blood samples were obtained at the end of each 3-min stage and analysed for lactate concentration using an automatic analyser (Lactate Pro 2, Arkray, Tokyo, Japan). When blood lactate concentration exceeded 4.0 mmol.L^{-1}, step duration was reduced to 1 min until attainment of volitional exhaustion. $\dot{V}O_{2max}$ was accepted as the highest 15-s average oxygen consumption providing two of the following three criteria were met: respiratory exchange ratio >1.10, heart rate ± 10 b.min^{-1} of the age-predicted maximum heart rate (205.8 – 0.685[age (y)]) (222), and attainment of volitional exhaustion. The first ventilatory threshold (VT_1) was identified as the work rate at which the ventilatory equivalent for oxygen ($\dot{V}E.\dot{V}O_2^{-1}$) began to increase in the absence of changes in the ventilatory equivalent for carbon dioxide ($\dot{V}E.\dot{V}CO_2^{-1}$), the second ventilatory threshold (VT_2) was identified as the first work rate at which $\dot{V}E.\dot{V}O_2^{-1}$ and $\dot{V}E.\dot{V}CO_2^{-1}$ increased alongside a reduction in $PetCO_2$ (296). Two blinded expert observers determined ventilatory thresholds. Power output at 2 and 4 mmol.L^{-1} blood lactate concentrations were calculated using available software (356). Following the incremental exercise tests, participants rested for ~20 min before completing a self-paced 30-min TT, to act as familiarisation prior to the experimental TT. Following completion of the familiarisation TT, participants were instructed on how to use a smartphone application for measurement of waking HRV (HRV4Training, Amsterdam, NET), and instructed to complete it each morning for the duration of the study. Participants were provided with 'base' CHO (e.g. pasta, rice, noodles, rolled oats) to provide 1 g.kg^{-1} for dinner at ~20:00 the evening before, and 1 g.kg^{-1} for breakfast two hours prior to, the experimental TT. Participants were instructed to record any additions they made to the base CHO for these meals, photograph their prepared and finished meal, and send these photographs to the primary researcher such that these meals could be replicated precisely in advance of the post-training experimental TT. Lastly, participants were instructed to precisely record all exercise they performed throughout the pre-training assessment week, such that this could be repeated in the post-training assessment week. The replication of these training records was verified.

6.4.1.4 Muscle microbiopsies

Participants arrived at the laboratory ~48 h following the incremental exercise test at ~9:00 having fasted overnight. A muscle microbiopsy was then obtained from the *vastus lateralis* using the microbiopsy technique (184). Briefly, local anaesthesia was applied to the skin and superficial muscle fascia, after which a microbiopsy needle was inserted into the mid-belly of the *vastus lateralis* to a depth of ~2 cm to recover ~15-20 mg of tissue using a spring-loaded mechanism (14G Ultimate Biopsy Needle, Zamar Care, Croatia). Muscle tissue was immediately frozen using dry ice, and stored at -80°C until further analysis. The site of the pre-training microbiopsy was recorded by an International Society of Kinathropometry accredited anthropometrist such that the post-training microbiopsy occurred ~2 cm from the site of the pre-training biopsy.

6.4.1.5 Time-trial performance assessments

Participants arrived at the laboratory for performance TTs at ~8:00, having been asked to avoid high-^{13}C foodstuffs (e.g. corn-derived sources) and vigorous exercise for 48 h, and having ingested their standardised pre-trial dinner at ~20:00 the previous evening and standardised pre-trial breakfast ~2 h beforehand. Sum of eight skinfold thickness was then determined by an International Society of Kinathropometry accredited anthropometrist (triceps brachii, biceps brachii, subscapular, iliac crest, supraspinale, abdominal, anterior thigh, posterior shank). Participants were fitted with an antecubital venous cannula, from which a 5-mL resting blood sample was drawn, and heart rate monitor (RS800, Polar Electro Oy, Kempele, Finland). Resting expired breath samples were collected into duplicate 10-mL evacuated, plain tubes to correct exercise samples for background expired ^{13}C.

Cycling then commenced for two hours in a temperature-controlled laboratory (18°C and 60% rH) on an ergometer (Excalibur Sport, Lode, Groningen, NET) at 80% of the workload eliciting VT_1 in the pre-training IET. Convective airflow was provided by an industrial fan (FS-75, FWL, Auckland, NZ). Participants consumed 60 $g.h^{-1}$ of glucose in 7.5% liquid solutions at 15-min intervals throughout the constant-load phase. Beverages were enriched with ~47 $mg.L^{-1}$ of isotopically labelled [U-^{13}C]glucose (Cambridge Isotope Laboratories, Cambridge, MA, USA), such that the ingested solution was high in ^{13}C (~50 δ‰ vs. Pee Dee Bellemnitella [PDB]). Expired gases were collected for 4 min every 15 min using a metabolic cart (TrueOne2400, ParvoMedics, Sandy, UT, US), 5-mL venous blood samples were obtained every 30 min, heart rate was recorded continuously, and expired breath samples were collected into duplicate 10-mL evacuated plain tubes at 60, 90, and 120 min.

Upon completion of the two-hour constant-load phase, participants commenced a 30-min, maximal-effort TT (IndoorTrainer, SRM, Jülich, Germany). During the TT participants were blinded to power and heart rate, but informed of the time remaining every 10 min and with 5 and 1-min remaining.

6.4.1.6 Training protocol

Following completion of the pre-intervention TT, participants began an individualised three-week training programme, having being randomly allocated to either a temperate (TEMP) or moderate environmental heat stress (HEAT) training intervention. Training interventions were based on heart rate at the ventilatory thresholds in the pre-training IET (Table 18), and included five supervised training sessions in the laboratory each week in either 18°C (TEMP) or 33°C (HEAT), both at 60% rH. Water was consumed *ad libitum* and convective fan cooling was provided during all training sessions. Training sessions were programmed according to heart rates measured at ventilatory thresholds obtained in the pre-training IET such that the relative cardiovascular demand of the training programme was equal between-subjects and between-groups, with an acceptable range for target heart rate of ± 2 $b.min^{-1}$. When attempting to match for relative cardiovascular demand, data collected in our laboratory indicates measures of heart rate at individual physiological thresholds are stable between temperate and moderate heat stress environments (317). Therefore, there was considered no requirement for an IET to be performed under heat stress to derive these measurements for HEAT.

Subjective wellness using five-point Likert scale questionnaires assessing fatigue, sleep quality, muscle soreness, stress, and mood (327) were assessed prior to each training session, along with the duration and perceived exertion of any additional training performed since the previous session using Borg's 1-10 scale (48). Participants were asked to limit training outside of the study to a maximum of three-hours per week, all at low intensity. This was considered necessary to allow training adaptations from swimming, running, and resistance exercise to be maintained, thus reducing the possibility of detraining in other exercise modalities impacting cycling performance. Following each training session, duration, power, heart rate, and perceived session exertion using Borg's 1-10 scale were recorded. Training load was calculated using the session-RPE model (81).

Table 18. Training intervention in Training Study 3, which took place in 18°C (TEMP) or 33°C (HEAT) and 60% relative humidity. Heart rate at the first (VT_1) and second (VT_2) ventilatory thresholds was quantified in the pre-intervention incremental exercise test

	Type	Session
1	Heavy-severe	4-6 x 8 min at HR at VT_2, 2 min recovery
2	Moderate	90 min at 95 % of HR at VT_1
3	Heavy	3 x 25 min at midpoint of HR at VT_1 and VT_2, 5 min recovery
4	Moderate	90 min at 95 % of HR at VT_1
5	Severe	6-10 x 3 min at 'best session effort', 2 min recovery

Abbreviations: HR = heart rate, VT_1 = first ventilatory threshold, VT_2 = second ventilatory threshold.

6.4.1.7 Gas analyses

Indirect calorimetry was performed using a metabolic cart (TrueOne2400, ParvoMedics, Sandy, UT, US). Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) values was averaged for the final 1-min of each step during the incremental exercise tests, and 3-min prior to each measurement time-point during the TT. Whole-body rates of energy expenditure, CHO oxidation, and fat oxidation were calculated according to standard non-protein equations (Equation 2) (246), and exogenous (CHO_{exo}) and endogenous (CHO_{end}) CHO oxidation rates during the 60-120 min constant-load phase of the experimental trials were estimated using gas chromatography continuous-flow mass spectrometry (Equation 5) and standard equations (Equation 6) (89).

$$\delta^{13}C = [(^{13}C{:}^{12}C \text{ sample}/^{13}C{:}^{12}C \text{ standard}) - 1] \times 10^3 \text{ mL}^{-1}$$

Equation 5 where isotopic enrichment was expressed as $\delta.mL^{-1}$ and related to an international standard (PDB).

$$CHO_{exo} = \dot{V}CO_2 \times [(\delta Exp - Exp_{bkg})/(\delta Ing - Exp_{bkg})] \times (1/0.7467)$$

$$CHO_{end} = CHO_{tot} - CHO_{exo}$$

Equation 6 where δExp = ^{13}C enrichment of expired gas sample, δIng = ^{13}C enrichment of ingested carbohydrate, Exp_{bkg} = 13C enrichment of expired gas sampled at rest, 0.7467 = $\dot{V}CO_2$ of 1 g glucose oxidation, and CHO_{tot} = total carbohydrate oxidation rate.

6.4.1.8 Muscle analysis

Frozen muscle samples were rinsed using cold phosphate-buffered saline (PBS) and ~5 mg samples were suspended to 25 $mg.mL^{-1}$ in PBS and ground manually using a pre-cooled glass Dounce homogeniser. Resulting homogenate was solubilised with extraction buffer (ab260490, Abcam®) to 5 $mg.mL^{-1}$ and incubated on ice for 20 min prior to centrifugation at 16000 g for 20 min at 4°C. Supernatant was extracted and stored at -80°C prior to further analysis. A Bradford assay for sample protein concentration was subsequently performed in duplicate. Maximal citrate synthase activity, via a kinetic immunocapture assay (ab119692, Abcam®), and cluster of differentiation 36 (CD36) concentration, via an enzyme-linked immunosorbent assay (ab267614, Abcam®), were determined using commercially-available kits in duplicate according to the manufacturer's instructions.

6.4.1.9 Statistical analyses

Sample data is expressed mean ± standard deviation unless otherwise stated. Two-way analyses of variance were used to assess within-group, between-group, and interaction effects in measured variables, with the location of significant effects detected post-hoc using Holm-Bonferroni stepwise correction. Specific comparisons were also made using paired *t*-tests where appropriate. Significance was inferred when $P \leq 0.05$.

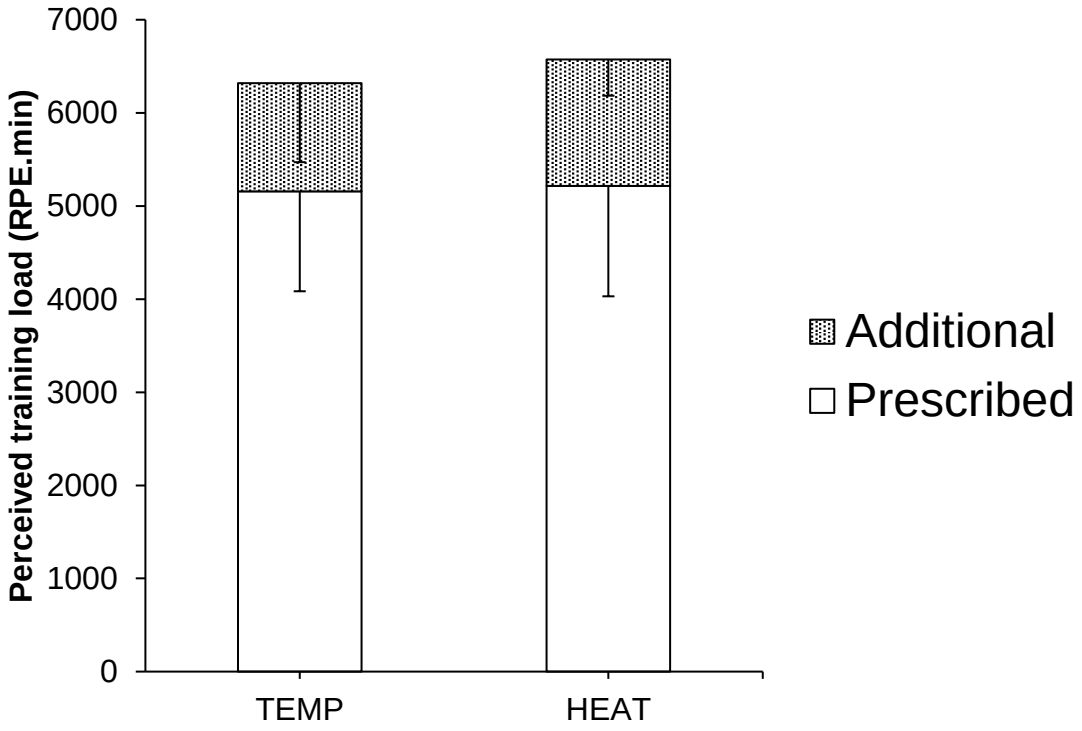
6.4.2 Results

6.4.2.1 Training interventions

All subjects completed all 15 prescribed training sessions. Total ($P = 0.67$), prescribed ($P = 0.92$), and additional ($P = 0.54$) RPE-based training load did not significantly differ between-groups (Figure 11). Corrected pairwise comparisons revealed power output achieved during the moderate-intensity training sessions, expressed relative to each individual's power at VT_1 in the pre-training IET, was significantly lower in HEAT vs. TEMP in session one (76 ± 14 vs. 91 ± 8%, $P = 0.04$), and approached significance in session two (79 ± 11 vs. 94 ± 13%, $P = 0.06$, Figure 11). Average power output during the 3-min 'best effort' repetitions in the severe-intensity training sessions, expressed relative to each individual's power at 4 $mmol.L^{-1}$ blood lactate concentration in the pre-training IET, was significantly lower in HEAT vs. TEMP ($P = 0.03$, Figure 11). No significant between- or within-group differences were observed for overall or weekly absolute HRV (expressed as the root mean square of the sum of successive

differences in R-R intervals [rMSSD]). Differences in day-to-day variation in HRV were not significant between-groups (29 ± 17 vs. 39 ± 15% in TEMP and HEAT, respectively, *P* = 0.20) although this comparison approached significance in the second training week (23 ± 14 vs. 39 ± 19% in TEMP and HEAT, respectively, Figure 11). Self-report wellbeing data throughout the training period did not differ between-groups (17.3 ± 1.5 vs. 16.9 ± 1.1 AU in TEMP and HEAT, respectively, *P* = 0.55).

(a)



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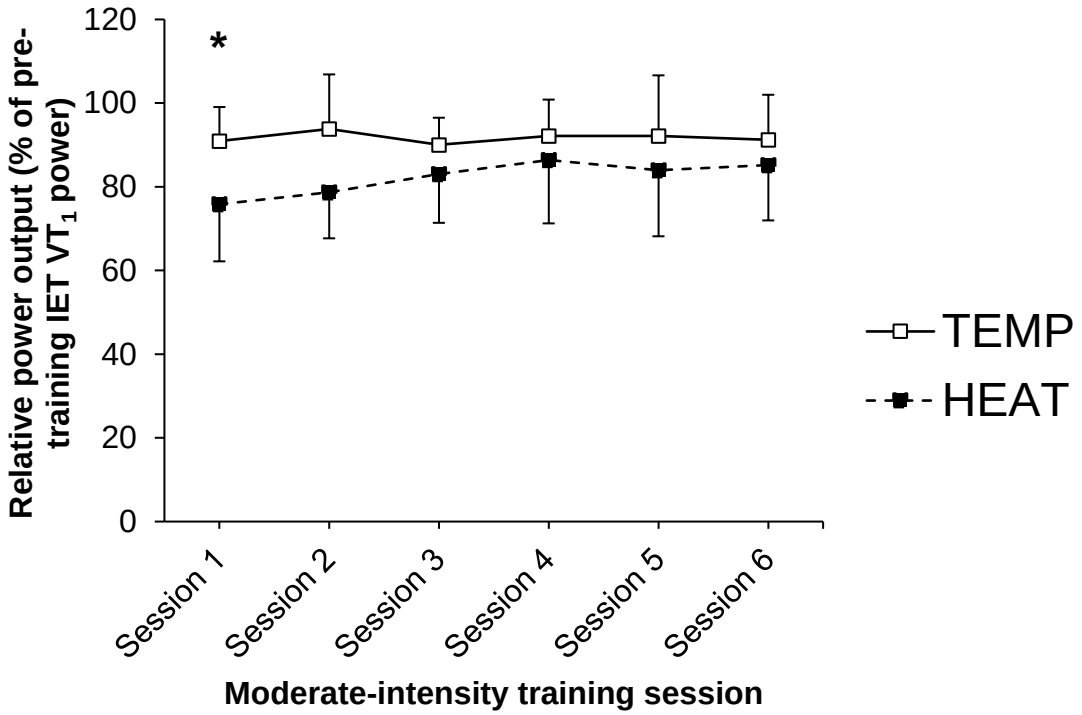
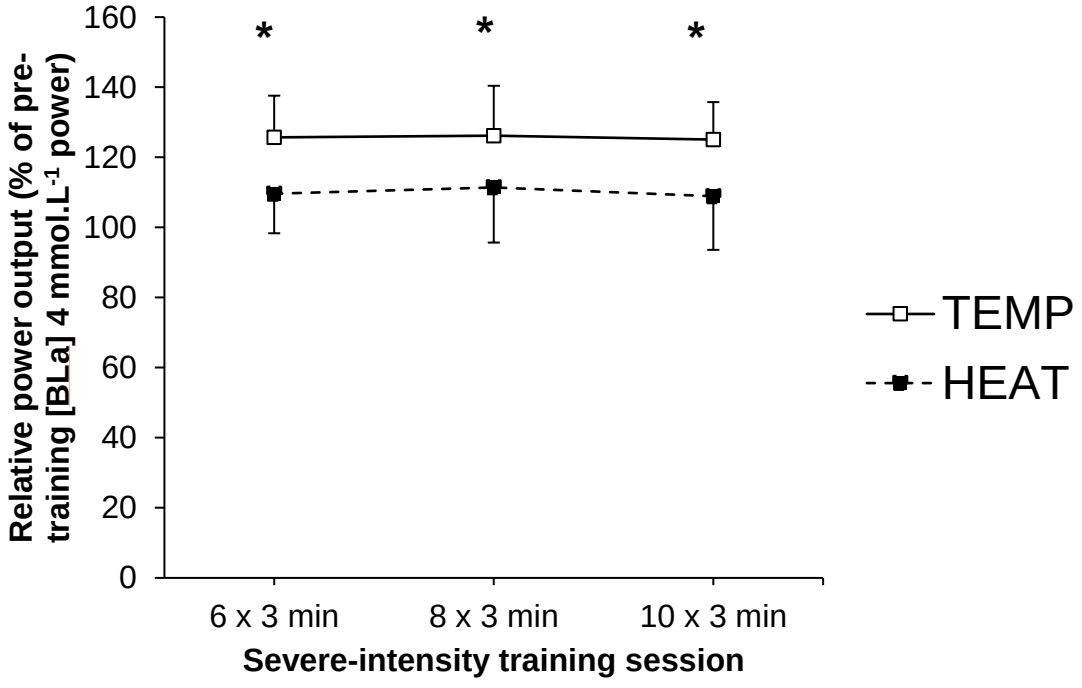

120
100
80
60
40
20
0
*
Relative power output (% of pre-training IET VT1 power)
Session 1
Session 2
Session 3
Session 4
Session 5
Session 6
Moderate-intensity training session
TEMP
HEAT

(c)


160
140
120
100
80
60
40
20
0
*
*
*
Relative power output (% of pre-training [BLa] 4 mmol.L-1 power)
6 x 3 min
8 x 3 min
10 x 3 min
Severe-intensity training session
TEMP
HEAT

(d)

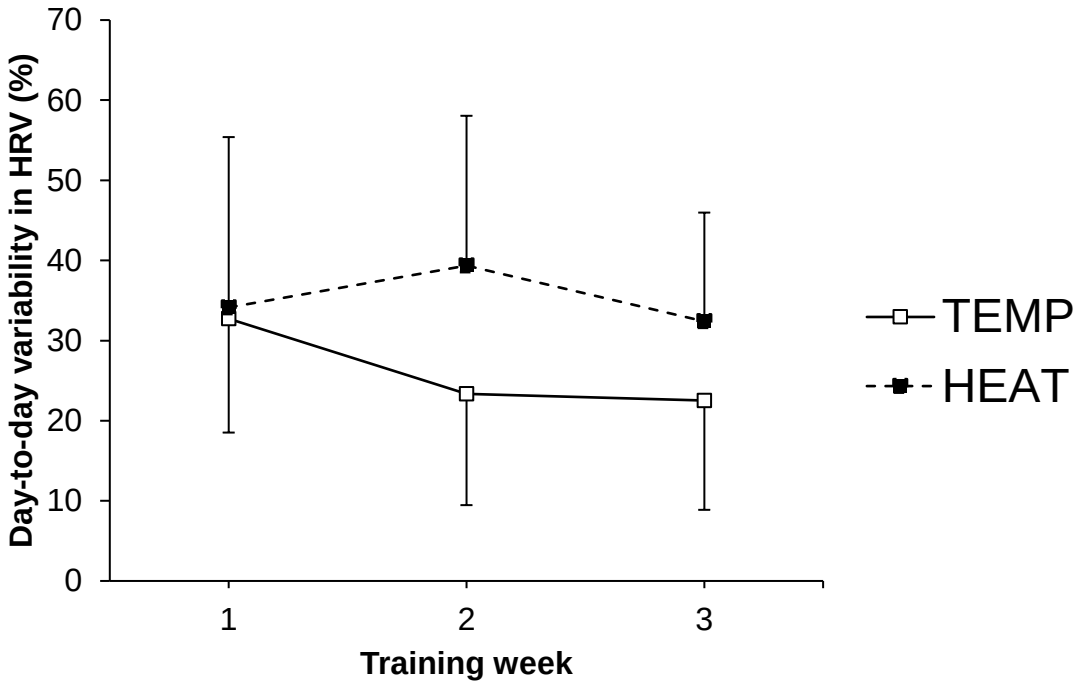


Figure 11. Characteristics of the three-week training interventions in Training Study 3. (a) RPE-time training load, (b) power output during the 90-min constant heart rate moderate-intensity training sessions relative to power output at the first ventilatory threshold (VT_1) in the pre-training incremental exercise test, (c) average 3-min repetition power output during the 6 x 3 min, 8 x 3 min, and 10 x 3 min 'best effort' severe-intensity training sessions relative to power output at 4 $mmol.L^{-1}$ blood lactate concentration in the pre-training incremental exercise test, and (d) coefficient of variation in weekly resting heart rate variability (rMSSD) in the three training weeks. '*' indicates $P \leq 0.05$ between-groups

6.4.2.2 Performance metrics

A Grubb's test revealed a significant outlier for training-induced change in pre-loaded 30-min TT performance in TEMP (+90 W, Z = 2.23, $P < 0.05$). With this outlier removed, pre-loaded 30-min TT significantly improved pre-to-post training in both groups, but to a significantly greater magnitude in HEAT (30 ± 13 vs. 16 ± 5 W, $P = 0.04$, Figure 12, Table 19). Significant main effects for time were observed for power at 2 and 4 $mmol.L^{-1}$ blood lactate concentration, VT_1, and VT_2, but significant group x time interactions were not observed (Table 19). Individual between-group pairwise comparisons for training-induced changes in these IET variables were also not significant ($P > 0.07$).

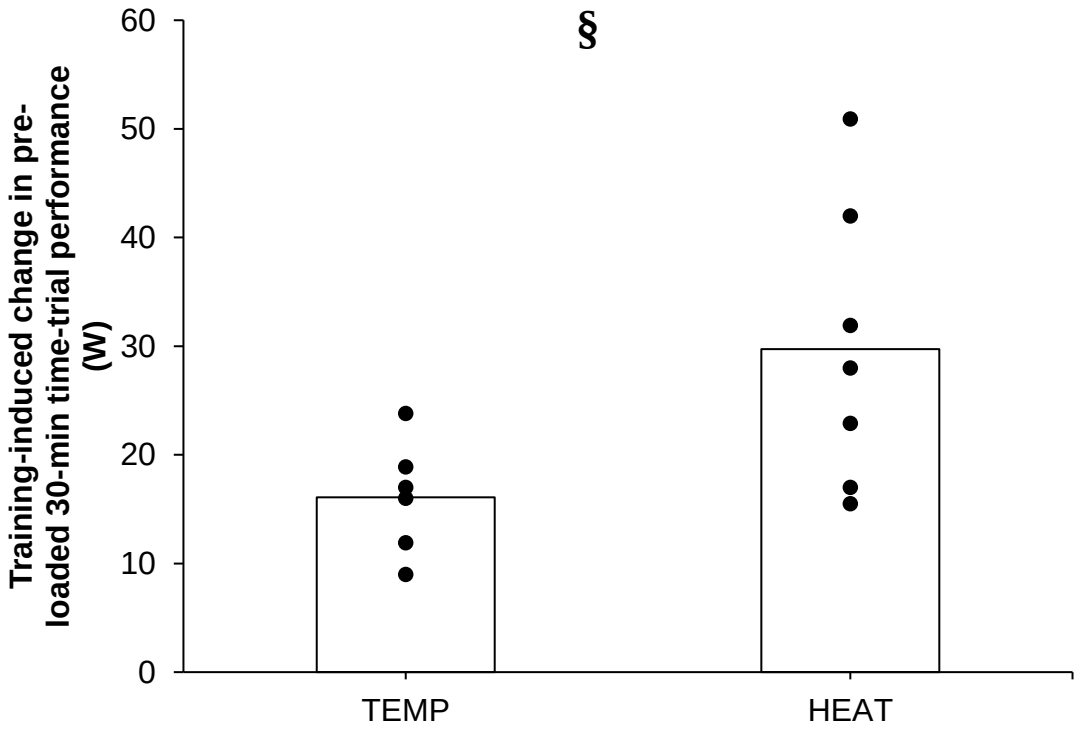


Figure 12. Training-induced changes average power output during the pre-loaded 30-min time-trial performance assessment in TEMP (N = 6) and HEAT (N = 7). Bars indicate group mean changes and dots indicate the results of individual participants. '§' indicates $P \leq 0.05$ between-groups.

Table 19. Functional response data with statistical comparisons. '*' indicates significantly different vs. pre-training. '§' indicates significantly different between-groups. Significance is inferred when $P \leq 0.05$. Performance time-trial data compares 6 subjects from the temperate training group, 7 subjects from the heat training group. Remaining data is derived from the incremental exercise tests features 9 subjects in the temperate training group, 8 subjects from the heat training group

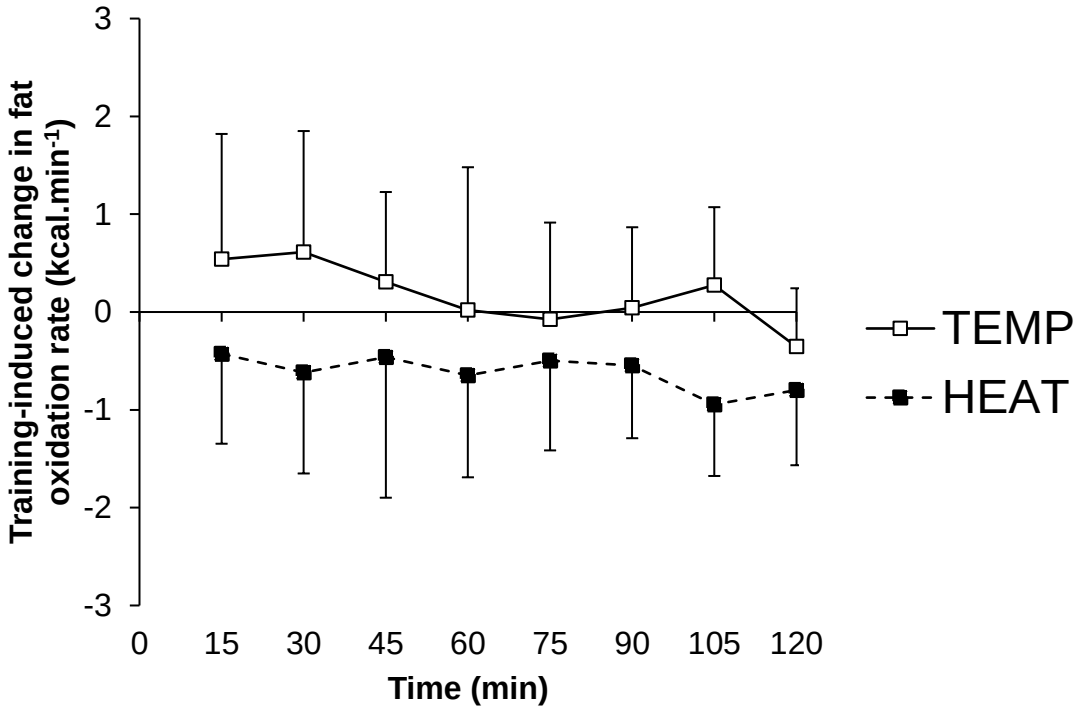
	Temperate training group			Heat training group			ANOVA		
	Pre	Post	Δ	Pre	Post	Δ	Group	Time	G x T
30-min TT (W)	253 ± 54	269 ± 57*	16 ± 5§	240 ± 38	270 ± 47*	30 ± 13§	.813	<.001	.038
[BLa] 2 mmol.L^{-1} (W)	192 ± 62	203 ± 61*	12 ± 14	198 ± 66	226 ± 50*	28 ± 33	.618	.008	.221
[BLa] 4 mmol.L^{-1} (W)	241 ± 53	251 ± 54*	9 ± 8	256 ± 55	275 ± 58*	19 ± 20	.472	.002	.219
VT_1 (W)	190 ± 48	197 ± 34	7 ± 11	191 ± 48	213 ± 44*	22 ± 21	.657	.003	.082
VT_2 (W)	232 ± 45	251 ± 48*	20 ± 8	253 ± 56	276 ± 59*	23 ± 18	.388	<.001	.590
$\dot{V}O_{2max}$ (L.min^{-1})	4.25 ± 0.81	4.29 ± 0.82	0.04 ± 0.15	4.29 ± 0.70	4.31 ± 0.66	0.02 ± 0.16	.930	.465	.800

Abbreviations: [BLa] = blood lactate concentration, 30-min TT = pre-loaded 30-min time-trial, $\dot{V}O_{2max}$ = maximum oxygen consumption, VT_1 = first ventilatory threshold, VT_2 = second ventilatory threshold

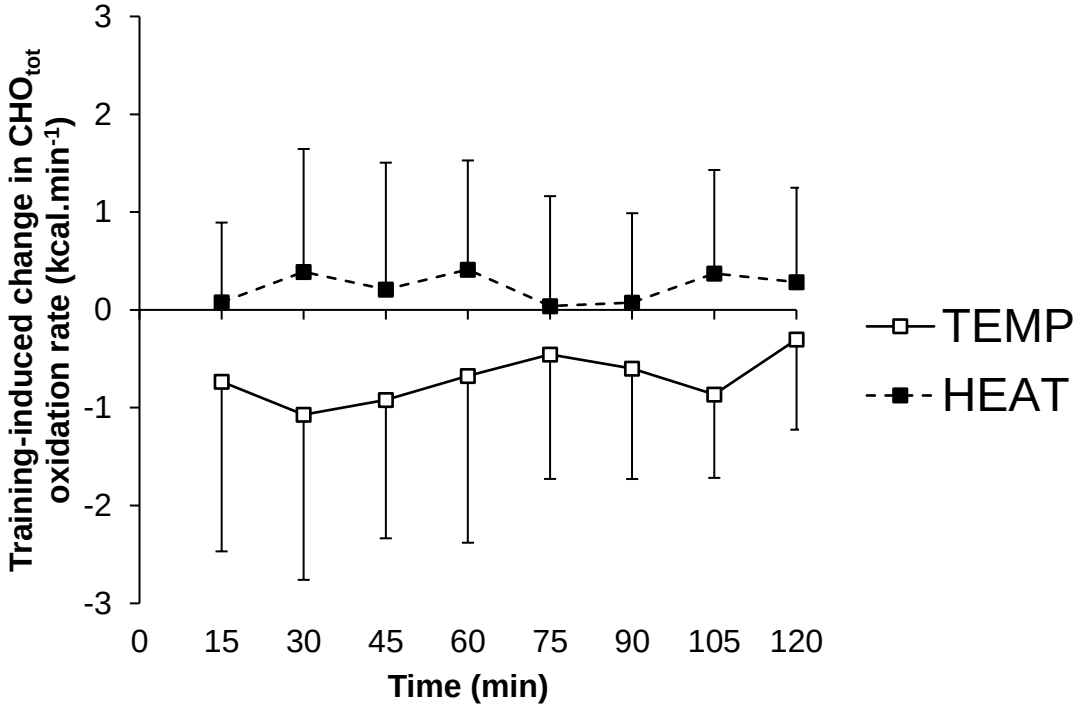
6.4.2.3 Metabolic responses

During IET, a significant group x time interaction was for the observed maximal fat oxidation rate (MFO) whereby the training-induced change in MFO was significantly lower in HEAT vs. TEMP (Table 20). During the constant-load phase of the TT, gross efficiency (GE) significantly improved in TEMP, but not HEAT, but significant group x time interactions were not observed for fat, total CHO, endogenous CHO, or exogenous CHO oxidation rates (Figure 13). Significant main effects of time were observed for mean HR during, and HR at 120 min of, the constant-load phase of the TT, though no significant group x time interactions were observed (Table 20).

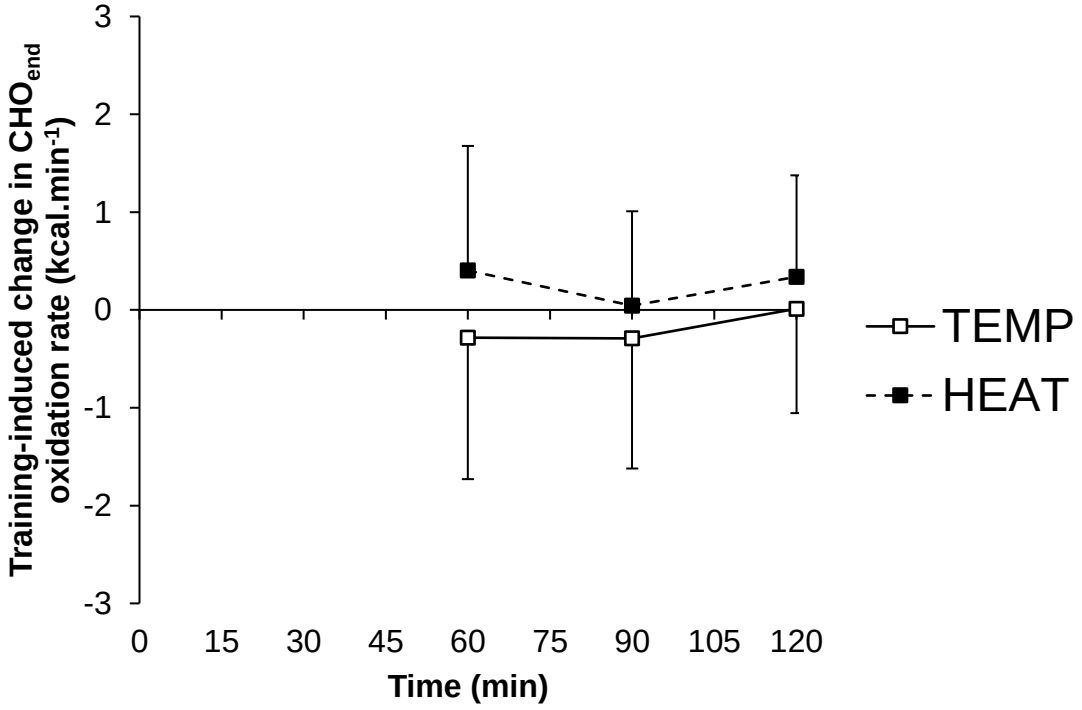
(a)



(b)


Training-induced change in CHOtot oxidation rate (kcal.min-1)
3
2
1
0
-1
-2
-3
0
15
30
45
60
75
90
105
120
Time (min)
TEMP
HEAT

(c)


Training-induced change in CHOend oxidation rate (kcal.min-1)
3
2
1
0
-1
-2
-3
0
15
30
45
60
75
90
105
120
Time (min)
TEMP
HEAT

(d)

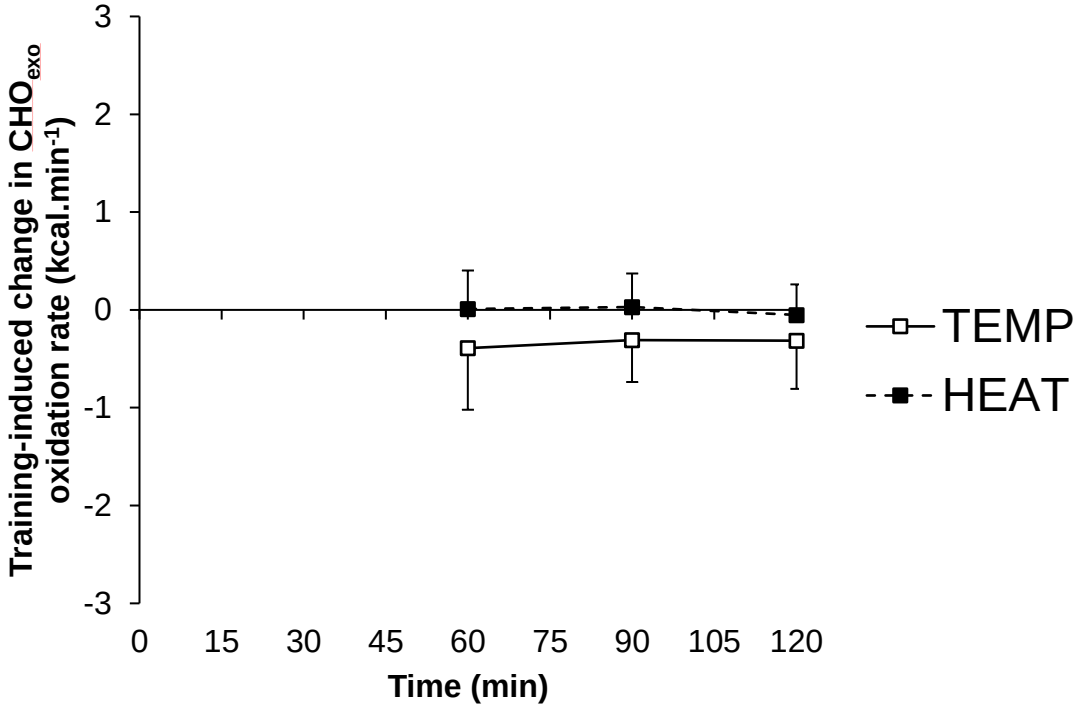


Figure 13. Training-induced changes in (a) fat, (b) total carbohydrate (CHO_{tot}), (c) endogenous carbohydrate (CHO_{end}), and (d) exogenous carbohydrate (CHO_{exo}) oxidation rates during the 120-min constant-load phase of the time-trial. For all variables 7 subjects are included in the temperate and heat training groups.

Table 20. Metabolic data from Training Study 3 with statistical comparisons. '*' indicates significantly different vs. pre-training. '§' indicates significantly different between-groups. Significance was inferred when $P \leq 0.05$. Data derived from the incremental exercise tests features 9 subjects in the temperate training group, 8 subjects from the heat training group. Performance time-trial data compares 7 subjects from the temperate training group, 7 subjects from the heat training group

	Temperate training group			Heat training group			ANOVA		
	Pre	Post	Δ	Pre	Post	Δ	Group	Time	G x T
GE (%)	19.7 ± 1.1	20.6 ± 1.2*	1.0 ± 0.9	18.9 ± 0.9	19.6 ± 0.7	0.7 ± 0.9	.080	.006	.574
Fat oxidation (g)	36 ± 23	39 ± 26	3 ± 10	40 ± 19	33 ± 23	-7 ± 11	.936	.447	.104
CHO_{tot} (g)	254 ± 38	233 ± 39	-22 ± 36	228 ± 68	235 ± 50	6 ± 29	.650	.411	.139
Mean CHO_{exo} ($g.min^{-1}$)	0.63 ± 0.08	0.55 ± 0.13	-0.08 ± 0.12	0.49 ± 0.06	0.49 ± 0.06	0.00 ± 0.07	.025	.126	.138
Mean CHO_{end} ($g.min^{-1}$)	1.46 ± 0.22	1.41 ± 0.35	-0.05 ± 0.28	1.42 ± 0.55	1.49 ± 0.43	0.06 ± 0.25	.932	.899	.459
Mean HR ($b.min^{-1}$)	133 ± 14	128 ± 11	-5 ± 9	123 ± 7	119 ± 7	-4 ± 5	.078	.036	.838
HR at 120 min ($b.min^{-1}$)	137 ± 14	131 ± 11	-6 ± 11	126 ± 8	120 ± 7	-6 ± 8	.038	.035	.978
MFO ($g.min^{-1}$)	0.57 ± 0.20	0.61 ± 0.30	0.05 ± 0.14§	0.60 ± 0.20	0.50 ± 0.14	-0.09 ± 0.12§	.762	.567	.046

Abbreviations: [BLa] = blood lactate concentration, CHO_{tot} = total whole-body carbohydrate oxidation, CHO_{end} = endogenous carbohydrate oxidation, CHO_{exo} = exogenous carbohydrate oxidation, GE – gross cycling efficiency during the 120-min constant-load phase of the time-trial, MFO = highest observed rate of fat oxidation during the incremental exercise test.

6.4.2.4 Muscle adaptations

A significant main effect of time was observed for maximal *vastus lateralis* citrate synthase activity (P = 0.007), but not group x time interaction (P = 0.149). Pairwise comparisons detected a significant increase in maximal *vastus lateralis* citrate synthase activity in HEAT (fold-change, 1.25 ± 0.25, P = 0.03) but not in TEMP (fold-change, 1.10 ± 0.22, P = 0.22). The magnitude of the training-induced change in maximal *vastus lateralis* citrate synthase activity was not significantly different between-groups (P = 0.25, Figure 14). No significant main effects of group (P = 0.59) or time (P = 0.09), or group x time interaction (P = 0.97), were observed for CD36 concentration per unit of muscle protein. Specific comparisons also did not reveal significant training-induced changes in muscle CD36 concentration in TEMP (pre-training, 15.7 ± 16.3; post-training, 19.1 ± 20.3 pg.μg^{-1} protein, P = 0.14) or HEAT (pre-training, 20.1 ± 11.5; post-training, 23.3 ± 13.1 pg.μg^{-1} protein, P = 0.34).

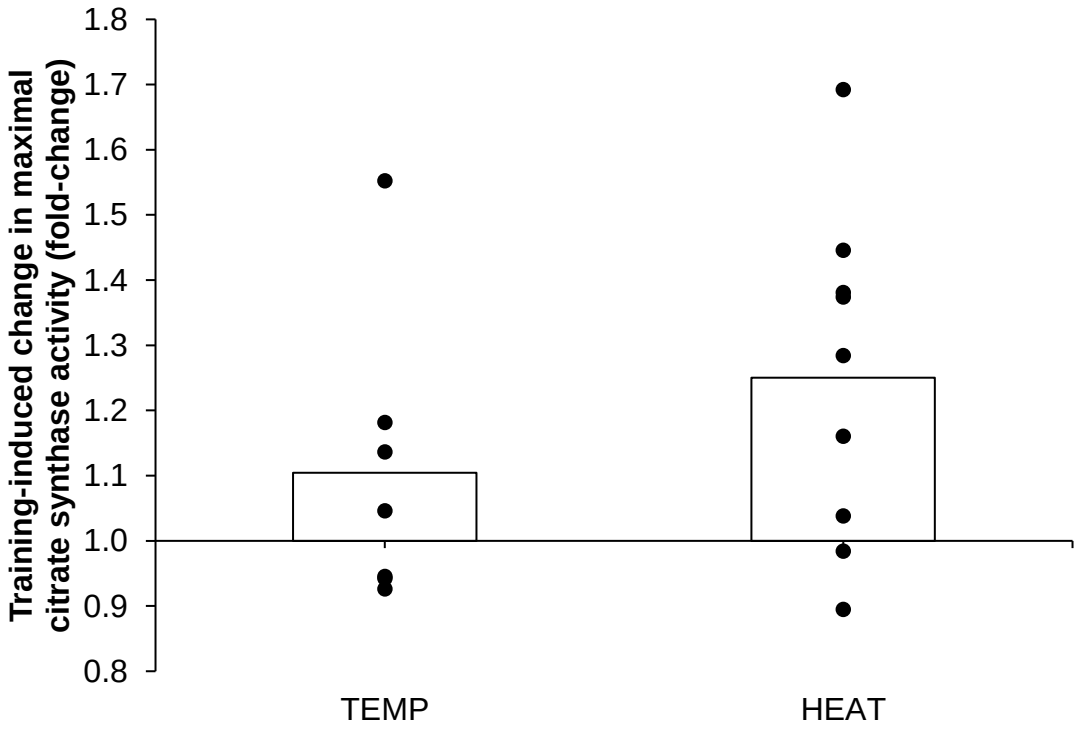


Figure 14. Training-induced change in maximal citrate synthase activity in TEMP (N = 7) and HEAT (N = 9). Bars indicate mean pre- to post-training fold-change values and dots indicate individual responses.

6.4.3 Discussion

The primary aim of Training Study 3 was to determine the effect of a three-week endurance training intervention performed under moderate environmental heat stress on metabolic adaptations and performance in a temperate environment. The main

findings were that (i) training performed in 33°C improved pre-loaded 30-min TT performance in temperate conditions to a significantly greater extent than matched training performed in 18°C, (ii) maximal *vastus lateralis* citrate synthase activity significantly increased following training when training was performed in 33°C but not 18°C, and (iii) the effect of training under moderate environmental heat stress for whole-body metabolic responses during exercise was unclear.

Using the information discerned from Training Study 1 and Training Study 2, training interventions in Training Study 3 were completed successfully, with all participants completing all 15 prescribed sessions. As expected, relative power output achieved during moderate- and severe-intensity training sessions was lower in HEAT compared to TEMP (Figure 11), although by the end of the intervention power output during moderate-intensity sessions was not significantly different between-groups (Figure 11). Reduced mechanical training stress when under moderate environmental heat stress can be easily explained by the increased heart rate observed at a given power output (369, 418), and reduced power output achieved at given physiological thresholds (232, 292), when under moderate environment heat stress, effects observed in Training Study 2. Therefore, in Training Study 3, where training interventions in TEMP and HEAT were performed at matched relative cardiovascular load alongside 'best-effort' severe-intensity interval training sessions, reduced mechanical training stress was expected in HEAT. Importantly, RPE-based training load and self-reported wellbeing were not significantly different between-groups (Figure 11), which indicates the training interventions were successfully matched, therefore allowing between-group effects to be attributed to the addition of moderate environmental heat stress in HEAT. Interestingly, there appeared a tendency towards increased day-to-day variability in HRV in the second training week in HEAT vs. TEMP (39 ± 19 vs. 23 ± 14%, respectively, P = 0.08, Figure 11), which may be indicative of greater modulation of cardiac-autonomic balance, and therefore a physiological stress response, in HEAT (129, 235).

In line with the pre-trial hypothesis, power output during a 30-min TT preceded by two hours of constant-load cycling and performed in a temperate environment significantly increased in TEMP and HEAT, but to a significantly greater magnitude in HEAT (30 ± 13 vs. 16 ± 5 W, P = 0.04, Figure 12, Table 19). This aligns with incremental exercise test data collected in this study showing significant training effects for power output at 2 and 4 mmol.L^{-1} blood lactate concentrations, although interactions between group and time were not significant (Table 19). A significant training-induced increase in maximal *vastus lateralis* citrate synthase activity, a marker of mitochondrial protein content and therefore interpreted in this context as training-induced mitochondrial biogenesis (273),

occurred in HEAT but not TEMP (Figure 14). This may indicate the effect of moderate environmental heat stress during endurance training on temperate performance is at least partially attributable to effects on mitochondrial adaptations to training. The performance data aligns with recent research reporting significant improvements in 15-min temperate TT performance in a group of elite cyclists performing 4-5 sessions per week for five weeks in 37.5-38.5°C, but not 15.5°C (413). In that study, performance-enhancing effects of training under heat stress were partially attributed to significant positive effects on haemoglobin mass not observed in the temperate training group. Increased haemoglobin mass therefore increased $\dot{V}O_{2max}$ (413), given oxygen delivery appears limiting to $\dot{V}O_{2max}$ (345). In the present study, training-induced changes in $\dot{V}O_{2max}$ were not observed (Table 19), possibly due to the limited number of training sessions performed in the severe-intensity domain (once per week), given time >90%$\dot{V}O_{2max}$ during training appears to be influential for training-induced changes in $\dot{V}O_{2max}$ (414, 416). Therefore, as an increase in haemoglobin mass and oxygen-carrying capacity would be expected to increase $\dot{V}O_{2max}$, haematological adaptations may not have made a substantial contribution to the observed greater improvement in 30-min TT performance in HEAT in the present investigation, which would further suggest the positive mitochondrial adaptations observed in HEAT contributed to the observed performance responses. However, haematological parameters were not measured and therefore cannot be dismissed.

Increased mitochondrial protein content would be expected to increase the rate of oxidative metabolism, and therefore external workloads, at which a metabolic steady-state can be achieved (29, 200, 254). Alongside the possibility of increased metabolically-sustainable rates of oxidative metabolism, the increase in gross cycling efficiency (GE) observed during the 2-h constant-load phase (Table 20), may have contributed to an increase in the external workloads achieved for a given rate of oxidative metabolism, assuming this effect was observed at greater workloads. However, this effect is unlikely to explain the significantly greater increase in TT performance in HEAT, given a significant increase in constant-load GE was observed in TEMP and not HEAT, with the interaction between group and time for GE not significant (Table 20). As such, it is likely the greater improvement in temperate 30-min TT performance in HEAT is at least partially attributable to adaptations related to mitochondrial protein content, and therefore metabolic steady-state workloads.

The mechanism by which mitochondrial adaptation to training may have been augmented by moderate environmental heat stress cannot be confirmed in the present investigation, and is intriguing in the context of the lower relative mechanical workloads during training in HEAT (Figure 11). Endurance training performed under moderate

environmental heat stress augments expression of heat shock proteins (452), which chaperone newly-synthesised mitochondrial proteins from the nucleus to the mitochondria (518), as well as circulating catecholamines (122, 177), which are implicated in PGC-1α mRNA expression (13, 57), a transcriptional co-activator involved in the coordination of mitochondrial adaptation to training (513). Moderate environmental heat stress may also increase muscle glycogenolysis (122) and interleukin-6 expression (461) in response to endurance training, both of which are implicated in the activation of AMPK, a signalling kinase upstream of PGC-1α (302, 509). Lastly, the greater lactate accumulation in muscle and blood when exercise is performed under moderate environmental heat stress (122, 318) may exert direct effects on mitochondrial adaptation (469). Accordingly, it could be hypothesised that the addition moderate environmental heat stress during training in HEAT interacted with gene expression, protein activation, and protein chaperoning implicated in the adaptive mitochondrial response to exercise. However, previously it was reported that transcription of several genes related to mitochondrial biogenesis was blunted 3-h following 1-h constant-load cycling in 33 vs. 20°C, with no effect on nuclear PGC-1α translocation (186). Reconciling these results with the present investigation, it is possible moderate environmental heat stress alters the time-course of post-exercise signalling responses related to mitochondrial adaptation, or that an interaction between the specific exercise training parameters and moderate environmental heat stress alters the adaptive consequences. It is indeed also possible our incomplete understanding of adaptive signalling pathways coordinating mitochondrial adaptation to endurance training makes translation of acute signalling studies to longitudinal training adaptation difficult (226). It is recommended that future studies seek to verify our evidence of increased mitochondrial adaptation to endurance training performed under moderate environmental heat stress, and investigate the acute signalling mechanisms that regulate this effect.

In terms of adaptations to whole-body metabolic responses to fasted incremental and fed, constant-load exercise, the effect of environmental heat stress during training was unclear (Figure 13, Table 20). Adaptation to the maximum rate of fat oxidation observed during the fasted incremental tests was significantly different between-groups, with the training-induced change significantly lower in HEAT (Table 20). This aligns with the 2-h constant-load data, where mean responses tending to indicate a possible reduction in fat oxidation with HEAT, though this was not statistically significant (Figure 13, Table 20). The effect of exercise training interventions on whole-body fat oxidation rates during exercise is mixed (20, 440, 484), although trained athletes tend to exhibit greater fat oxidation rates than untrained individuals (316), and thus the prevailing paradigm is that endurance training increases fat oxidation rates at

given workloads. The possibility of blunted exercise fat oxidation in response to HEAT is therefore interesting, and could plausibly relate to lower utilisation of and therefore adaptation to fatty acid metabolism pathways during training (122, 177). Adaptations to resting muscle CD36 protein content, a sarcolemmal fatty acid transport protein, were not observed in either group, although it should be acknowledged that between-subject variability in training-induced fold-changes for CD36 were substantial in both groups (TEMP, 0.78-2.17; HEAT, 0.58-2.20), and that it remains possible adaptations may have been observed at other levels of fatty acid metabolism. Alternatively, differences in glycogen storage following training may have been influential here, given glycogen availability exerts an autoregulatory effect on substrate metabolism during exercise (179), however, resting glycogen content was not measured. Therefore, the effects of endurance training performed under heat stress for acute metabolic responses to exercise in temperate conditions, and whether they are ergogenic or maladaptive, is not easily discernible from the present data. These effects therefore require further investigation in mechanistic studies with larger sample sizes and therefore statistical power.

From an applied perspective, the results of Training Study 3 provide tentative support for use of block periods of moderate environmental heat stress during training as a means of up-regulating adaptations relevant to endurance performance in temperate conditions (183). The perceptual and HRV data presented here also indicate this training methodology can be effectively utilised without large perturbations to athlete wellbeing or inducing maladaptation, which has been shown previously (406). It is important to recognise the specific duration, training programme, athletic population, and magnitude of environmental heat stress may have been critical to the adaptive responses observed here, and therefore longer training blocks or hotter conditions may not produce the same responses. Accordingly, it is suggested that future studies should look to assess these responses using different training programmes, elite and female athletes, and in different magnitudes of environmental heat stress.

In summary, a three-week endurance training intervention performed in 33°C produced significantly greater positive effects on pre-loaded 30-min TT performance in a temperate environment than a matched training programme performed in 18°C. This effect may have been at least partially attributable to beneficial effects of moderate environmental heat stress on mitochondrial adaptations to training. These results therefore provide support for the use of moderate environmental heat stress during a well-controlled endurance training intervention for up-regulating adaptations relevant to performance in a temperate environment. It is recommended that future studies assess

the effect of different combinations of training and environmental heat stress on temperate performance adaptations including mitochondrial biogenesis

6.5 Summary of training studies

The primary aims of the training-related studies presented in this thesis were to (i) describe how heat stress training camps are used by elite endurance athletes in the real-world, (ii) investigate whether heart rates at individual physiological thresholds are consistent between temperate and moderate heat stress environments, and (iii) determine if environmental heat stress exerts an additive effect on the adaptive metabolic response to endurance training in humans. The main findings were that:

1. Elite endurance athletes were able to 'successfully' undertake heat stress training camps without compromising self-report wellbeing, HRV, or training intensity distribution, and primarily used temperate-measured threshold heart rates to guide training intensity (Table 14, Table 15, Table 16).
2. Heart rates corresponding to individual physiological thresholds appear to be relatively consistent between temperate and moderate heat stress environments (Figure 9).
3. A simulated three-week heat stress training camp in recreationally-trained males produced significantly greater increases in endurance performance than a three-week training programme matched for cardiovascular demand in temperate conditions (Figure 12), an effect which may have been explained by augmented mitochondrial adaptation to training (Figure 14).

These findings therefore support the use of specific blocks of endurance training under moderate environmental heat stress for purposes of up-regulating training adaptations relevant to performance in temperate conditions. Specifically, the data presented in this chapter suggests that well-controlled heat stress training camps can elicit positive mitochondrial adaptations to training, and exert greater beneficial effects on performance in temperate conditions without compromising athlete wellbeing or inducing maladaptation. In the next chapter, the acute and chronic metabolic implications of environmental heat stress for endurance athletes will be discussed in the context of the addition of the present thesis to the pre-existing literature.

7 Discussion: What are the metabolic implications of environmental heat stress for endurance athletes?

As discussed throughout this thesis, many endurance athletes are exposed to environmental heat stress during training and competition. This can be through incidental exposure, such as on hot days during summer months, in planned training sessions for heat acclimatisation or in an effort to up-regulate the adaptive response to training, or during competition (71, 183, 403, 404). With exposure to environmental heat stress during endurance training, greater increases in core and muscle temperatures (122, 369), peripheral blood flow (160, 434), evaporative sweat loss (242, 474), dehydration (195), heart rates (369, 418), and circulating catecholamines (121) are observed compared to when the same exercise is performed in temperate conditions. As has been the focus of this thesis, the physiological perturbations evoked by exercise-heat stress have the potential to impact acute substrate metabolism during endurance exercise (118) (

Table 4, Acute Studies 1-3, Chapter 5), and metabolic adaptations with repeated exercise-heat stress during endurance training (183), a topic that had seen limited systematic evaluation prior to this thesis (Training Studies 1-3, Chapter 6).

Understanding the acute effects of exposure to environmental heat stress on substrate metabolism during endurance exercise has implications for athletes, practitioners, and researchers. During prolonged exercise, CHO and fat are the primary substrates oxidised to resynthesise ATP and support energy metabolism (290, 412). The total endogenous CHO pool is finite, typically <3000 kcal (162), with ~80% in skeletal muscle and ~10-15% in the liver (236). In contrast, human fat storage is vast, and primarily situated in subcutaneous and visceral adipose tissue cells as triacylglycerol (143), although intramuscular triacylglycerol is also present (496). These inter-substrate disparities in human energy storage have implications during prolonged exercise. Prolonged exercise of sufficient length and intensity will eventually deplete working skeletal muscle glycogen to very low concentrations (7, 35, 36, 189, 217, 218), with depletion of muscle glycogen sequestered in the intramyofibrillar compartment specifically associated with impaired fatigue resistance (361) and tetanic Ca^{2+} handling (359, 375), implicating a role for these stores in excitation-contraction coupling and therefore their depletion in muscle fatigue. Evidence for the applied relevance of endogenous CHO availability in endurance capacity, performance, and fatigue is provided by the observed ergogenic effects of pre-exercise CHO loading and exogenous CHO ingestion during exercise (16, 35, 69, 95, 101, 269, 275, 324, 370, 420, 462), and the model of substrate metabolism during Ironman Triathlon presented in this thesis suggesting CHO oxidation in these events is likely close to, or exceeds, pre-exercise endogenous CHO availability (Chapter 2.2.4.1) (Table 2). This is particularly notable in the present context, given the annual Ironman World Championships take place in Kaliua-Kona, Hawaii at high environmental temperatures. Therefore, understanding the likely acute metabolic implications of exposure to environmental heat stress during endurance exercise may help inform fuelling strategies, training programming when focused on stimulating a particular metabolic pathway, and the likely limiting factors to performance in given combinations of exercise-heat stress.

Similarly, understanding the metabolic adaptations to repeated exercise-heat stress during endurance training also has implications for athletes, practitioners, and researchers, given this practice is common-place in endurance sport (71, 183, 314). Elite endurance athletes are defined by their ability to sustain high metabolic rates over prolonged periods in order to sustain high external workloads (296, 298), with this physiological trait partly underpinned by skeletal muscle mitochondrial protein content

and activity (29, 200). Mitochondria are the sites of β-oxidation of fatty acids to acetyl CoA, oxidation of acetyl CoA from fatty acid and non-fatty acid-derived sources in the citric acid cycle, and oxidative phosphorylation along the electron transport chain (199, 322, 512). Greater mitochondrial protein content and activity would result in lower ADP concentrations in response to given workloads (29, 200), thus attenuating stimulation of phosphofructokinase, glycolysis, and lactate formation, as well as creatine kinase and therefore phosphocreatine (PCr) degradation (29, 200, 254). Therefore, greater mitochondrial protein content and activity would be expected to increase the external workloads at which a metabolic steady-state can be attained, therefore increasing the external workloads that can be sustained for prolonged periods in training and competition. Importantly, skeletal muscle mitochondrial properties are responsive to endurance training (166, 167, 209, 339, 436, 458). Therefore, understanding the effect of repeated exposure to environmental heat stress during endurance training for adaptations to skeletal muscle mitochondria has strong implications for endurance athletes considering undertaking heat stress training camps or specific blocks of exercise-heat stress in preparation for events in temperate or hot conditions.

Therefore, the purpose of this chapter is to discuss the metabolic implications of environmental heat stress for endurance athletes in the context of previous literature and the addition of the data collected and presented in this thesis. This chapter is divided into three parts; discussion of the acute metabolic effects of environmental heat stress, discussion of the effects repeated exposure to environmental heat stress during endurance training on metabolic adaptations relevant to performance in temperate conditions, and an overall summary with proposed future research directions.

7.1 Implications of environmental heat stress for substrate metabolism during acute endurance exercise

A number of well-controlled investigations have shown the physiological perturbations evoked by exposure to environmental heat stress during endurance exercise can manifest metabolically as a shift in metabolism towards CHO oxidation and away from fat oxidation (118) (

Table 4). Studies have demonstrated enhanced net muscle glycogenolysis (121, 122, 124, 126, 242), whole-body CHO oxidation (104, 121, 122, 124, 242, 307, 318), and hepatic glucose output (177) during prolonged exercise performed under environmental heat stress (>30°C) compared to equivalent exercise in temperate conditions, with reduced whole-body fat oxidation rates (104, 121, 122, 318). The mechanisms regulating this heat stress-induced shift in substrate metabolism during endurance exercise have been independently related to the increases in core temperature (123), muscle temperature (119, 460), circulating catecholamines (120, 212, 213, 234, 495), and dehydration (178, 287, 288) observed when exercise is performed under heat stress (Figure 1). Furthermore, when considering exercise with concomitant exogenous CHO feeding, it appears heat stress blunts the rate at which exogenous CHOs are oxidised (242), an effect plausibly mediated by reduced splanchnic blood flow (268), or inhibition of muscle glucose uptake via greater build-up of glycogenolytic intermediates such as glucose-6-phosphate, secondary to the accelerated rates of muscle glycogenolysis (121, 122, 124, 126, 242). Accordingly, the reduced capacity for exogenous CHO oxidation during exercise under heat stress demonstrates the observed heat stress-induced stimulation of endogenous CHO metabolism is present in fasted (122) and fed (242) conditions.

However, it must be acknowledged that a number of studies making measurements of substrate metabolism during acute endurance exercise performed under environmental heat stress have not reported these changes (1, 148, 455, 514, 516) (

Table 4). The addition of the present thesis to the literature helps reconcile these apparently contradictory results (Acute Studies 1-3, Chapter 5), as the data presented here suggests regulatory effects of exercise intensity and the specific magnitude of the environmental heat stress (Table 7, Table 10, Figure 7), with the regulatory effects of exercise intensity replicated in a recently published study (421). Indeed, in this thesis, it was demonstrated that the magnitude of the change in substrate oxidation rates evoked by heat stress was larger at higher absolute workloads, with the workload at which heat stress-induced changes in substrate oxidation rates was observed lowered by increasing environmental temperature. Specifically, heat stress-induced changes in CHO oxidation rates at moderate exercise intensities (the absolute workload at which the first ventilatory threshold is observed in temperate conditions) were not observed under moderate environmental heat stress (34-36°C, Acute Studies 1-3, Table 7, Table 10, Table 13, Figure 7), whereas at 40°C heat stress-induced stimulation of CHO oxidation rates at this intensity was observed (Acute Study 2, Table 10, Figure 7). The mechanism regulating these effects is not clear, although interactive effects of environmental temperature and exercise intensity on plasma adrenaline concentrations may partially mediate this response (Table 9). Accordingly, it is suggested that previous studies failing to observe an effect of environmental heat stress on substrate metabolism during acute endurance exercise may be explained by use of an exercise-heat stress stimulus that was insufficient to induce the necessary physiological perturbations required to augment CHO metabolism (1, 148, 455, 514, 516).

Therefore, it may be inferred that moderate environmental heat stress-induced changes in substrate oxidation rates at moderate exercise intensities are perhaps not as substantial as previously thought (118, 121, 122), given many of the previous studies showing substantial heat stress effects employed more extreme combinations of environmental heat stress (temperature and the absence of convective fan cooling) and/or exercise intensity (104, 121, 122, 124, 126, 177, 242, 307), which have been shown to be influence the magnitude of heat stress effects on substrate oxidation rates (Table 7, Table 10, Figure 3, Figure 7). Of specific relevance when seeking to apply these findings to the real-world is the likely reduction in absolute external workloads achieved during exposure to environmental heat stress, given hot temperatures lower work outputs at given heart rates, physiological thresholds, and perceived exertions (232, 292, 369, 418). Lower absolute workloads necessitate lower overall energy expenditures and substrate fluxes, and data presented in this thesis tentatively suggests the reduced energy expenditures achieved under moderate environmental heat stress may actually be supported by *reduced* whole-body CHO oxidation rates (Figure 5). This has implications for athletes training or competing under heat stress, as a specific session type, such as a training session designed to take place within the

moderate-intensity domain, may actually utilise lower absolute rates of energy expenditure and CHO oxidation compared to that same session performed in temperate conditions. These effects warrant further investigation in specific studies adopting an experimental design appropriate for discerning the effects of environmental heat stress on substrate metabolism during prolonged endurance exercise at workloads matched for physiological or perceptual demand.

A further critical variable to consider here is the potential for the heat acclimation status of the athlete to impact the effect of heat exposure on substrate metabolism during acute endurance exercise. With repeated heat exposure, thermoregulatory adaptations are accrued that reduce the physiological disturbance evoked by a given bout of exercise under heat stress (71). Following a period of heat acclimation or acclimatisation training, an increased sweat rate and earlier onset of sweating, increased plasma volume, and lower resting core temperature have all been observed (262, 292, 351), thus facilitating a reduced thermoregulatory perturbation when training or competing under heat stress (435). Accordingly, it would be expected that the effect of heat stress on substrate metabolism during acute endurance exercise would be at least partially diminished after a period of heat acclimation training. Indeed, this has been observed, with classic work reporting a reduced respiratory exchange ratio (RER) during exercise in 40°C before and after acclimation, although differences in net muscle glycogenolysis were not significant (182 ± 27 vs. 154 ± 28 mmol glycosyl units.kg^{-1} dw in pre-acclimation and post-acclimation trials, respectively), though this may have been related to low statistical power (N = 6) (121). Regardless, the reduction in RER indicates a reduced whole-body CHO oxidation rate, which may be partially explained by the reduced plasma adrenaline concentrations during exercise. Therefore, any effects of heat stress on substrate metabolism during acute endurance exercise are likely at least partially blunted by acclimation.

Thus, presently the literature (Figure 15) suggests moderate environmental heat stress-induced changes in substrate oxidation rates at moderate exercise intensities are less substantial than indicated by the classic work in this field, which utilised extreme exercise-heat stress stimuli (118, 121, 122), particularly when the blunting effect of heat acclimation is considered (121). The effects of heat stress on substrate oxidation rates during acute endurance exercise appear to be exacerbated by increasing exercise intensity and environmental temperature (Table 7, Table 10, Figure 7).

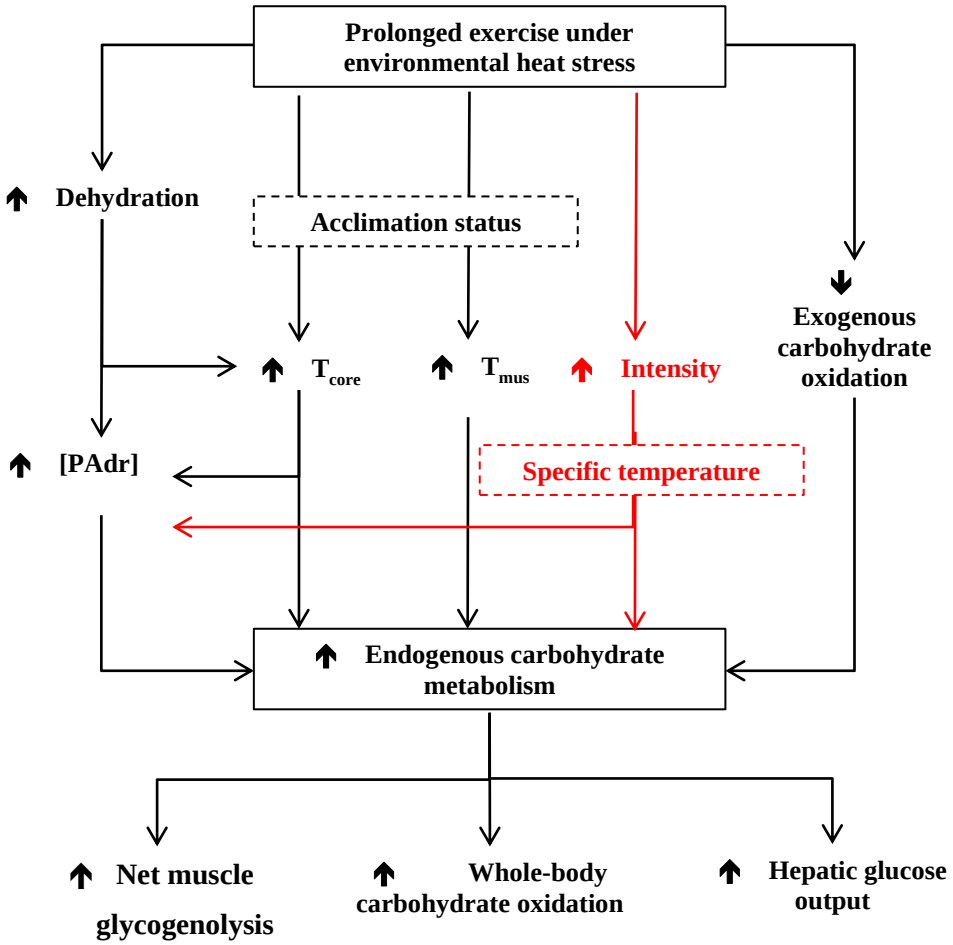


Figure 15. Acute effects of environmental heat stress during prolonged exercise on substrate metabolism. Abbreviations: PAdr = plasma adrenaline, T_{core} = core temperature, T_{mus} = working skeletal muscle temperature. Red indicates additions to the evidence-base from this thesis.

7.2 Exposure to environmental heat stress during endurance exercise and metabolic adaptations to training

It is well-established that repeated exposure to environmental heat stress during endurance training elicits thermoregulatory adaptations that blunt the physiological disturbances associated with exercise-heat stress (435), and accordingly performing specific heat acclimation training prior to competitions taking place in thermally stressful conditions is recommended and commonplace (71, 403). Recently, researchers have discussed the potential for exposure to heat stress during endurance training to up-regulate metabolic adaptations to training, such as mitochondrial biogenesis (183). As a physiological stressor, it is intuitive that environmental heat stress may play a role in metabolic training adaptation; however, little systematic evaluation of this effect has taken place.

Our understanding of mitochondrial adaptations to training dates back to 1967, when John Holloszy observed mitochondrial biogenesis and improved mitochondrial oxidative capacity in response to exercise training in Wistar rats (197). These findings have been replicated and extended in subsequent studies in humans, with increases in mitochondrial volume density (209, 339), mitochondrial oxidative capacity (166, 167), and mitochondrial enzyme content and activity (167, 436, 458) all observed in response to various training programmes. Mitochondrial adaptation to endurance training requires transcription and translation of mitochondrial proteins from the nuclear and mitochondrial genomes, and nuclear-mitochondrial chaperoning of newly-synthesised nuclear-encoded mitochondrial proteins (198, 199). Transcription of genes encoding for mitochondrial proteins in response to endurance training is thought to coordinated by the transcriptional co-activator PGC-1α (171, 176, 513), which is both activated and transcribed following exercise (237, 394, 490, 510).

There are many components of the mitochondrial-endurance training signalling cascade at which exposure to environmental heat stress may interact (Figure 2). Briefly, it can be hypothesised that exercise performed under heat stress may increase PGC-1α phosphorylation and therefore activation via heat stress effects on muscle osmolality, and therefore activation of upstream factor p38 MAPK (448), and muscle glycogenolysis (118) and IL-6-induced (461) activation of upstream factor AMPK (392); as well as increased PGC-1α mRNA expression via increased expression of heat shock proteins (285, 352, 470) and circulating catecholamines (13, 57, 79, 336). The increased heat shock protein expression observed with exercise-heat stress may also assist with the chaperoning of newly-synthesised nuclear-encoded mitochondrial proteins from the nucleus to the mitochondria (518). Lastly, a direct effect of lactate, which circulates in blood and resides in muscle at greater concentrations during

exercise-heat stress (104, 121, 122, 124, 192, 242, 514, 517), has also been proposed in mitochondrial adaptation (469). An up-regulatory effect of heat stress on signalling related to mitochondrial biogenesis, and therefore mitochondrial adaptation to training, is also supported by several *in vitro* experiments conducted in various experimental models reporting positive effects of passive heat exposure for acute and chronic mitochondrial changes (284, 385, 470). Given the conclusions of the previous chapter, namely that the effects of environmental heat stress on CHO metabolism – and therefore muscle glycogenolysis and lactate production – and circulating catecholamines are likely influenced by regulatory effects of exercise intensity, the specific environmental temperature, and acclimation status, it is possible that the effect of heat stress on mitochondrial adaptations to endurance training is similarly influenced by these variables.

In vivo studies published prior to the completion of this thesis did not appear to support the hypothesis presented above. These studies reported blunted transcription of genes related to mitochondrial biogenesis immediately and 3 h post-exercise performed in 33°C compared to exercise performed in a temperate environment (186), and no evidence of mitochondrial biogenesis after 10-d low-intensity heat acclimation (90 min daily treadmill walking in 42°C, 30-50% relative humidity) (304). These findings were in contrast to the authors' hypotheses, which were based on the *in vitro* studies reporting positive effects of passive heat exposure for acute and chronic mitochondrial changes (284, 385, 470), and the basic training principle that greater homeostatic disturbances lead to greater training adaptations (127, 183). Translation of these findings to endurance athletes undertaking training in hot environments is, however, challenging, given the low-intensity training used in the longitudinal study (304), and the acute signalling responses measured in the acute study (186). It is possible moderate environmental heat stress alters the time-course of post-exercise signalling responses related to mitochondrial adaptation such that muscle biopsies obtained immediately and 3 h post-exercise do not capture the beneficial effects, or that an interaction between the specific exercise training parameters and moderate environmental heat stress alters the adaptive consequences, meaning that the effect of environmental heat stress on mitochondrial adaptation to training is not uniform across different training environments. It is indeed also possible our incomplete understanding of adaptive signalling pathways coordinating mitochondrial adaptation to endurance training makes translation of acute signalling studies to longitudinal training adaptation difficult (226). Accordingly, a more appropriate means of assessing the adaptive effects of environmental heat stress for skeletal muscle mitochondria in response to endurance training is measurement of mitochondrial protein content, activity, or density before and after an extended period of heat stress training.

This was the study design adopted in the final experimental chapter of this thesis (Training Study 3, Chapter 6.4). To interrogate the hypothesis that exposure to environmental heat stress impacts metabolic adaptations to endurance training, a cohort of trained males performed three-weeks of training in either 18 or 33°C, with *vastus lateralis* biopsies and assessments of exercise metabolism and endurance performance in temperate conditions before and after the intervention. It was shown that training under moderate environmental heat stress resulted in significantly greater improvements in 30-min time-trial (TT) performance preceded by two hours of constant-load exercise (Figure 12), an effect plausibly mediated by temperature-dependent mitochondrial adaptations (Figure 14). Specifically, maximal *vastus lateralis* citrate synthase activity, a surrogate for mitochondrial protein content (273), significantly increased in the heat stress training group, but not in the group training in temperate conditions (Figure 14). The temperate performance-enhancing effects of moderate environmental heat stress during training align with recent research reporting significant improvements in 15-min temperate TT performance in a group of elite cyclists performing 4-5 sessions per week for five weeks in 37.5-38.5°C, but not 15.5°C (413). Whilst the effects of training under moderate environmental heat stress for substrate metabolism during acute endurance exercise in temperate conditions were unclear in the present thesis (Table 20, Figure 13), a significant interaction between training group and time was observed for the maximum observed rate of fat oxidation during incremental exercise in temperate conditions (Table 20). Specifically, mean responses indicated a decrease in the heat stress training group and increase in the temperate training group, with the training-induced change values significantly different between-groups (Δ 0.05 ± 0.14 vs. -0.09 ± 0.12 $g.min^{-1}$ in the temperate and heat stress training groups respectively, $P = 0.05$). This result is interesting in the context of mean data suggesting fat oxidation during two-hours of constant-load exercise with CHO feeding may have reduced following training in the heat stress group, though this was not significant (40 ± 19 vs. 33 ± 23 g, $P = 0.13$). These results add to the existing literature making measurement of endurance performance in temperate conditions following a period of training under environmental heat stress (253, 255, 292, 323, 333, 486), and provides the first systematic evaluation of the effects of moderate environmental heat stress during a realistic simulation of endurance training on mitochondrial adaptations and temperate performance. Much of the existing research utilised training programmes not reflective of real-world endurance training (255, 292, 333, 355, 456, 486), did not control training (253), or did not include a control group (355, 407, 456), and none of these studies measured mitochondrial adaptations. The results presented here warrant verification in elite and female populations, as well as in

response to alternative training methodologies and at different magnitudes of environmental heat stress.

Temperate performance-enhancing effects of training under moderate environmental heat stress may also be mediated by positive effects on haematological parameters and oxygen-carrying capacity. Previously, Rønnestad and colleagues reported performing 4-5 sessions per week for five weeks in 37.5-38.5°C, but not 15.5°C, induced significant positive effects on haemoglobin mass and therefore increased $\dot{V}O_{2max}$ (413), given oxygen delivery appears limiting to $\dot{V}O_{2max}$ (345). Whilst adaptations to haematological parameters were not measured in the present thesis, training-induced changes in $\dot{V}O_{2max}$ were not observed, possibly due to the limited number of training sessions performed in the severe-intensity domain (once per week) given time >90%$\dot{V}O_{2max}$ during training appears to be influential for training-induced changes in $\dot{V}O_{2max}$ (414, 416). Therefore, it is possible the addition of exposure to moderate environmental heat stress during endurance training has the potential to augment endurance performance in temperate conditions via adaptations to both oxygen-carrying capacity *and* skeletal muscle mitochondria. The existing research therefore supports the use of moderate environmental heat stress during an endurance training intervention for up-regulating adaptations relevant to performance in a temperate environment.

In order to realise these positive effects, it may also be important that any heat stress training intervention is performed in a well-controlled manner, with attention paid to regulation of training load and relative physiological stress. Indeed, a previous study reported maladaptive effects of short-term, high-intensity heat acclimation training, plausibly due to failure to appropriately manage training stress (406). When training under environmental heat stress, the increased heart rates (369, 418) and circulating catecholamines (104, 121, 177) at given external workloads are likely indicative of greater sympathetic and reduced parasympathetic activation, and may chronically disturb cardiac-autonomic balance when not managed effectively. Suppressed parasympathetic activation, measured using heart rate variability (HRV) upon waking, may ultimately predict negative performance consequences such as fatigue and non-functional overreaching (396). Therefore, management of training load during periods of endurance training under heat stress may be critical to achieving the aforementioned positive adaptations and avoiding non-functional overreaching. In the present thesis, it was shown that, despite increased heart rates for given power outputs and decreased power outputs at given physiological intensity thresholds (

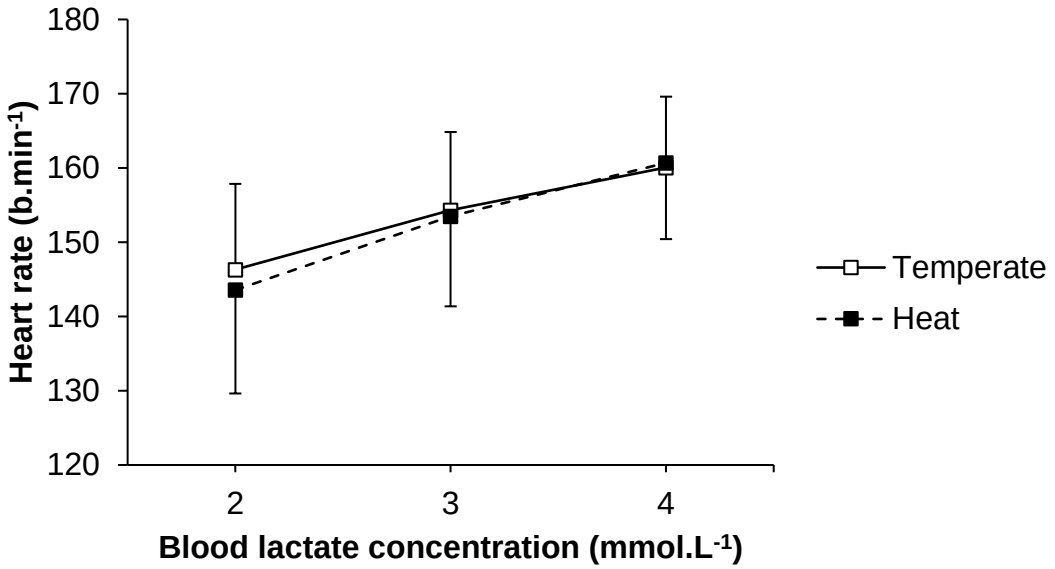


Figure 8), heart rates measured at specific intensity thresholds were consistent between 18 and 35°C (Chapter 6.3) (Figure 9). These results support use of heart rate thresholds measured in temperate conditions for regulation of training intensity, and therefore load, when embarking on a period of endurance training under moderate environmental heat stress, a practice used with apparent success by the elite Ironman triathletes in Kaliua-Kona, Hawaii in the descriptive case study in Chapter 6.2. Indeed, this methodology was used to successfully control training load and match training interventions in the heat stress training intervention in this thesis (Chapter 6.3), in which positive adaptations were observed (Figure 12) alongside undisturbed perceived wellbeing and HRV responses (Figure 11).

Therefore, in summary, exposure to moderate environmental heat stress during endurance training may offer temperate-performance enhancing effects via up-regulation of metabolic and haematological adaptations, although further research in diverse athlete populations, training methodologies, and environmental temperatures is warranted to verify these effects (Figure 16). The implications of exposure to environmental heat stress during endurance training on substrate metabolism during acute endurance exercise in temperate conditions remains unclear. In contrast, the effects of exposure to environmental heat stress during endurance training for acute substrate metabolism during endurance exercise performed under heat stress are apparent, with the thermoregulatory adaptations associated with heat acclimation appearing to reduce the burden on CHO metabolism. Whilst exposure to environmental heat stress during endurance training has the potential to induce maladaptive responses if not managed effectively, careful attention to regulation of training intensity and load may be useful in mitigating these risks.

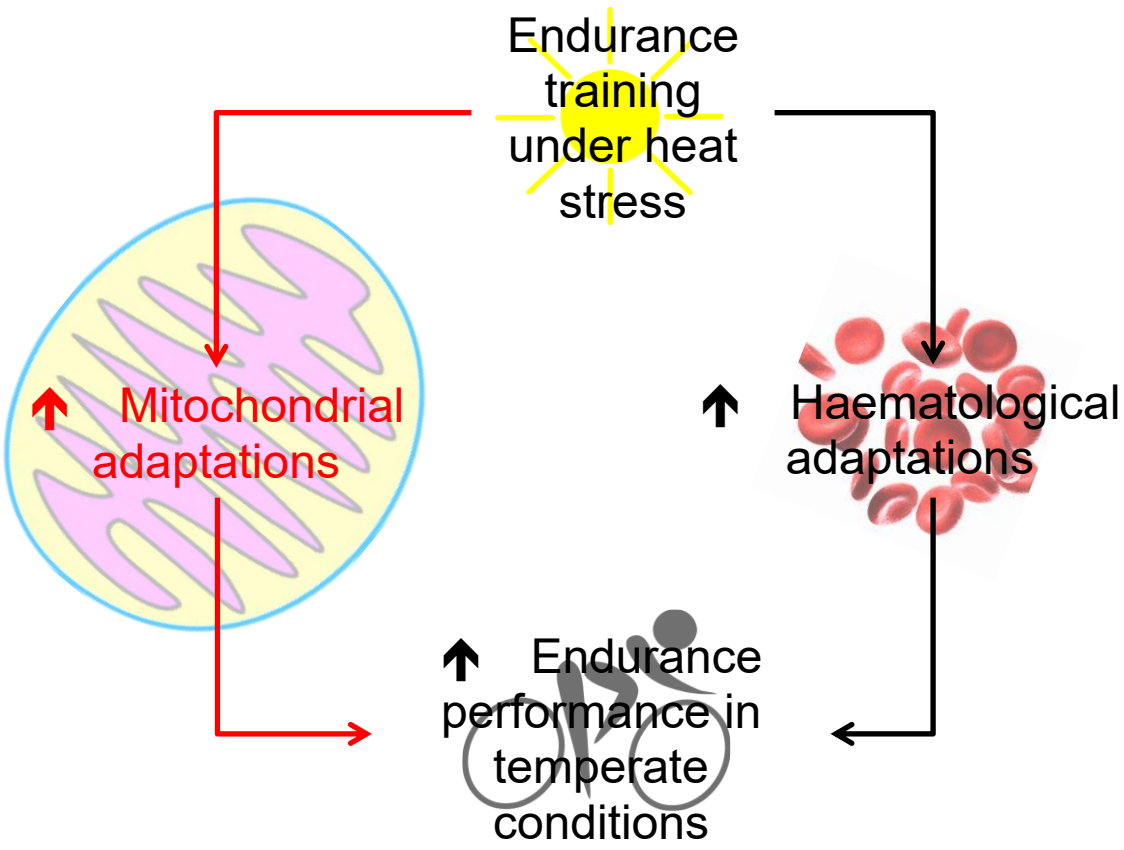


Figure 16. Performing endurance training under environmental heat stress may up-regulate performance in temperate conditions via effects on mitochondrial and haematological parameters. The novel observations presented in this thesis are indicated in red.

7.3 Moving forward

7.3.1 Practical applications

The literature (118, 121, 122), combined with the studies presented in this thesis (Table 7, Table 10, Figure 3, Figure 7), suggest that whilst endurance exercise performed under heat stress certainly has the potential to stimulate CHO metabolism to a greater extent than equivalent temperate exercise, practitioners and athletes concerned with the potential acute metabolic effects of environmental heat stress need to consider the specific combination of exercise-heat stress they are working with, and the acclimation status of the athlete. Specifically, low external workloads and only moderate environmental heat stress (~28-34°C) appear unlikely to effect a dramatic shift in substrate utilisation, whilst more extreme combinations of exercise intensity and environmental temperature may effect more substantial changes, with heat acclimation blunting these changes. Interestingly, when the likely reduction in external workloads achieved for given physiological thresholds, heart rates, or perceived exertion when exercise is performed under environmental heat stress is considered (232, 292, 369, 418), and accompanying reduction in exercise energy expenditure, it is quite possible that 'equivalent' sessions actually induce less CHO oxidation when performed under heat stress. Practitioners working with athletes performing training and/or competition in hot environments and considering altering fuelling strategies or training programming when seeking to target a particular metabolic pathway based on expected heat-stress induced metabolic changes must therefore consider these variables.

From a training adaptation perspective, the results of this thesis support the use of three-week blocks of training under environmental heat stress in the preparation of endurance athletes for competition in hot or temperate conditions, with the caveat that careful attention must be paid to management of training intensity and load to avoid non-functional overreaching and maladaptation, as observed elsewhere (406). Given the positive mitochondrial adaptations observed to endurance training performed under moderate environmental heat stress in this thesis (Figure 14), and the potential for positive haematological adaptations as observed in recent studies (323, 413), practitioners have physiological rationale for recommending specific moderate environmental heat stress training camps, despite the loss of external workloads produced when exercise is performed in these conditions (Figure 11). It should be acknowledged that the data presented in this thesis comes from a three-week heat stress training programme performed in 33°C (60% rH), with previous research observing positive haematological adaptations lasting 3-5 weeks in 33-38.5°C (323, 413). Therefore, it is not presently possible to make recommendations to practitioners regarding the effectiveness of longer, hotter, or cooler heat stress training

programmes. It is possible that the specific combination of training programme duration and temperature have influence on the adaptive outcomes.

7.3.2 Future research directions

Regarding the effects of acute exposure to environmental heat stress on substrate metabolism during endurance exercise, it is recommended that future studies should investigate the effects of exposure to environmental heat stress at the specific intensities training sessions or competitions are likely to take place in; that is, acknowledging the likely reduction in external workloads achieved for given heart rates, physiological thresholds, and perceived exertion under environmental heat stress (232, 292, 369, 418). Studies adopting this design may help to further inform practitioners and athletes regarding the likely metabolic implications of a hot temperature on substrate metabolism for a given moderate-, heavy- or severe-intensity training session or competition. Ideally, these studies would utilise experimental techniques, such as muscle biopsy sampling and stable isotope tracer methodologies, that allow quantification of the contribution of fuel sources derived inside (i.e. muscle glycogen and triacylglycerol) and outside (i.e. hepatic glycogen and adipose tissue-derived fatty acids) skeletal muscle tissue. Such an approach, which was not adopted in the acute studies due to budgetary and logistical limitations, would allow for a more detailed assessment of the acute metabolic implications of environmental heat stress for endurance athletes, as well as limiting factors in specific circumstances. For instance, it is theoretically possible that a given rate of whole-body CHO oxidation is comprised of greater contributions of from muscle glycogen and less from liver glycogen, or *vice versa*.

Regarding the effects of exposure to environmental heat stress during endurance training on metabolic adaptations, it is recommended that future studies should investigate mitochondrial adaptive responses in elite and female populations, as well as in response to alternative training methodologies, at different magnitudes of environmental heat stress, and in response to shorter (<3 weeks) and longer (>5 weeks) training programmes. Similarly, the implications of exposure to environmental heat stress during endurance training on substrate metabolism during acute endurance exercise in temperate conditions remains unclear, and warrants specific investigation in appropriately-powered studies as the intervention study in the present thesis may have not been sufficiently powered to discern differences in training-induced substrate metabolism during acute endurance exercise between-groups.

Lastly, an additional direction for future research may be the application of locally-applied heat stress during endurance training for up-regulating metabolic adaptations.

A likely effect of training under environmental heat stress (1, 54), verified in this thesis (Training Study 3, Chapter 6.4, Figure 11), is the loss of mechanical power outputs during training sessions. It is possible that part of the mechanism behind the stimulatory effects of environmental heat stress on metabolic adaptations to training is the increased muscle temperature (183); indeed, elevated muscle temperature has been shown to influence substrate metabolism during acute endurance exercise (119). Given it is possible to manipulate muscle temperature without increasing core body temperature (119), it might be hypothesised that artificially increasing muscle temperature before and/or during endurance training might stimulate mitochondrial adaptations, but crucially without lowering external workloads achieved during training itself. This hypothesis warrants investigation in a series of studies moving from acute to chronic observations. Given how unpleasant performing endurance exercise under environmental heat stress can be, it is similarly possible that locally-applied heat stress has better translatory potential to recreational exercisers seeking to maximise the metabolic health benefits of individual exercise bouts.

7.3.3 Final thesis conclusions

In conclusion, substrate metabolism and endogenous CHO availability may be important variables for consideration in ultra-endurance athletes. Exposure to environmental heat stress has the potential to influence substrate metabolism during acute endurance exercise, with the acute studies presented in this thesis demonstrating that the specific external workload and environmental temperature appear to regulate this effect. Specifically, the heat stress-induced stimulation of CHO metabolism during acute endurance exercise is more pronounced with increasing exercise intensity, with increasing environmental temperature lowering the intensity at which the stimulation of whole-body CHO oxidation rates is observed. However, it is possible that the reduced external workloads, and therefore absolute rates of energy expenditure, achieved during training and racing when under environmental heat stress may reduce absolute CHO oxidation rates compared to exercise of the same relative intensity, and therefore at higher absolute workloads, in temperate conditions. Performing a block of endurance training under environmental heat stress may blunt heat stress effects on acute substrate metabolism, and, shown for the first time in this thesis, may up-regulate mitochondrial adaptations to training that translate into improved endurance performance in temperate conditions. The implications of performing training under moderate environmental heat stress for substrate metabolism during acute endurance exercise in temperate conditions remains unclear and warrants further investigation in appropriately-powered studies. Future research directions, including those that may improve the translational potential of heat stress as

a stimulus for up-regulating mitochondrial adaptations to exercise in the general population, have been suggested.

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Appendices

Appendix 1: Wellbeing scale used in Training Study 3

	5	4	3	2	1
FATIGUE	Very fresh	Fresh	Normal	More tired than normal	Always tired
SLEEP QUALITY	Very restful	Good	Difficulty falling asleep	Restless sleep	Insomnia
GENERAL MUSCLE SORENESS	Feeling great	Feeling good	Normal	Increase in soreness and/or tightness	Very sore
STRESS LEVELS	Very relaxed	Relaxed	Normal	Feeling stressed	Highly stressed
MOOD	Very positive mood	Generally good mood	Less interested in others than usual	Snappiness at others	Highly annoyed, irritable/down

Appendix 2: Consent forms

Acute Study 1 and 3, Training Study 2

Project title: ***Acute effects of environmental heat stress on the relationships between exercise intensity, exercise duration, and whole-body substrate oxidation rates in endurance-trained males***

Project Supervisor: ***Dr. Andrew E. Kilding***

Researcher: ***Ed Maunder***

- ○ I have read and understood the information provided about this research project in the Information Sheet dated 6th December 2018
- ○ I have had an opportunity to ask questions and to have them answered.
- ○ 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.
- ○ 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.
- ○ 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.
- ○ I agree to provide blood samples.
- ○ I agree to take part in this research.
- ○ I wish to receive a summary of the research findings (please tick one): Yes○ No○
- ○ 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○ No○

Participant's signature : .....……

Participant's name: .....……

Participant's Contact Details (if appropriate):

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Date:

Approved by the Auckland University of Technology Ethics Committee on 16th January 2018 AUTEC Reference number 17/409

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

Acute Study 2

Project title: ***Identifying the threshold thermoregulatory changes at which environmental heat stress impacts whole-body substrate metabolism during prolonged exercise in endurance-trained males***

Project Supervisor: ***Dr. Andrew E. Kilding***

Researcher: ***Ed Maunder***

- ○ I have read and understood the information provided about this research project in the Information Sheet dated 1st June 2018
- ○ I have had an opportunity to ask questions and to have them answered.
- ○ 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.
- ○ 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.

- ○ 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.
- ○ I have not been resident in a hot country for a period of three months or more in the last year, or engaged in specific heat acclimatisation training in the last six months.
- ○ I agree to provide blood samples.
- ○ I agree to provide urine samples.
- ○ I agree to self-insert a rectal thermometer for measurement of core temperature.
- ○ I agree to take part in this research.
- ○ I wish to receive a summary of the research findings (please tick one): Yes○ No○
- ○ 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○ No○

Participant's signature : .....……

Participant's name: .....……

Participant's Contact Details (if appropriate):

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Date:

Approved by the Auckland University of Technology Ethics Committee on 6th June 2018 AUTEC Reference number 18/186

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

Training Study 1

Project title: ***Metabolic effects of a heat stress training camp: A case study of elite male Ironman triathletes***

Project Supervisor: ***Dr. Andrew E. Kilding***

Researcher: ***Ed Maunder***

- ○ I have read and understood the information provided about this research project in the Information Sheet dated 6th March 2018
- ○ I have had an opportunity to ask questions and to have them answered.
- ○ 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.
- ○ 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.
- ○ 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.
- ○ I agree to provide blood samples.
- ○ I agree to provide a urine sample on arrival
- ○ I agree to take part in this research.
- ○ I wish to receive a summary of the research findings (please tick one): Yes○ No○

○ 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○ No○

Participant's signature : .....……

Participant's name: .....……

Participant's Contact Details (if appropriate):

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Date:

Approved by the Auckland University of Technology Ethics Committee on 31st March 2018 AUTEC Reference number 18/101

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

Training Study 3

Project title: ***Implications of prolonged exercise performed under environmental heat stress for the adaptive response to training: A randomised controlled trial in endurance-trained males***

Project Supervisor: ***Dr. Andrew E. Kilding***

Researcher: ***Ed Maunder***

○ I have read and understood the information provided about this research project in the Information Sheet dated 12th April 2019

○ I have had an opportunity to ask questions and to have them answered.

- ○ 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.

- ○ 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.

- ○ 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.

- ○ I have not been resident in a hot country for a period of three months or more in the last year, or engaged in specific heat acclimatisation training in the last six months.

- ○ I agree to provide blood samples.

- ○ I agree to provide muscle samples.

- ○ I agree to take part in this research.

- ○ I wish to receive a summary of the research findings (please tick one): Yes○ No○

- ○ I wish to have my blood and/or muscle samples returned to me in accordance with right 7 (9) of the *Code of Health and Disability Services Consumers' Rights* (please tick one): Yes○ No○

Participant's signature : .....……

Participant's name: .....……

Participant's Contact Details (if appropriate):

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Date:

Approved by the Auckland University of Technology Ethics Committee on 7th June 2019 AUTEC Reference number 19/146

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

Appendix 3: Health screening questionnaire

Please answer the following questions. If you have any questions feel free to ask any of the investigators. You answers will be kept strictly confidential.

1.	Gender….	Male	Female
2.	What is your date of birth? So your age is..... years	…..…../……/……	
3.	Are you currently taking any medication?	YES	NO
4.	Have you ever been advised you not to take vigorous exercise?	YES	NO
5.	Do you suffer from any chronic disease? E.g. Cardiovascular, kidney, or thyroid disease, diabetes, etc.	YES	NO
6.	Have you ever been diagnosed with high blood pressure?	YES	NO
7.	Have you ever taken medication for blood pressure or your heart?	YES	NO
8.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
9.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO
10.	Have you ever been diagnosed with high blood cholesterol?	YES	NO
11.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
12.	Have you had a cold or feverish illness in the last month?	YES	NO
13.	Have you had a musculoskeletal injury in the last	YES	NO

	month?		
14.	Do you suffer from asthma?	YES	NO
15.	Have you ever suffered from exertional heat stroke?	YES	NO
16.	Are you accustomed to vigorous endurance based exercise (over an hour a week)?	YES	NO
17.	Do you participate in cycling-based endurance competition (e.g. cycling, triathlon, etc)?	YES	NO
18.	Have you recently (< 6 months) undertaken heat acclimation training or been permanently resident in a hot environment (>30°C)	YES	NO

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Participant signature: .....

Date: ..... /..... /.....

Researcher signature: .....

Date: ..... /..... /.....