Central fatigue during exercise: potential manuipulations and limiting factors

CENTRAL FATIGUE DURING EXERCISE: POTENTIAL MANIPULATIONS AND LIMITING FACTORS.

By

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ABSTRACT

The development of fatigue has been an area of interest to athletes and scientists alike for many years. Often, particularly during prolonged exercise in the heat, there is no obvious peripheral reason for fatigue and the central nervous system is cited as the source. The mechanisms and potential manipulations of this fatigue remain largely unclear.

Chapters Three and Four attempted to reduce the transport of the serotonin precursor tryptophan into the brain in order to reduce or delay serotonin synthesis and therefore increase exercise capacity. In Chapter Three branched-chain amino acid drinks were fed before and during prolonged cycling to exhaustion in the heat on two occasions and control drinks were fed on two other occasions. There was no effect of the branched-chain amino acids on exercise capacity and the intra-individual variability in seven of the eight participants was small. One participant did appear to cycle for longer on the branched-chain amino acid trials compared to the control trials. In Chapter Four a 104 g bolus of amino acids, designed to deplete plasma tryptophan concentration, was fed seven hours before a prolonged cycle to exhaustion in the heat. There was no difference in exercise capacity between the tryptophan depletion trial and the control trial in which tryptophan was also ingested. These findings suggest that the delivery of tryptophan to the central nervous system is not the only factor influencing the onset of fatigue.

The investigation undertaken in Chapter Five looked at the serotonin transporter density on the blood platelets of current and retired international level athletes competing in either endurance or sprint running events and a sedentary control group. Using the platelet as an accessible and reliable model for the serotonergic neuron, the maximum number of binding sites was assessed using the radio-labelled serotonin reuptake inhibitor [^{3}H]Paroxetine. Those currently training for endurance events had a greater number of binding sites than any of the other groups. This supports previous findings and suggests that endurance training can increase the number of serotonin transporters on blood platelet membranes.

During resting heat exposure in Chapter Six, the application of a 1 % menthol solution to the skin of the forearms, back and forehead elicited a warming sensation in some individuals and a cooling sensation in other individuals, but never any change in skin or core temperature nor skin blood flow. A small proportion of individuals did not perceive any change in skin thermal sensation. Chapter Seven applied these findings to a pre-loaded twenty minute exercise performance test in the heat. It was hypothesised that those who perceived a warming effect may perform worse when a menthol solution was applied compared to a control solution and conversely, those who perceived a cooling sensation may perform better with a menthol solution than with a control solution. There was no difference in exercise performance between those who felt a warming sensation and those who felt a cooling sensation. Those who felt a warming sensation felt significantly warmer on the menthol trial than the control trial but this did not affect their performance. However, those who reported a cooling sensation tended to feel cooler on the menthol trial than the control trial, and there was a tendency for an improvement in performance on the menthol trial compared to the control trial.

Due to the experimental protocols adopted in this thesis it was possible to assess the reliability of an exercise capacity test compared to an exercise performance test. Chapter Three showed a coefficient of variation of 11.0 ± 11.2 % and Chapter Four showed a 11.5 ± 12.4 % variability for exercise capacity tests. Chapter Seven showed a coefficient of variation in a pre-loaded time-trial exercise performance test of 3.9 ± 9.6 % suggesting that an exercise performance test may be more reliable than an exercise capacity test. However, the aims of an investigation are still likely to be the main factor influencing the choice of protocol.

It seems likely that no single mechanism will be responsible for the cessation of exercise. The investigations undertaken in this thesis also highlight many avenues for future exploration.

KEY WORDS: Central fatigue; Tryptophan; Serotonin transporters; Thermal sensation.

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CHAPTER ONE

General introduction.

1.1 Definition of fatigue

For many years the development of fatigue during exercise has received considerable interest from athletes, coaches and scientists alike. From as far back as the late 19th century it has been suggested that the central nervous system plays a key role in the development of exercise fatigue, to the extent that central fatigue is a protective mechanism over peripheral fatigue (Waller, 1891). Several years later, Bainbridge took this further and stated that there were two types of exercise fatigue: one that was entirely centrally mediated and the other which was both central and peripheral in origin (Bainbridge, 1919).

Since that date, fatigue during exercise has generally been defined as an inability to maintain the required force or power output for a given task, leading to a reduction in the performance of the task (Edwards, 1981). To this day fatigue is still subdivided in relation to the location of the inhibition to perform a task, i.e. centrally mediated or peripherally mediated fatigue. Central fatigue can be defined as any exercise-induced reduction in maximal voluntary contraction force that is not accompanied by the same reduction in maximal evocable force (Astrand *et al.,* 2003A). Peripheral fatigue encompasses all events leading to fatigue that occur independently of the central nervous system. This includes, but is not restricted to, disturbances to neuromuscular transmission, sarcolemma excitability and excitation-contraction coupling (Meeusen *et al.*, 2006) along with substrate depletion, metabolic acidosis (Maughan *et al.,* 1997A) and the peripheral effects of hypohydration and elevated core temperature (Maughan *et al.,* 1997B).

1.2 Central fatigue during prolonged exercise

1.2.1 Introduction

Often, when no peripheral reason for fatigue during prolonged exercise is apparent, the fatigue is said to be centrally mediated. Indeed, one of the first symptoms of fatigue is an increase in the perception of the effort required to perform a task (Davis and Bailey, 1997). This is followed by a decrement in the performance of the task, which will inevitably occur if the task is continued.

Centrally mediated fatigue has come under more scrutiny in recent decades with many contemporary theories being proposed to explain the possible mechanisms of central fatigue. However, no definitive single cause has ever been found. This thesis will focus on the role of serotonin in centrally mediated fatigue even though it is widely accepted that this is just one of many avenues of exploration into the aetiology of fatigue during exercise.

1.2.2 Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) has been implicated in many physiological and psychological sensations and processes. It has been shown to be involved in feelings of tiredness, lethargy and sleep, sensory perception of pain, and thermoregulation (for a full review see Jacobs and Azimita, 1992). Manipulation of various aspects of the serotonergic system therefore offer appealing theories as to how exercise performance and capacity can be improved through increased resistance to, or the delayed on-set of, fatigue.

1.2.2.1 The serotonergic system

Serotonin is just one of approximately sixty neurotransmitters within the nervous system. Of all the neurons in the central nervous system, the complex and vast network of serotonergic neurons constitutes only about one in every one million neurons (Jacobs and Azimita, 1992). The cell bodies of the serotonergic neuronal network tend to originate in the lower brain stem, with dense collections in or near the raphe nuclei (Struder and Weicker, 2001B). The neurons have been subdivided into two functionally significant groups: the superior group which supplies the forebrain and the inferior group which generally projects into the spinal cord (Jacobs and Azimita, 1992).

Serotonin neuronal firing, release and metabolism are, sometimes at least, independent of each other. Crespi and colleagues (1990) showed this through *in vivo* administration of a 5-HT agonist which decreased 5-HT neuronal firing and extracellular 5-HT but not extracellular serotonin metabolite 5-HIAA concentrations in rats. Aghajanian (1972) proposed that serotonin release operates on a negative feedback system, which works to

regulate and normalise serotonin levels. A simplistic and idealised overview of a single serotonergic neuron can be seen in *Figure 1.1*.

- Tryptophan from the blood
- Large neutral amino acid transporter
- Nucleus
- Cell body
- Tryptophan Hydroxylase
- 5-Hydroxytryptophan
- 5-Hydroxytryptophan Decarboxylase
- Serotonin
- Axon
- Synaptic vesicle
- Serotonin uptake
- Serotonin release
- Mitochondria
- Monoamine Oxidase
- Nerve ending
- Serotonin transporter
- Presynaptic autoreceptor
- Postsynaptic receptor
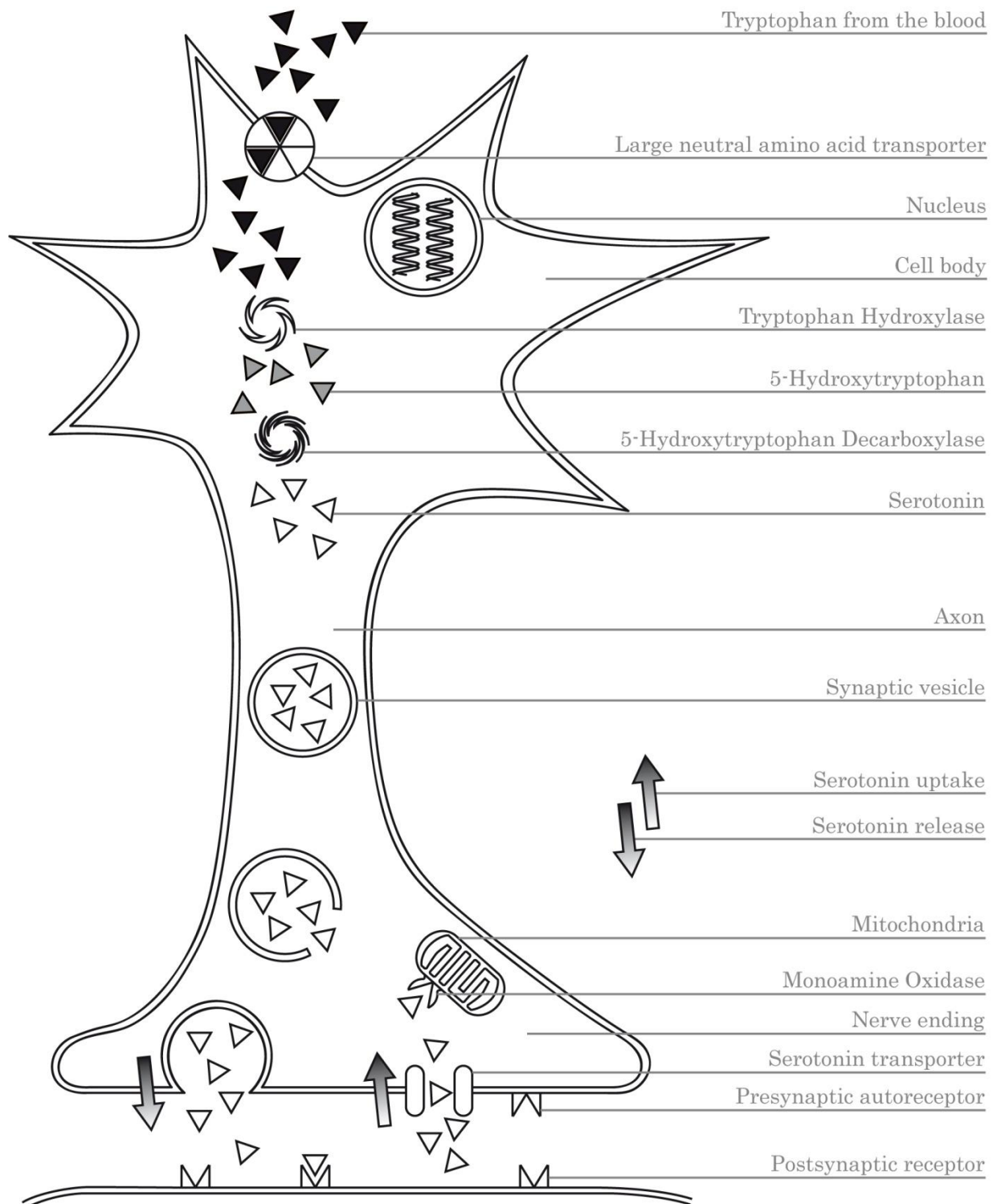
Figure 1.1. A simplistic diagram of a single serotonergic neuron, annotated.

1.2.2.2 Serotonin synthesis

Only about 1 – 2 % of the serotonin in the body is found in the brain (Cooper *et al.,* 2003A). Mast cells, enterochromaffin cells in the gastrointestinal tract and blood platelets, none of which are involved with the nervous system, are all able to either synthesise and/or transport serotonin (Struder and Weicker, 2001A). Serotonin is not able to cross the blood brain barrier, nor is it easily able to diffuse across cell membranes. Therefore, the synthesis of serotonin must take place within the neurons from its precursor, tryptophan. This process requires several enzymes, and their presence is one of the defining characteristics of the serotonergic system (Cooper *et al.*, 2003A).

Tryptophan, an essential amino acid with a hydrophobic aromatic R-group, must be consumed in the diet. Generally, foods that are high in protein are also high in tryptophan. When eaten, these foods release tryptophan and the amino acid enters the circulation. Originally it was thought that the plasma concentration of tryptophan was the only determinant of serotonin synthesis (Fernstrom and Wurtman, 1971). It is now apparent however, that the situation is far more complex, with several factors influencing serotonin synthesis, including competition from other compounds which compete with tryptophan for the same transporter across the blood brain barrier.

By using ^{14}C labelling of individual amino acids, Oldendorf and Szabo (1976) showed that there are three amino acid transporters across the blood brain barrier and that, other than proline, alanine and glycine, each amino acid could only be transported by one of the three. The neutral transporter, which is temperature dependent and stereospecific but not sensitive to external sodium or potassium concentrations, is responsible for transporting, among others, leucine, isoleucine, valine and tryptophan. Therefore, these amino acids are in direct competition for transport into the brain, meaning that the ratio of tryptophan to competing amino acids is an important determinant of the rate of uptake of tryptophan across the blood brain barrier and subsequently the rate of serotonin production.

An important and potentially confounding factor, which must be considered from this point onwards, is the relatively recent finding that during exercise in a warm environment, the integrity of the blood brain barrier may be compromised (Watson *et al.,* 2005B). The blood brain barrier is characterised by the presence of tight junctions and selective, specific molecular carrier systems. It acts as a protective mechanism maintaining a stable environment for the central nervous system despite changes in the peripheral circulation. However, after a protocol involving passive heating and exercise in a warm environment, the peripheral concentration of s100β, a protein found predominantly in the central nervous system at a concentration of around eighteen times that found in the peripheral circulation, was significantly elevated compared to the same protocol which did not induce a thermal strain. This finding suggests that thermal strain and exercise lead to an increase in the permeability of the blood brain barrier. In a subsequent investigation, the same investigators found that the increase in the serum concentration of s100β could be attenuated by the ingestion of water, in volumes similar to the body mass lost due to the exercise protocol, which was again performed in the heat (Watson *et al.,* 2006).

To further complicate the relationship of circulating plasma tryptophan concentration to serotonin production, approximately 80 – 90 % of circulating tryptophan is loosely bound to albumin (McMenamy and Oncley, 1958). Originally, it was thought that bound tryptophan was not available for uptake into the central nervous system due to the size of the albumin molecule (Knott and Curzon, 1972). However, subsequent investigations failed to find any relationship between the concentration of free tryptophan in the plasma and the concentration of tryptophan in the brain (Fernstrom *et al.,* 1976; Madras *et al.*, 1974). Pardridge (1983) suggested that the large neutral amino acid transporter at the blood brain barrier may be able to disassociate tryptophan from albumin, thereby making tryptophan available for transport into the brain across the blood brain barrier.

Once tryptophan does cross the blood brain barrier, it is delivered to the serotonergic neurons where the enzyme tryptophan hydroxylase hydroxylates

the 5 carbon position to create 5-hydroxytryptophan. Although the action of tryptophan hydroxylase is the rate-limiting step in the synthesis of serotonin, in a healthy, resting individual this enzyme is only around 50 % saturated. This means that an increase or decrease in the rate of tryptophan transport across the blood brain barrier will lead to a corresponding increase or decrease in the rate of serotonin synthesis within the central nervous system (Hamon *et al.,* 1981). The 5-hydroxytryptophan is then rapidly decarboxylated into 5-hydroxytryptamine (serotonin; 5-HT) by 5-hydroxytryptophan decarboxylase. The chemical pathways involved in this are shown in the top half of *Figure 1.2.*
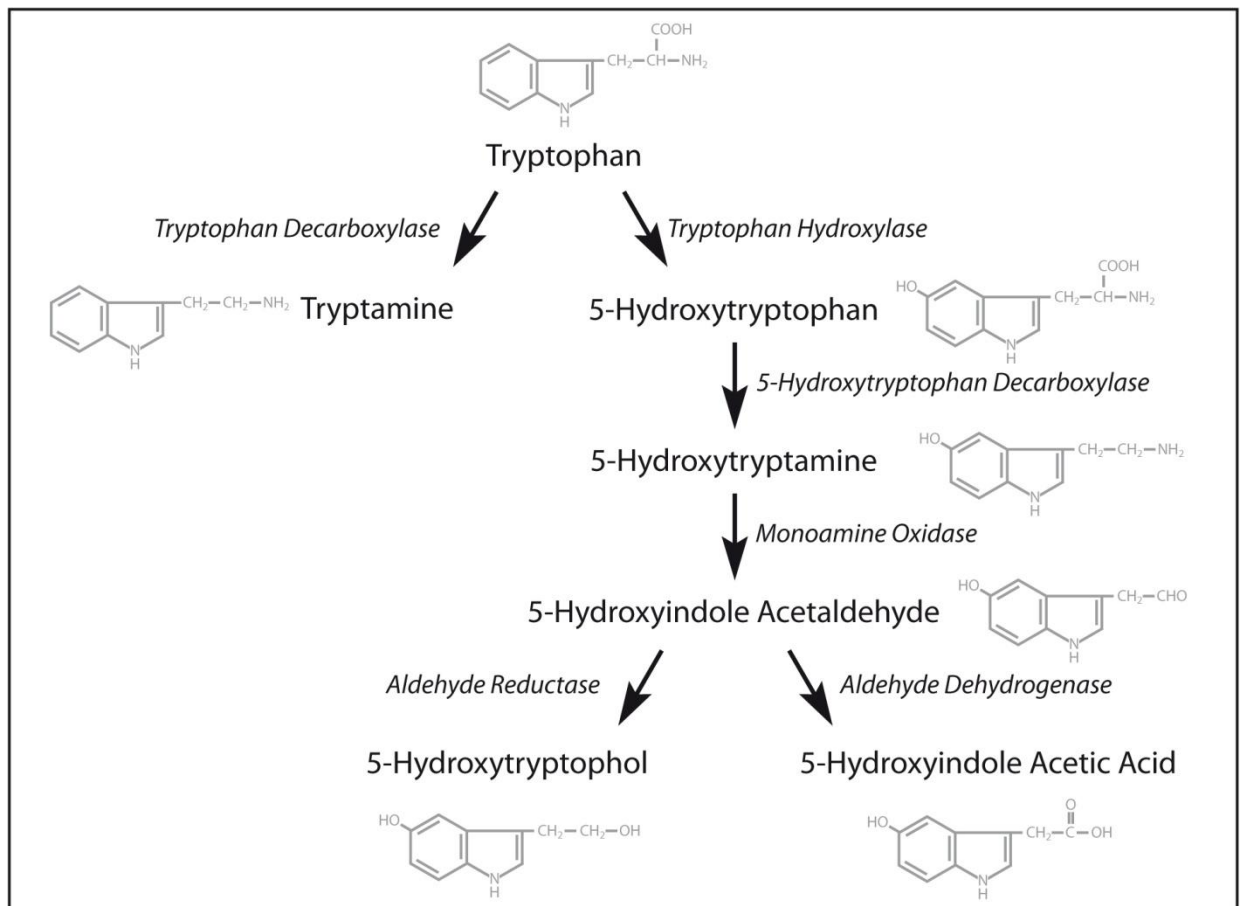
Figure 1.2. The chemical pathways involved in the synthesis and metabolism of serotonin.

The reactions synthesising serotonin from tryptophan occur in both the perikarya and the nerve terminals. If the serotonin is synthesised in the

perikarya it then travels down the axon to where it is stored, at the nerve terminals.

1.2.2.3 Serotonin storage and release

Serotonin is maintained at the nerve terminals of the dendrites and axons in vesicles so that there is a readily available store. By being stored in the vesicles the serotonin is protected from further metabolism. The serotonin content of the vesicles is maintained by H^+-ATPase (Moriyama and Futai, 1990). The serotonin is released into the synaptic cleft from the pre-synaptic vesicles in response to voltage sensitive calcium conductance (Cooper *et al.,* 2003A). Newly synthesised serotonin is preferentially released (Elks *et al.,* 1979). What remains unclear is whether the vesicle undergoes rapid fusion with the pre-synaptic specialised membrane, allowing the stored neurotransmitter to diffuse out into the synaptic cleft, or whether the vesicle lyses whilst still inside the nerve ending, and the release of the neurotransmitter evokes a rapid insertion of the vesicle into the plasma membrane, with the reverse occurring later for endocytosis (Cooper *et al.,* 2003C).

1.2.2.4 Serotonin reuptake

Once in the synaptic cleft, serotonin activates both pre- and post-synaptic receptors. The pre-synaptic auto-receptors have a limited effect at modulating serotonin release (Cooper *et al.,* 2003A). The action of serotonin is terminated by its reuptake into the presynaptic nerve terminal (Cooper *et al.,* 2003A). Serotonin transporters actively reduce the synaptic concentrations of serotonin to levels that are not capable of maintaining the activation of post-synaptic receptors. Many pharmacological agents have been developed which can selectively inhibit the reuptake of serotonin into the pre-synaptic nerve terminal, thereby maintaining the concentration of serotonin at the post-synaptic receptors. These pharmacological agents, often referred to as selective serotonin reuptake inhibitors (SSRIs), include the antidepressant drugs Citalopram, Paroxetine and Fluoxetine.

Serotonin is transported back into the neuron by a high affinity and low capacity carrier which can reach saturation and which also relies on sodium and chloride ions being co-transported with the serotonin and potassium or hydrogen ions being counter-transported (Talvenheimo *et al.,* 1983). This carrier is energy, temperature and sodium dependent (Lawrence, 1991). Serotonin may be returned to the synaptic vesicles for reuse or may be metabolised.

1.2.2.5 Serotonin metabolism

Serotonin is initially metabolised into 5-hydroxyindole acetaldehyde by monoamine oxidase A and then converted to either 5-hydroxyindoleacetic acid (5-HIAA; the main metabolite) by aldehyde dehydrogenase, or 5-hydroxytryptophol (5-HTOH; a minor metabolite) by aldehyde reductase. Which of these metabolic pathways is taken is determined by the ratio of NAD^+ to NADH in the tissue (Cooper *et al.,* 2003A). These pathways are shown in the bottom half of *Figure 1.2*.

Serotonin is implicated in many psychiatric disorders, and therefore much of the research into its role within the body is from a pharmacological stand point. The concentration of 5-HIAA in the brain has been used in the assessment of psychiatric disorders as a marker of serotonergic activity (Roy, 1999; Gjerris *et al.,* 1987).

1.2.2.6 Peripheral models of the serotonergic neuron

For obvious reasons, the direct examination of the central serotonergic system is restricted in humans. However, as mentioned previously, the blood platelets are able to transport serotonin and share several commonalities with the serotonergic neuron. Therefore, it is possible to use the blood platelet as a simple and accessible peripheral model for the examination of the serotonergic neuron and investigate the development of fatigue within individuals.

In humans, the serotonin transporters in both brain tissue and blood platelets are encoded by a single gene on the chromosome 17q11.2. Platelets and

serotonergic neurons both actively transport serotonin and are also both storage vesicles for serotonin; their serotonin uptake kinetics are similar, as are their release mechanisms (Da Prada *et al.,* 1988). Although there are many similarities between the blood platelets and the serotonergic neurons which can be exploited to study the central nervous system, there are also several differences, including the platelet's inability to synthesise serotonin. Despite their dissimilarities, the relative simplicity and accessibility of a platelet compared to a neuron may be an advantage to investigations and the dense, complex network of interconnecting cells in neuronal tissue is not an interfering influence in platelets (Pletscher, 1988).

Blood platelets vary in size and age; younger platelets are larger, denser and more metabolically active. However, the functional properties of the platelets are believed to be independent of size and density (Thompson *et al.,* 1983). Acute, strenuous exercise increases blood platelet counts by the alpha-adrenergic stimulation of the spleen. The spleen contains approximately one third of the exchangeable platelet population (Chamberlain *et al.,* 1990), and the age of these platelets is similar to that of the population in the circulation (Chamberlain *et al.,* 1990).

There is previous research in healthy athletic populations investigating platelet serotonin transporter density. Strachan and Maughan (1998) showed that the density of the serotonin reuptake transporter is greater in the platelet membranes of endurance trained participants compared to sedentary controls. As discussed above in Section 1.2.2.4, the reuptake of serotonin reduces the concentration in the synaptic cleft to levels incapable of maintaining activation of post-synaptic receptors. Therefore, an increased number of serotonin reuptake transporters could lead to a faster reduction in the synaptic concentration of serotonin, which in turn would theoretically lead to a delay in the onset, or a blunting in the degree, of fatigue. Wilson and Maughan (1992) indirectly supported this theory by showing that administering a serotonin reuptake inhibitor reduced prolonged exercise capacity. Furthermore, Struder *et al.* (1999) then found that after a bout of acute endurance exercise (five hours cycling on a cycle ergometer at a heart

rate corresponding to a blood lactate concentration of 2 mmol / L) the 5-HT_{2A} receptor density on blood platelets was reduced.

If these peripheral changes are reflected in the central nervous system it may imply that individuals who regularly undertake endurance exercise have more serotonin transporters and also less serotonin receptors which could alter their perception of fatigue and pain. Unfortunately, due to the nature of these investigations it can only be hypothesised that the changes which occur peripherally will also occur within the brain. This being said, research into depression has frequently used the blood platelet as a peripheral model for investigating the serotonergic neuron, and the potency of tricyclic antidepressants to inhibit serotonin uptake in platelets and the brain are highly correlated (Da Prada *et al.,* 1988).

Genetic differences have been identified within healthy human participants for the expression and function of serotonin transporters in platelets. The short form allele is dominant in healthy males, but the genotype has no effect on platelet [^{3}H]Paroxetine binding (Bmax), which is a predictor of the number of transporters on the membrane of a serotonergic neuron (Greenberg *et al.,* 1999). As suggested above, the density of transporters could alter the perception of fatigue. Park *et al.* (2004) suggest that the short form was found more in elite long distance runners compared to either elite short distance runners or control participants. Greenberg *et al.* (1999) found that the long form allele was associated with a rapid initial platelet serotonin uptake (Vmax). Once again, a transporter with an elevated rate of uptake could also influence the effects of the released neurotransmitter.

1.2.2.7 Serotonin overview

Numerous proposals have been put forward as to how the synthesis and release of serotonin can be manipulated, and the effects of such interventions have been hypothesised. As discussed above, tryptophan must be hydroxylated, and vital components of this process are pteridine and oxygen. Therefore, the availability of these substrates is also vital.

1.2.3 Dopamine

The possible involvement of dopamine in the central fatigue process was initially discovered through studies into the effects of amphetamines in animals (Chaouloff *et al.,* 1987). These studies showed that during prolonged activity there are increases in dopamine activity within the brain. Amphetamines act to enhance exercise performance through their influence as potent dopamine releasers (Adams *et al.,* 2002) and reuptake inhibitors (Azzaro *et al.,* 1974). The elevation of catecholaminergic neurotransmission is generally linked to feelings of reward, arousal, attention and motivation. Amphetamines are well known to enhance exercise performance (see Laties and Weiss, 1981, for a review) although, in sport, this knowledge is of no use due the inclusion of amphetamines, and related substances, on the list of prohibited substances in sport as set by the World Anti-Doping Agency (WADA).

Subsequently, since pharmacological manipulation of dopamine using amphetamines is not an option for athletes, nutritional manipulation has also received attention. The amino acid tyrosine is the precursor to dopamine production. Unlike tryptophan however, it is not an essential amino acid and it can be synthesised in the liver from phenylalanine. Tyrosine is transported across the blood brain barrier by the same transporter as tryptophan and other large neutral amino acids, as discussed above. However, the rate limiting enzyme in the metabolic pathway transforming tyrosine to dopamine is saturated at rest (Cooper *et al.,* 2003B), meaning increased tyrosine delivery will not increase dopamine synthesis (Davis and Bailey, 1997).

Adenosine inhibits the release of excitatory neurotransmitters, such as dopamine. Caffeine is a strong adenosine antagonist and produces a marked reduction in the central adenosine neurotransmission when it crosses the blood brain barrier, which it does with ease. Caffeine therefore enhances the release of dopamine and acts in a similar way to amphetamine and cocaine. It has been shown to significantly improve exercise capacity in rats when infused directly into the brain (Davis *et al.,* 2003) and in humans when ingested orally (Graham and Spriet, 1995). Conversely, the infusion of an

adenosine agonist drug reduced exercise capacity, and when the antagonistic caffeine and the agonistic drug were infused concurrently there was no change in the exercise capacity (Davis *et al.,* 2003).

Bupropion, as well as being widely used as an aid in the cessation of smoking, is an anti-depressant drug that acts to inhibit dopamine reuptake. Watson and colleagues (2005A) found that in warm (30 °C) environmental conditions, the administration of Bupropion improved pre-loaded time-trial cycling performance. It has no significant effects on the serotonergic system, either clinically or on post synaptic receptors, making it distinctly different from many antidepressant drugs which cause a variety of side effects including sedation, weight gain and sexual dysfunction (Stahl *et al.*, 2004). However, Watson and colleagues (2005A) did not find an improvement in the performance of the same task with Bupropion supplementation in cooler ambient conditions (18 °C).

1.3 Newsholme's theory of central fatigue

1.3.1 Newsholme's theory

The most investigated and best known theory proposed as a possible explanation for central fatigue during exercise was put forward over twenty years ago. Newsholme and colleagues (1987) suggested that increased brain serotonin levels during physical activity reduces exercise capacity and is therefore detrimental to performance. This theory has received a great deal of attention and has been the reasoning behind many investigations into the role of the brain during fatigue.

The precursor for serotonin synthesis, as explained above in section 1.2.2.2, is tryptophan. The majority of tryptophan (80 – 90 % depending upon various factors including recent diet and exercise) circulates in the blood loosely bound to albumin. Only free tryptophan can cross the blood brain barrier for use in the synthesis of serotonin. Even free tryptophan, which is not bound to albumin, must compete with branched-chain amino acids for transport into the central nervous system, also explained in section 1.2.2.2. The Newsholme

theory of central fatigue is based on these situations. A simplistic diagram of the resting situation at the blood brain barrier is presented in *Figure 1.3*.
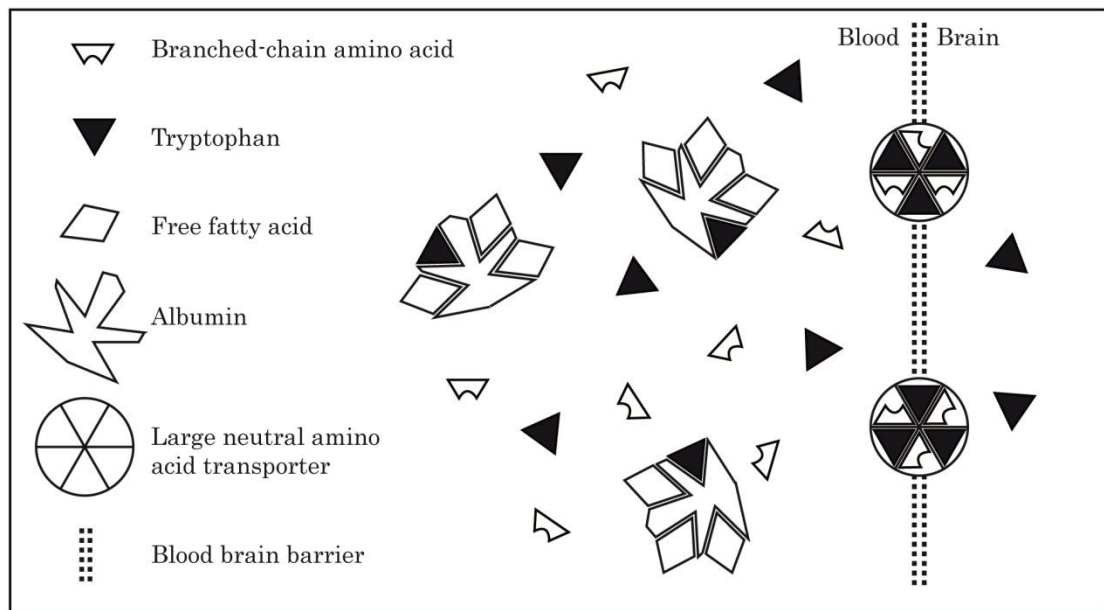
Figure 1.3. A simplistic view of the situation at the blood brain barrier at rest.

As discussed above, free fatty acids are carried through the circulation by albumin. At the onset of exercise there is an initial fall in the plasma concentration of free fatty acids due to the rate of uptake into the muscle increasing disproportionately to the rate of mobilisation from adipose tissue. As exercise progresses however, there is often a gradual increase in the circulating concentration of free fatty acids due to adrenaline stimulated lipolysis, without a concurrent increase in the rate of uptake into the working muscle (Spriet, 2002). As exercise progresses, and muscle and liver glycogen stores become depleted, the mobilisation of free fatty acids increases disproportionately compared to the rate at which they can be transported into the muscle, resulting in further elevations in circulating free fatty acid levels (Newsholme and Castell, 2000). The increase in plasma free fatty acid concentration leads to an increase in free tryptophan concentration in the circulation as they are both predominantly transported bound to albumin. However, albumin has a greater affinity for free fatty acids and in the process of attaching to the free fatty acid, the albumin undergoes a

conformational change which results in the release of the tryptophan from its binding site into the circulation. Plasma free tryptophan and free fatty acid concentrations have been shown to be highly related during exercise (Davis *et al.,* 1992). This increase in free tryptophan means that more is available to be transported across the blood brain barrier to form serotonin.

Concurrently during prolonged exercise, plasma branched-chain amino acid concentrations fall as they are taken up from the blood and metabolised in working muscles to provide energy (Blomstrand *et al.,* 1988). This means there is less competition for tryptophan to be transported across the blood brain barrier. Therefore, as exercise duration increases there is an increase in tryptophan availability and transport into the central nervous system becomes less competitive. A simplistic diagram of the situation during prolonged exercise is presented in *Figure 1.4.*
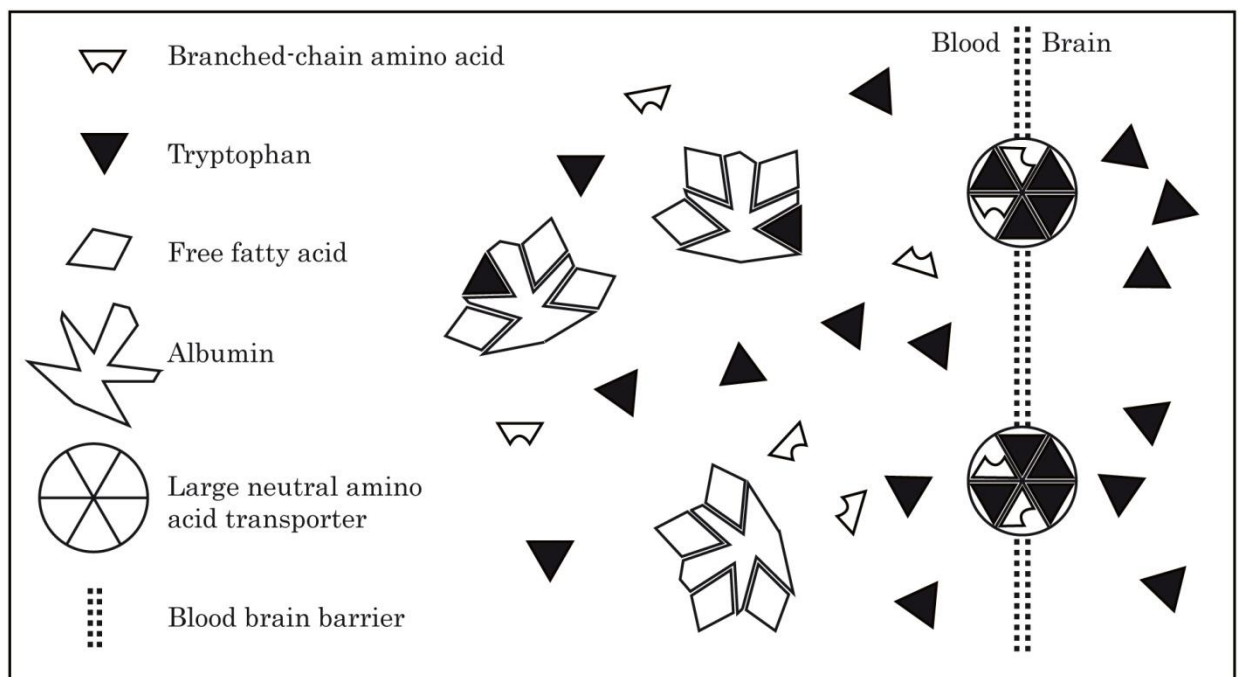
Figure 1.4. A simplistic view of the situation at the blood brain barrier during prolonged exercise.

Newsholme's theory suggests that this leads to increased levels of serotonin in the brain, increased perception of the effort required to sustain an exercise

intensity, loss of drive and motivation and ultimately fatigue and the cessation of exercise. Evidence for this chain of events exists in animal studies. Prolonged exercise increased plasma free tryptophan levels and also brain tryptophan and 5-hydroxyindoleacetic acid levels in rats (Chaouloff *et al.,* 1985; Chaouloff *et al.*, 1986A; Chaouloff *et al.*, 1986B). Further studies in rats have shown that the brain concentration of serotonin and its metabolites increases during prolonged exercise and peaks at the point of exhaustion (Bailey *et al.*, 1993).

One of the reasons for the popularity of the Newsholme theory is undoubtedly the opportunity it offers for manipulation in order to improve exercise capacity and/or performance. The theory has been extensively investigated, yet contrasting and inconsistent results mean that its accuracy remains unclear.

1.3.2 Pharmacological evidence

By artificially increasing brain serotonin levels in rats through the intraperitoneal administration of various doses of a specific serotonin agonist (m-chlorophenyl piperazine), treadmill exercise time to exhaustion decreased in a dose response manor, with the biggest dose resulting in the shortest run time to exhaustion (Bailey *et al.,* 1992). Conversely, intraperitoneal administration of a serotonin antagonist (LY 53857) increased time to exhaustion compared to a control injection (Bailey *et al.,* 1993).

The principles employed in the aforementioned investigations in animals have also been studied in human participants, where drugs designed to inhibit the removal of serotonin from the synaptic cleft (serotonin agonists) were administered. Increased effort and reduced exercise capacity were reported in these trials, supporting Newsholme's theory of central fatigue (Wilson and Maughan, 1992; Davis *et al.,* 1993; Marvin *et al.,* 1997). However, other studies have failed to replicate these findings (Meeusen *et al.*, 1997; Meeusen *et al.*, 2001; Strachan *et al.,* 2004), and further experiments designed to improve exercise performance through altering catecholamine or brain dopamine concentrations have also shown little success (Meeusen *et al.,* 1997; Piacentini *et al.*, 2004; Watson *et al.,* 2005A).

1.3.3 Nutritional evidence

Although there is evidence supporting the Newsholme theory in animal models, the picture in humans is less clear. Well controlled laboratory based experiments largely discredit the theory regarding the role of fatty acids and branched-chain amino acids regulating tryptophan production in the brain.

1.3.3.1 Branched-chain amino acid supplementation

As discussed above it is well established that the branched-chain amino acids compete with tryptophan for transport across the blood brain barrier. By increasing the concentration of branched-chain amino acids in the plasma through supplementation, the ratio of free tryptophan to branched-chain amino acids is also decreased. This would result in a reduced uptake of tryptophan into the brain and therefore possibly a reduction in the synthesis of serotonin, thereby delaying the onset of fatigue. The ratio of tryptophan to branched-chain amino acids in plasma has been shown to be correlated with the brain tryptophan concentration (Leathwood, 1987). This ratio is likely to be more important than the absolute concentrations of free tryptophan.

Figure 1.5 shows a simplistic view of the situation at the blood brain barrier during prolonged exercise when amino acids are supplemented. Only three studies in the published literature find that the administration of branched-chain amino acids to human participants improves exercise performance.

Chronic branched-chain amino acid supplementation has been shown to improve exercise performance by Hefler *et al.* (1995). They put participants on a supplementation program feeding 16 g of branched-chain amino acids per day for fourteen days. They found a 12 % improvement in performance of a forty kilometre time-trial in temperate conditions compared to a placebo treatment. Unfortunately, little more is known about the study as this investigation was merely presented as an abstract and never published as a full article. Supporting data and detail of the methodology are not available, making the findings hard to interpret and highly questionable.
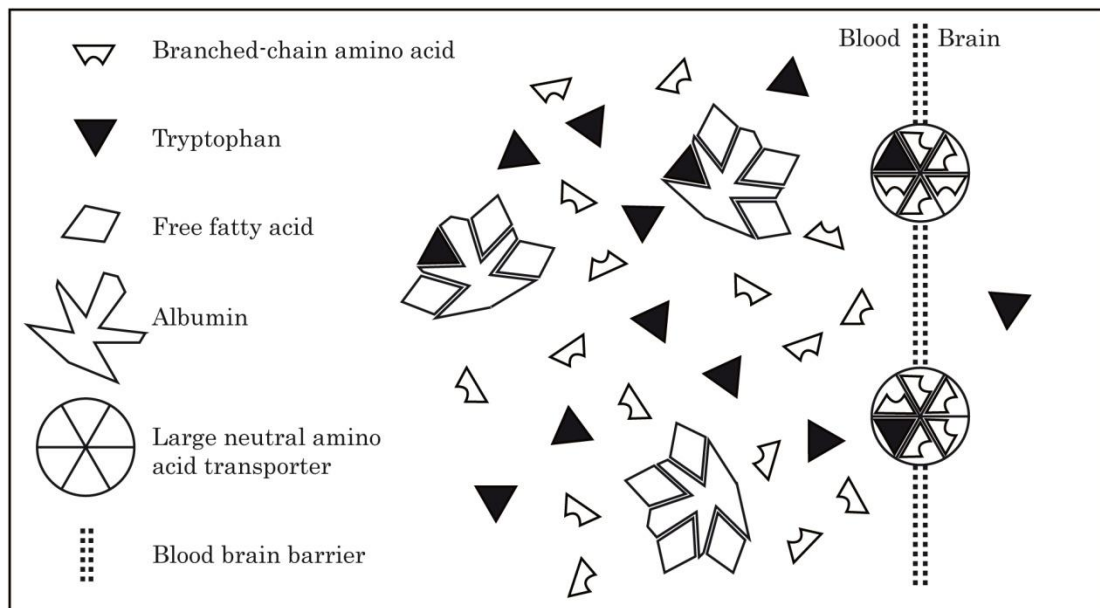
Figure 1.5. A simplistic view of the situation at the blood brain barrier during prolonged exercise when branched-chain amino acids are supplemented.

Mittleman and colleagues (1998) performed an apparently well-controlled laboratory based study into the effects of supplementation of branched-chain amino acids on exercise (40 % $\dot{V}O_2$max) capacity in the heat (34.4 ± 1.8 °C). They found that exercise time to exhaustion was longer (153.1 ± 13.3 vs. 137.0 ± 12.2 min) when participants were given drinks (5 ml / kg body mass) containing 5.88 g / L of BCAA (54 % leucine, 19 % isoleucine and 27 % valine) compared to when they were given the same volume of a placebo drink. They reported no difference in any peripheral markers of fatigue between the trials and therefore concluded that their supplementation regimen was suitable for delaying the onset of central fatigue.

1.3.3.1.2 Evidence against an ergogenic effect of branched-chain amino acids

Despite the three studies which find a positive effect on exercise performance of branched-chain amino acid supplementation, there are many studies in the published literature that find no benefit. These include exercise capacity tests (Blomstrand *et al.*, 1995; Blomstrand *et al.*, 1997; Galiano *et al.*, 1991; Struder *et al.,* 1998; Van Hall *et al.,* 1995; Watson *et al.*, 2004), incremental tests (Varnier *et al.,* 1994), intermittent tests (Davis *et al.,* 1999) and performance tests (Hassmen *et al.,* 1994). Details of these studies are in *Table 1.1.*

Table 1.1. A review of studies involving branched-chain amino acid supplementation, listed according to main outcome.

STUDY	PARTICIPANTS	BCAA DETAILS	PROTOCOL	OUTCOME
Mittleman *et al.,* 1998	7 males and 6 females, moderate training	5.88 g / L BCAA or 5.88 g / L polydextrose placebo	Cycle time to exhaustion at 40 % $\dot{V}O_2$peak in 34 °C	INCREASED EXERCISE CAPACITY (153.1 ± 13.3 minutes BCAA, 137.0 ± 12.2 minutes placebo)
Blomstrand *et al.,* 1991A	107 (BCAA) and 111 (placebo) male runners	7.5 g (cross country) or 16 g (marathon) or a 5 % CHO placebo	30 km cross country running race or 42.2 km marathon running race	IMPROVED MARATHON PERFORMANCE (but in slower runners only)
Hefler *et al.,* 1995 (Abstract only)	10 competitive male cyclists	16 g / day BCAA for 14 days. Placebo used.	40 km cycling time-trial	IMPROVED EXERCISE PERFORMANCE (56.7 ± 1.6 minutes placebo, 51.4 ± 2.6 minutes BCAA)
Hassmen *et al.,* 1994	52 males	BCAA + CHO or CHO only	30 km cross country running race	IMPROVED COGNITIVE FUNCTION (in one test, prevented a drop in performance of two tests)
Blomstrand *et al.,* 1991B	6 female national level footballers	6 % CHO + 7.5 g / L BCAA or 6 % CHO	Stroop tests before and after a match	IMPROVED COGNITIVE FUNCTION (after match compared to before match)
Blomstrand *et al.,* 1997	7 endurance trained male cyclists	7 g / L or flavoured water	Pre-loaded (60 minutes at 70 % $\dot{V}O_2$max) 20 minute cycling time-trial	IMPROVED RPE (area under the curve smaller) NO CHANGE IN EXERCISE CAPACITY (Work done: 275 kJ BCAA, 250 kJ placebo)

STUDY	PARTICIPANTS	BCAA DETAILS	PROTOCOL	OUTCOME
Davis *et al.,* 1999	3 active males, 5 active females	20 % CHO or 20 % CHO + 7 g BCAA	Preloaded (75 min intermittent shuttle run) shuttle run to exhaustion	NO CHANGE IN EXERCISE CAPACITY (CHO+BCAA: 9 min, CHO alone: 9.66 min)
Blomstrand *et al.,* 1995	5 endurance trained males	6 % CHO or 6 % CHO + 7 g / L BCAA or placebo	20 minutes pre-loaded (60 minutes at 75 % $\dot{V}O_2$max) time-trial	NO CHANGE IN EXERCISE CAPACITY (work: 264 ± 11 kJ CHO+BCAA, 263 ± 13 kJ CHO, 231 ± 11 kJ placebo)
Galiano *et al.,* 1991	8 trained males	6 % CHO or 6 % CHO with 735 mg / L BCAA	Cycling time to exhaustion at 70 % $\dot{V}O_2$max	NO CHANGE IN EXERCISE CAPACITY (220 ± 11 min CHO+BCAA, 235 ± 10 min CHO)
Struder *et al.,* 1996	10 endurance trained males	26 g / L BCAA + 46.5 g / L other amino acids	90 minute treadmill run (2 mmol / L lactate)	NO CHANGE IN RPE
Struder *et al.,* 1998	10 endurance trained males	21 g of BCAA. Placebo used	Cycle time to exhaustion at 2 mmol / L lactate	NO CHANGE IN EXERCISE CAPACITY (Placebo 157 ± 53 min, BCAA 152 ± 41 min)
Van Hall *et al.,* 1995	10 endurance trained males	6 or 18 g / L BCAA 6 % sucrose placebo	Cycle time to exhaustion at 70 – 75 % Wmax	NO CHANGE IN EXERCISE CAPACITY (122 ± 3 min both conditions)
Varnier *et al.,* 1994	6 moderately trained males	260 mg / kg / h or saline placebo	Incremental cycle (35 W every 4 minutes) time to exhaustion	NO CHANGE IN EXERCISE CAPACITY (about 60 min exercise duration)
Watson *et al.,* 2004	8 recreationally active males	12 g / L BCAA or placebo	Cycle time to exhaustion at 50 % $\dot{V}O_2$ max in 30 °C	NO CHANGE IN EXERCISE CAPACITY (placebo 104 ± 27 min, BCAA 111 ± 29 min)

Despite the overwhelming evidence that branched-chain amino acid supplementation does not improve exercise performance or capacity, there is some evidence to suggest that it improves cognitive performance (Blomstrand *et al.*, 1991B; Hassmen *et al.*, 1994). Also, ratings of perceived exertion during exercise have been shown to both improve (Blomstrand *et al.*, 1997) and to not differ (Struder *et al.*, 1996) with the supplementation of branched-chain amino acids during exercise.

1.3.3.2 Tryptophan depletion

Completely removing tryptophan from the diet reduces plasma tryptophan by only around 20 %, and consequently has few behavioural consequences (Bell *et al.*, 2001). To induce greater falls in plasma tryptophan concentration, feeding an amino acid load, with the particular omission of tryptophan, achieves about an 80 % reduction in plasma free and total tryptophan levels five to seven hours after consumption, a technique known as rapid or acute tryptophan depletion (Delgado *et al.*, 1990).

Acute tryptophan depletion works by loading the system with amino acids, causing protein synthesis to be stimulated, a process which requires tryptophan (Bell *et al.*, 2001). Since it was not ingested, tryptophan is utilised from the stores within the body. Administering large neutral amino acids further reduces the amount of tryptophan transported across the blood brain barrier due to increased competition for the common transporter (Pardridge, 1983). Such a reduction may therefore inhibit the synthesis of serotonin (Bell *et al.*, 2001). *Figure 1.6* shows a simplistic view of the situation at the blood brain barrier with acute tryptophan depletion.

Acute tryptophan depletion is a popular, well documented technique which has been used extensively in clinical studies into: depression (Neumeister *et al.*, 2004); anxiety (Bell *et al.*, 2001); aggression (McCloskey *et al.*, 2009); sleep patterns (Bhatti *et al.*, 1998); memory (Schmitt *et al.*, 2000); eating disorders (Weltzin *et al.*, 1995) and cognitive function (Gallagher *et al.*, 2003). Through the combined effects of a tryptophan free diet and acute tryptophan

depletion, the lowered brain serotonin concentrations could, theoretically, lead to an improvement in exercise capacity.
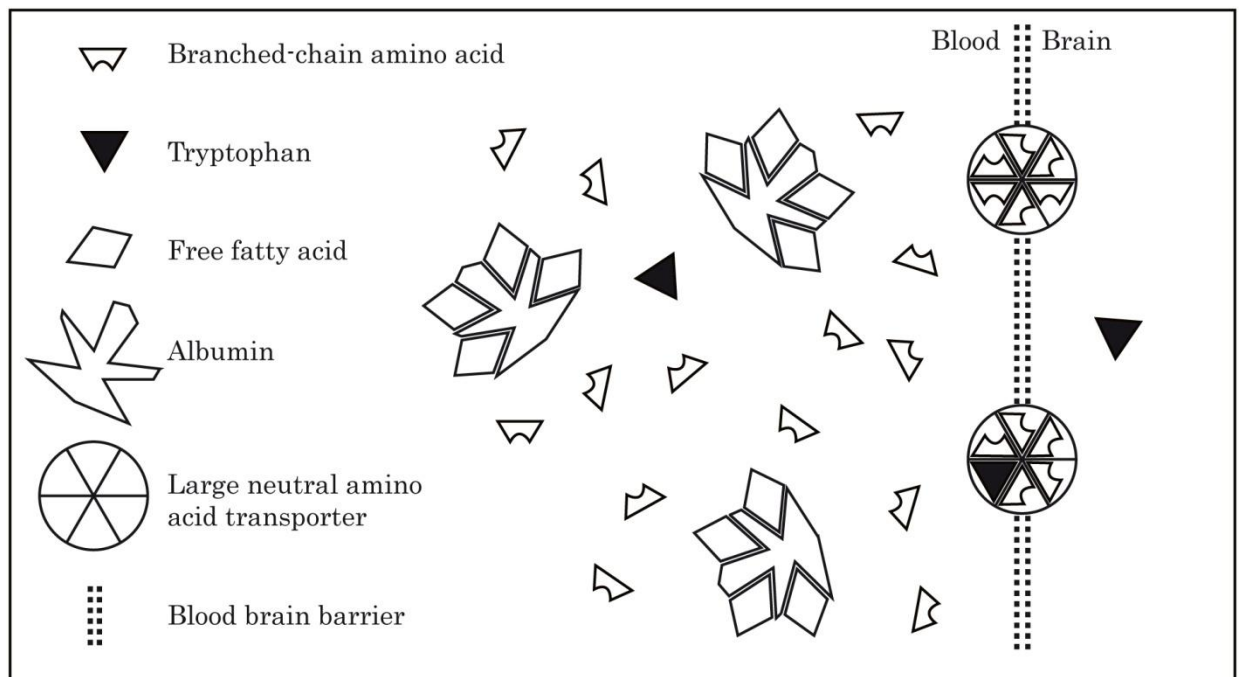
Figure 1.6. A simplistic view of the situation at the blood brain barrier when an acute tryptophan depletion protocol has been followed.

1.3.3.3 Tryptophan supplementation

Following the Newsholme theory of central fatigue, supplementing tryptophan should elicit a decrement in exercise capacity and this has been shown to be the case in horses (Farris *et al*., 1998) and rats (Soares *et al*., 2004; Soares *et al*., 2007).   In humans however, tryptophan supplementation (1.2 g over approximately fifteen hours) has been shown to improve exercise capacity (Segura and Ventura, 1988). However, it should be noted that the findings of an improvement in exercise capacity (running at 80 % $\dot{V}O_2$max) by on average 49 % (and 260 % and 160 % in two participants), combined with some incredulously short run times to exhaustion (placebo trial mean 335 ± 140 seconds, range 150 – 570 seconds) leaves these findings open to speculation.   Furthermore, when this protocol was repeated with forty-nine participants rather than just twelve, no effect on exercise capacity was seen (placebo group mean of 443.5 ± 100.2 seconds *vs*. tryptophan group mean of

451.4 ± 99.6 seconds; Stensrud *et al.,* 1992). This finding is also reflected in the results by Van Hall *et al.* (1995).

1.3.3.4 Free fatty acid supplementation

Huffman *et al.* (2004) investigated the Newsholme theory from another angle. They hypothesised that supplementation with omega-3 fatty acids would reduce free tryptophan concentration, reduce serotonin production and therefore increase exercise capacity. Omega-3 fatty acids cause an increase in the rate of free fatty acid oxidation through the activation of a nuclear transcription factor (Clarke, 2001). However, chronic supplementation of omega-3 fatty acids (4 g / day for four weeks) in five male and five female participants did not alter pre-loaded exercise capacity nor free tryptophan or free fatty acid plasma concentrations, although the average free tryptophan to branched-chain amino acid ratio over the exercise bouts was significantly lower after supplementation than before.

1.3.3.5 Ammonia

Ammonia has been suggested to have an influence on cerebral metabolism and on the development of central fatigue. Ammonia concentration increases with the deamination of branched-chain amino acids, as occurs during prolonged exercise. Therefore, when branched-chain amino acids are supplemented there is an even greater increase in the circulating concentration of ammonia. Indeed, Watson *et al.* (2004) found that branched-chain amino acid supplementation did not change plasma ammonia concentration at rest. During subsequent exercise to exhaustion at 50 % $\dot{V}O_2$peak in 30 °C, the plasma ammonia concentration on the branched-chain amino acid trials was significantly greater than on the placebo trials (Watson *et al.,* 2004).

This exercise-induced hyperammonaemia has been hypothesised to be one of the reasons for the failure to see an improvement in exercise capacity after supplementation of branched-chain amino acids (Nybo and Secher, 2004). Ammonia and glutamate are the precursors for the synthesis of glutamine.

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The brain has no mechanism for the removal of excess ammonia, which can cross the blood brain barrier, other than glutamine synthesis. Glutamine synthesis may therefore lower the concentration of glutamate in the brain, and glutamate is a precursor for the synthesis of gamma-aminobutyric acid (GABA), meaning that this could also be lowered as a result of increased ammonia concentrations (Nybo and Secher, 2004). GABAergic neurons are the major source of synaptic inhibition in the nervous system (Bear *et al.,* 2001). Therefore, reduction in GABA could be involved in central fatigue. However, this theory has been sparsely investigated in humans and therefore it is impossible to conclude whether or not this plays a role in the failure to see an ergogenic effect of branched-chain amino acid supplementation protocols.

1.3.3.6 Carbohydrate supplementation

Carbohydrate supplementation has been suggested to influence central fatigue through its actions on the serotonergic system because carbohydrate attenuates the transportation of tryptophan across the blood brain barrier. Exogenous carbohydrate suppresses lipolysis, thereby reducing circulating free fatty acid concentration in the plasma. With less free fatty acids displacing the albumin-bound tryptophan, carbohydrate limits the rise in circulating free tryptophan concentration seen during prolonged exercise. This reduces the level of tryptophan uptake into the brain compared to when plain water is given (Blomstrand *et al.,* 2005). This may subsequently lead to a reduction in serotonin synthesis, thereby improving exercise performance.

Indeed, plasma branched-chain amino acid concentrations fell from around 400 µmol / L at rest to around 325 µmol / L and 275 µmol / L at the point of exhaustion when 6 % and 12 % carbohydrate drinks respectively were administered. Branched-chain amino acid concentration was maintained throughout the exercise on the placebo trial. There was also a five- to seven-fold increase in the ratio of free tryptophan to branched-chain amino acids in plasma during the 6 % and 12 % carbohydrate trials compared to the placebo trial (Davis *et al.,* 1992). Exercise capacity, assessed as time to exhaustion on a cycle ergometer at approximately 68 % $\dot{V}O_2$max, was increased in the

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carbohydrate trials (average (mean ± SEM) of 235 ± 10 and 234 ± 9 minutes for the 6 % and 12 % carbohydrate drinks respectively) compared to the placebo trials (average of 190 ± 4 minutes), suggesting that the supplementation of exogenous carbohydrate delayed the onset of central fatigue (Davis *et al.,* 1992).

Rats, however, showed no additive effect of carbohydrate with branched-chain amino acid supplementation (171 ± 12 minutes) compared to when the two supplements were administered individually, in an exercise to exhaustion test following a glycogen depletion protocol (Calders *et al.,* 1999). There was an improvement in the time to exhaustion in the branched-chain amino acid trial (158 ± 26 minutes) and in the carbohydrate trial (179 ± 21 minutes) compared to the saline trial (118 ± 35 minutes), probably resulting from their contribution as a fuel source. The lack of an additive effect was purported to be due to the presence of carbohydrate inhibiting amino acid catabolism.

Further evidence for the effect of carbohydrate on the brain during exercise comes from studies which have investigated the effects of mouth rinsing carbohydrate. The presence of 6.4 % carbohydrate (glucose or maltodextrin) in the mouth activates reward related regions of the brain, including the frontal operculum and the striatum (Chambers *et al.,* 2009). Interestingly, the artificial sweetener sucralose does not elicit such a strong response in these regions as sucrose (Frank *et al.,* 2008). This activation of reward related brain regions by the presence of carbohydrate in the mouth without ingestion has been shown to result in improvements in exercise performance in cycling (Carter *et al.,* 2004; Chambers *et al.,* 2009) and running (Rollo *et al.,* 2010), when participants were fasted for six or more hours. However, the same improvements were not seen in a running time-trial performance conducted after a four hour fast (Whitham and McKinney, 2007).

Unfortunately, due to the well reported ergogenic effect of carbohydrate supplementation on peripheral causes of fatigue (Neufer *et al.,* 1987; Jeukendrup *et al.,* 1997; Coggan and Coyle, 1991), it is very difficult to

interpret the extent of the central element of improvements in exercise performance or capacity as a result of exogenous carbohydrate supply.

1.3.4 Overview

It is important to consider the limitations of all investigations in order to determine whether or not their findings can be applied to alternative situations. Much of the early research on the role of the serotonergic system in fatigue used brain homogenates and therefore may not represent true *in vivo* situations. Often, research is not done using human participants and inter-species differences in substrate supply and neurotransmitter metabolism, storage and release, are likely to exist.

It has been hypothesised that neurotransmitters, other than serotonin, may also have roles to play in the onset of central fatigue. It is likely that there is not just a single mechanism of fatigue. Rather, an interdigitated response of several neurochemicals, which act in concert to bring about fatigue and the cessation of exercise, is a more plausible theory. However, the serotonin system will still undoubtedly play a role in the central response. Furthermore, the precise role and influence of each neurochemical may be dependent upon many other factors such as core temperature and individual fitness levels.

1.4 Prolonged exercise in the heat

1.4.1 Overview

Mechanically, humans are at most 20 – 30 % efficient; the majority of energy is lost as heat (Powers and Howley, 2007). Exercise and physical activity can substantially increase metabolic heat production, but due to effective heat loss mechanisms (radiation, conduction, convection and evaporation) the human core body temperature remains relatively stable over a large range of ambient temperatures (Astrand *et al.,* 2003B). Autonomic temperature regulation is under the control of the central nervous system and involves the activation of heat loss mechanisms such as sweating or heat retention mechanisms such as peripheral vasoconstriction (Parsons, 2003B).

1.4.1.1 Performance

Unless endurance exercise is undertaken in cool conditions or at low intensities then a steady rise in core body temperature occurs. In some power based events an elevated core temperature is thought to be of benefit, since this increases the rates of the actin / myosin interaction within the muscle fibre (Astrand *et al.,* 2003B) and also increases mitochondrial ATPase activity (Brookes *et al.,* 1971). However, it is generally accepted that during prolonged exercise a rising core temperature, due to a reduction in the body's ability to lose heat, will result in a decrement in performance due to the onset of hyperthermia.

High ambient temperatures (31 °C) can reduce the capacity to perform prolonged exercise, compared to a cooler environment (11 °C), by just below 50 % (Galloway and Maughan, 1997). A 6.5 % reduction in mean power output during a thirty minute cycling time-trial was seen when ambient temperature increased from 23 °C to 32 °C, without a change in rectal temperature between trials (Tatterson *et al.,* 2000). Often, when ambient temperature is increased there is also an accompanying increase in the rating of perceived exertion for the task (Morris *et al.,* 2000). Indeed, Nybo and Nielsen (2001C) found a correlation between core temperature and RPE, and also electroencephalogram activity in the frontal, central and occipital cortex.

1.4.1.2 Substrate utilisation

Exercise during heat exposure has been suggested to up-regulate the flux through the pyruvate dehydrogenase pathway, possibly because some of the pyruvate formed is being oxidised (Febbraio *et al.,* 1994; Febbraio, 2001). While fatigue during prolonged exercise in temperate conditions is often attributed to depletion of muscle glycogen (Bergstrom *et al.,* 1967), this relationship does not appear to hold true at high ambient temperatures. Parkin *et al.* (1999) found that despite no differences in resting muscle glycogen concentration; at the point of exhaustion following cycling at 70 % $\dot{V}O_2$peak there was significantly more muscle glycogen remaining after the

trials that took place in 40 °C (299 ± 33 mmol / kg) compared to either 20 °C (153 ± 27 mmol / kg) or 3 °C (116 ± 28 mmol / kg).

Exercise in the heat per-se does not affect oxygen uptake in the muscle (Gonzalez-Alonso *et al.*, 1998). Indeed, even if blood flow to the working muscles is reduced, as seen during exercise when hypohydrated (Gonzalez-Alonso *et al.,* 1998), there does not appear to be a compromise in oxygen delivery or muscle metabolism, since the oxygen extraction by the working muscles can increase as a means of compensating for the reduced muscle blood flow (Gonzalez-Alonso *et al.,* 1999A).

Nielsen and colleagues have shown that at the point of fatigue there is no reduction in cardiac output, mean arterial pressure, muscle or skin blood flow (Nielsen *et al.*, 1993; Nielsen *et al.,* 1997) and in Galloway and Maughan's (1997) experiment investigating exercise capacity at varying ambient temperatures, the carbohydrate oxidation rate was lower after forty five minutes cycling in 31 °C, where fatigue occurred earliest, compared to the same exercise performed in 4 °C, 11 °C or 21 °C.

1.4.1.3 Thermoregulation and cardiovascular responses

Hyperthermia or heat strain during exercise in warm or hot ambient temperatures places a strain on the cardiovascular system. During exercise the volume of blood sent to the working muscles increases to provide them with fuel and oxygen, and to remove waste products. However, at a given cardiac output during exercise in the heat, more blood will flow to the skin and less to the muscles than in lower environmental temperatures (Astrand *et al.,* 2003B). Cardiac output increases, through an increase in heart rate, in an attempt to maintain muscle blood flow. Often added into this situation is a falling blood volume due to dehydration, resulting from the loss of body fluids through sweating if no, or insufficient, fluids are consumed. This reduces stroke volume leading to a compensatory increase in heart rate to maintain cardiac output (Wyndham, 1973).

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Although skin blood flow increases during exercise in the heat, the reduced gradient between the skin temperature and the environmental temperature means that the efficacy of this avenue of heat loss is reduced and core temperature rises. If core body temperature continues to rise, hyperthermia ensues. This is thought to be a key factor in the development of fatigue as it reduces the motivation to continue with exercise (Bruck and Olschewski, 1987). This can be viewed as the body's built-in safety mechanism to ensure that there is no further increase in core body temperature which could put the individual at risk of permanent damage.

There have been suggestions that exhaustion during prolonged exercise in the heat may be related to a critically high core temperature of around 40°C (39.7 ± 0.15 °C), above which exercise cannot be sustained (Nielsen *et al.,* 1993). It has been shown that by lowering initial core temperature, exercise capacity can equally also be increased, suggesting that time to fatigue is inversely related to initial core temperature (Gonzalez-Alonso *et al.,* 1999B).

However, it appears that critical core temperature, if such a phenomenon even exists, may be related to physical fitness and must vary between individuals, as core temperatures of well below 40 °C are frequently seen at the point of exhaustion, after prolonged exercise in the heat (Cheung and McLellan, 1998; Sawka *et al.*, 1992; Bridge *et al.,* 2003A; Bridge *et al.,* 2003B; Hobson *et al.,* 2009; Watson *et al.,* 2004). Furthermore, core temperature readings at the point of exhaustion after prolonged exercise in the heat have been reported to be in excess of 40 °C in some individuals (Pugh *et al.,* 1967). A confounding factor in this debate, however, is the range of techniques and sites used to measure core temperature. Recently it has also been shown that during hypohydration (4 %), every 1 °C increase in skin temperature results in a decrement in aerobic performance of around 1.6 % (Kenefick et al., 2010), despite no differences in core temperature.

1.4.2 The brain

1.4.2.1 Blood flow and activity

Exercise and heat stress, when combined, have serious implications for metabolism and circulation within the brain. Blood flow to the brain increases during exercise in normal, temperate environments (Ide and Secher, 2000), but declines with hyperthermia, which is likely to occur during prolonged exercise in the heat (Nybo and Nielsen, 2001A). Increased global cerebral metabolism in response to hyperthermia suggests that the overall levels of neuronal activity are increased (Nybo *et al.,* 2002) although the underlying causes of this effect remain unclear as it could be attributed to increased exertion during prolonged exercise, or to the elevated core body temperature leading to increased rates of enzymatic processes (the Van't Hoff Q_{10} effect), coupled with increasing cerebral metabolic rates of oxygen and glucose, both seen by Nybo and colleagues (2002).

Through the use of positron emission tomography scans, Nunneley *et al.* (2002) demonstrated that at rest the cerebral metabolic rate of several brain regions, including the hypothalamus, thalamus, cerebellum and corpus callosum, was increased by systemic hyperthermia eliciting core temperatures of 38.6 °C and skin temperatures of 39 – 40 °C, compared to during normothermic conditions. Within the central nervous system, temperature control is regulated by the hypothalamus, the brain stem and the spinal cord, and more specifically by the pre-optic area of the anterior hypothalamus (POAH; Boulant, 2000). It is likely that similar activation occurs during passive heat exposure and exercise. It is important to note however, that several brain regions showed increases in cerebral activity in the study by Nunneley and colleagues, not just the hypothalamus, implying that thermoregulation is not controlled by the hypothalamus alone and that several brain regions may co-operate in the perception of heat and the autonomic and behavioural responses to alterations in environmental and body temperature.

1.4.2.2 Neurotransmitters

Serotonin is known to be involved in thermoregulation (Jacobs and Azmitia, 1992). The 5-HT$_{2c}$ reuptake inhibitor Paroxetine increases core temperature

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compared to when a placebo is administered, both at rest and during exercise in the heat (32 °C), although this did not influence exercise time to exhaustion nor ratings of perceived exertion (Strachan *et al.,* 2004). Serotonin is a major excitatory neurotransmitter for prolactin release (Struder and Weicker, 2001A). Therefore, increases in serum prolactin levels can be associated with increases in serotonin release. Serum prolactin levels are higher during exercise in the heat (30 °C) than during exercise in the cold (10 °C; Pitsiladis *et al.,* 2002), but they do not correlate to plasma tryptophan, free tryptophan, branched-chain amino acids or the ratio of free tryptophan to branched-chain amino acids, all of which have been purported as peripheral modulators of the serotonin system. Serum prolactin concentration did however relate to rectal temperature during exercise in the heat (Pitsiladis *et al.,* 2002).

Increased ratings of perceived exertion during exercise in the heat are not related to the cerebral balance of dopamine or tryptophan assessed by arterial and venous sampling (Nybo *et al.,* 2003). However dopamine has also been shown to be involved in thermoregulation. Based on studies into the relationship between dopaminergic pathways and thermoregulation by Hasegawa *et al.* (2000) coupled with their own findings, Bridge *et al.* (2003A) suggest that an individual's ability to exercise in the heat is enhanced by a high activity level in the dopaminergic pathways of the preoptic anterior hypothalamus.

1.4.2.3 Muscle activation

In rats (Fuller *et al.,* 1998) and goats (Caputa *et al.,* 1986) brain temperature has been shown to be a major factor affecting movement. Although not directly related to endurance exercise in the heat, several studies have investigated the influence of hyperthermia on muscle activation.

Nybo and Nielsen (2001B) found that exercise-induced hyperthermia (core temperature 40.0 ± 0.1 °C) caused a marked reduction in force generation capacity, both in exercised muscle groups and non-exercised muscle groups. However, Martin *et al.* (2005) and Saboisky *et al.* (2003) both found that while

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hyperthermia caused a marked reduction in force generation capacity of exercised muscle, no such reduction was seen in non-exercised muscle.

These results suggest that although an elevated core temperature decreases the central drive to activate muscles, it is unlikely to be the sole factor. Once again, methodological differences between studies in the duration of activation required may also be the cause of the discrepancy in the findings. Either way, these studies offer clear evidence that hyperthermia, induced by exercise, reduces the brain's drive to recruit active skeletal muscle.

1.4.2.4 Brain temperature manipulation

Several animal models have been used to investigate the role of various brain regions in the autonomic control of thermoregulation. The temperature of the POAH has been reduced to induce heat saving (peripheral vasoconstriction) and heat producing (shivering) responses (Boulant and Gonzalez, 1977) or warmed to induce heat loss responses (increased skin blood flow; Jacobson and Squires, 1970). Local warming of the hypothalamus has also been shown to evoke panting and cause vasodilation in cats and dogs (Magoun *et al.,* 1938; Hemingway *et al.,* 1940).

The concept of being able to selectively cool the brain has been widely accepted in most mammals. However, the application of this theory to the human brain is a source of much debate. Cabanac (1993) suggests that selective brain cooling offers a better explanation of the benefits seen through head cooling, such as improved comfort, and increased exercise capacity, than does afferent nervous input to the brain from the skin.

Unfortunately, there are several theories as to why selective brain cooling cannot exist in humans. Physiologically, humans do not 'pant' as a thermoregulatory mechanism, neither do they have a carotid *rete*, which in some non-human mammals is a counter-current heat exchanger between arterial and venous bloods flowing to and from the brain (Brengelmann, 1993). Furthermore, selective brain cooling would effectively remove the protective error signal against potentially harmful hyperthermic conditions in the rest of

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the body. Also, the low tympanic temperatures, seen in hyperthermic individuals are purported not to be a sign of brain cooling but rather a consequence of low head skin temperature contaminating the temperature reading (Shiraki *et al.,* 1988).

1.4.3 The skin and the brain

1.4.3.1 Integration of information

The POAH is highly sensitive to changes both centrally (as evidenced above in section 1.4.2.4) and peripherally (Boulant, 2000). Although the core temperature seems to be the strongest stimulus for the initiation of thermoregulatory responses, there can be little doubt that the temperature of the skin also plays a significant role. The POAH receives afferent sensory input from the warm and cold receptors in the skin (Boulant and Hardy, 1974). Therefore, the POAH can affect the most appropriate thermoregulatory response considering the internal and external environmental temperatures.

Simmons *et al.* (2008) assessed several parameters including alertness and calmness whilst at rest, and demonstrated that heat-related fatigue is a function of a combination of an elevated core and skin temperature. They found that elevated skin temperature did not negatively influence cognitive performance (using measures of accuracy and reaction time assessed from a battery of cognitive tests), but when core temperature was also increased cognitive performance was negatively affected. They also found that cooling of the head and neck, through use of a cold water perfused balaclava, did not improve cognitive performance when skin temperature was elevated, nor did it blunt the decrement in cognitive function seen when skin and core temperatures were elevated.

1.4.3.2 Facial cooling

Lowering of skin temperatures and facial cooling during exercise in the heat greatly reduces the perception of effort associated with elevated core temperatures (Armada-da-Silva *et al.*, 2004). Lowered skin temperature has also been suggested to modulate the effects of high core temperatures on cardiovascular (Riggs *et al.*, 1981) and hormonal responses to exercise in the

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heat (Bridge *et al.*, 2003B; Ansley *et al.*, 2008), and possibly even improve endurance exercise capacity (Ansley *et al.,* 2008) as detailed below.

Riggs *et al.* (1981) found that facial cooling, using wind at 10 °C during progressively intense exercise lasting sixteen minutes, evoked a lower heart rate than when no facial cooling occurred. The blunted heart rate response appeared to be associated with a declining skin temperature: no differences were seen in the blood pressure or core temperature of the participants. The authors suggested that the heart rate response was not a reflex mediated by increased stroke volume, but was mediated through a central thermoregulatory response. There have since been many studies looking at the effects of cooling the skin during exercise, often performed in the heat. Brisson *et al.* (1989) showed that facial cooling, using wind at 10 °C, during submaximal exercise in the heat (27 ± 0.5 °C) caused bradycardia, to such an extent that workload had to be increased by an average of twenty watts to elicit the same response as when there was no facial cooling.

Facial cooling in the study by Brisson *et al.* (1989) also caused a blunting (31 % increase from basal resting values) in the plasma prolactin response seen with no facial cooling (450 % increase from basal resting values). Bridge and colleagues (2003B) investigated this further by comparing the prolactin response to exhaustive exercise at 73 % $\dot{V}O_2$max in 20 °C and 35 °C. The prolactin concentration was elevated from baseline levels after forty minutes of exercise and remained elevated at the point of fatigue when the exercise was done in 20 °C. In 35 °C the prolactin concentration was elevated from baseline levels by twenty minutes of exercise and was greater than the values in 20 °C after ten minutes of exercise (Bridge *et al.*, 2003B). Prolactin release has been purposed to be a marker of central serotonergic activity. These findings therefore suggest that central serotonergic activity is increased by exercise in the heat and suppressed by head cooling during exercise in the heat.

Head cooling was again found to attenuate the increase in prolactin release seen in response to exhaustive exercise at 75 % $\dot{V}O_2$max in a warm environment (29 ± 1 °C), reducing the elevation from a six-fold increase in the control condition to a two-fold increase in the head cooling condition (Ansley *et al.*, 2008). Their head cooling protocol, achieved through wind and cool water application, also resulted in a 51 % (21 – 65 %) improvement in exercise time to exhaustion at 75 % $\dot{V}O_2$max in 29.0 ± 1.0 °C from 24 (22 - 47) minutes in the control condition to 45 (29 – 61) minutes in the head cooling condition.

1.4.3.3 Thermal comfort

Weiss and Laties (1961) suggested long ago that thermal comfort is the factor that leads to the initiation of behavioural thermoregulation such as heat seeking behaviour. The integration of peripheral or cutaneous thermal information with core temperature has been shown to be a key factor in the determination of thermal comfort (Frank *et al.,* 1999; Gibson *et al.,* 1980). Frank and colleagues (1999) demonstrated that skin temperature was directly linked to thermal comfort and that lowering skin temperatures to ~30 °C lowered thermal comfort ratings compared to the ratings given at skin temperatures of ~34 °C while increasing skin temperatures to ~36 °C increased thermal comfort ratings, all whilst core temperatures were maintained at ~37 °C.

Frank *et al.* (1999) also showed that core temperature and skin temperature contribute equally to levels of thermal comfort in men, assessed by a ten point visual analogue scale. By artificially manipulating the perception of skin temperature to lower than the true physiological temperature, it may be possible to manipulate thermal comfort and, as a result, improve heat tolerance. If this hypothesis holds true during exercise in a warm environment, endurance exercise performance or capacity may be improved. Equally, by artificially manipulating the perception of skin temperature to higher than the true value, endurance exercise performance or capacity may be reduced.

1.5 Manipulation of thermal sensation using menthol

1.5.1 Mechanism of action

Cool sensations can be caused by either the stimulation of cold receptors or the inhibition of warm receptors. Conversely, warm sensations can be caused by either the stimulation of warm receptors or the inhibition of cold receptors (Eccles, 2000). According to Eccles (2000), as far back as the nineteenth-century the warming and cooling sensations elicited by the plant extract menthol were suggested to be the result of stimulation of sensory nerve endings (Goldscheider, 1886). Therefore, the application of menthol to the skin results in a cooling or a warming sensation without a concomitant alteration in actual skin temperature.

It is now known that menthol exerts its effects on cold receptors by interfering with the movement of calcium across the cell membrane in thermoreceptors (Schafer *et al.,* 1986). However, the mechanisms of the action of menthol are more complex than a simple stimulation of cold receptors, since menthol also modulates feelings of warmth and, in high concentrations, can cause irritation and local anaesthesia (Eccles, 1994).

The transient receptor potential melastatin-8 (TRPM8), also known as cold and menthol receptor one (CMR1) is the ion channel with the most advocated role in cold transduction (McKemy *et al.,* 2002). It is activated by voltage, low temperature and cooling compounds. Its expression is also thought to be restricted to small sensory neurons (Malkia *et al.,* 2007). Further support for the role of TRPM8 in cold transduction comes from the use of the compound BCTC as a potent and full blocker of these channels, resulting in a lack of a response to the application of menthol in mice and guinea pigs (Madrid *et al.,* 2006). However, menthol has also been shown to activate transient receptor potential vanilloid-3, a heat activated ion channel (Macpherson *et al.*, 2006).

1.5.2 Responses in animals

When mice had menthol (1 – 10 %) applied to the skin of their whole trunk they demonstrated both behavioural and autonomic responses. These included heat seeking behaviour, shivering-like muscle activity and peripheral

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(tail) vasoconstriction (Tajino *et al*., 2007), as well as increases in core temperature of 1 – 2 °C (Tajino *et al.,* 2008). This suggests that in mice, when a large percentage of TRPM8 receptors are activated a cooling response ensues. Indeed, TRPM8 deficient mice exhibit diminished responses to cold, assessed by measuring acute flinching responses using acetone-evoked evaporative cooling, and an impaired ability to distinguish between warm and cold surfaces (Bautista *et al.,* 2007).

1.5.3 Responses in humans

Existing research on topically applied menthol has predominantly been carried out in temperate environments of 18 - 23 °C (Yosipovitch *et al.,* 1996; Kozyreva and Tkachenko, 2008). It is probable however, that the actual skin temperature rather than the ambient temperature plays a greater role in the perception of skin temperature after an application of menthol. Kozyreva and Tkachenko (2008) found that some people show an increase in the number of functioning cold receptors, while others show a decrease and some show no change when applying a 1 % menthol solution to the forearm at a skin temperature of 32.3 ± 0.1 °C (30.5 – 33.7 °C).

The most comprehensive and systematic study into the sensory effects of menthol application to the skin at different temperatures was done by Green (1992). When skin temperature was maintained at 31 °C, the application of 5 % or 10 % menthol to the skin had an excitatory effect. He also showed that at lower skin temperatures (21 °C), menthol solutions had a proportionally smaller effect on the perceived intensity of cooling than at 29 °C, or than when they were compared to a 0 % menthol control solution. These findings were mirrored with higher skin temperatures where the proportional intensity of the warming sensation of menthol was reduced compared to a 0 % menthol control as skin temperature increased from 33 °C to 41 °C, but as skin temperature increased so did the perceived intensity of the warming sensation in all trials.

Different areas of the body surface have different densities of thermoreceptors and Watson and colleagues (1978) showed that the eyes and buccal region

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are most sensitive to the effects of menthol, while the palms and soles are least sensitive. Despite this, Nakamura *et al.* (2008) suggest that the qualitative differences in the thermal comfort of various body areas seen in their study (preferential cooling of the head in the heat and maintaining the warmth of the core in the cold) cannot be solely explained by the distribution of peripheral thermoreceptors, thereby implicating processing mechanisms within the central nervous system.

1.5.4 Performance responses

In a recent study by Mundel and Jones (2010) it was shown that cycling exercise capacity in the heat (34 °C) was improved by 9 ± 12 % when swilling a menthol solution around the oral cavity (63 ± 14 minutes) compared to swilling a placebo solution (58 ± 16 minutes). Central RPE (defined by the authors as the rating of perceived exertion for the cardiopulmonary system) was significantly lower for the initial forty minutes of exercise, and pulmonary ventilation was greater after twenty and forty minutes of exercise on the menthol trials compared to the placebo trials.

Mundel and Jones (2010) postulated that the mechanism of the improvement in exercise capacity was an alteration in the sensation of oropharyngeal temperature through alterations in psychophysical processes. The reduction in central RPE was likely due to the improved ventilation, as there were no differences in any of the circulatory or metabolic parameters measured. As suggested by Watson *et al.,* (1978), the oropharyngeal cold receptors of the mouth are highly sensitive to menthol and this may have altered the perception of the effort required to breath. Anecdotal evidence from comments made by their participants also suggested that the menthol caused a sensation that was described as 'refreshing' and 'stimulating' (Mundel and Jones, 2010).

1.5.5 Overview

There are clear physiological and performance effects of actual skin, head and facial cooling. In recent years there has been an increase in the availability of commercially available, menthol infused, sports clothing

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claiming to improve performance. Further to this there is also evidence for alterations in thermal sensation evoked by cutaneous menthol application, coupled with the improvements in exercise capacity found when mouth-rinsing with menthol. These factors all contribute to the necessity to further investigate the effects of manipulating thermal sensation without altering body temperature during exercise in the heat.

1.6 Assessment of the efficacy of interventions

There has always been a need within exercise physiology to perform reproducible, accurate and precise exercise tests to determine changes induced by various interventions. Indeed, within sports science research, exercise tests are one of the most commonly used tools (Hinkson and Hopkins, 2005). Therefore, it is important to use the most appropriate test to enable the correct interpretation of results.

Two main distinctive types of protocol are employed to assess possible changes in the outcome of endurance exercise. They are exercise capacity tests (time to fatigue at a constant workload), and exercise performance tests (time taken to complete a set amount of work, or completing as much work as possible in a set duration). Both of these methods offer useful opportunities to investigate the efficacy of various interventions upon exercise, but equally both have drawbacks. It is therefore important to understand these so that the most appropriate method can be employed.

The more traditionally used test involves exercise at a fixed workload, maintained to the point of exhaustion. This is a useful tool for the measurement of changes that may occur within the physiology of the participant due to various pharmacological, training, nutritional or dietary manipulations over the course of an exercise bout (Bergstrom *et al.,* 1967; Cogan and Coyle, 1987; Gleeson *et al.,* 1986; Coyle *et al.,* 1986; Wilson and Maughan, 1992). However, time to exhaustion tests are really tests of exercise capacity or fatigue, rather than being a direct assessment of performance.

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Historically, time to exhaustion was the test of choice and was widely utilised. However, as reports began to appear from some researchers presenting very large variations within individual performances of the protocol (up to 56 %, Krebs and Powers, 1989; up to 40 % Jeukendrup *et al.,* 1996), there has been a shift in favour of time-trial protocols This was despite further researchers finding much lower variations in performance measured by time to exhaustion than these reported values (3 ± 15 %, Watson *et al.,* 2004; 7.0 ± 5.1 %, Maughan *et al.,* 1989; 7.9 % Coyle *et al.*, 1986 according to Hopkins *et al.*, 2001).

Time-trials are direct measures of exercise performance and involve the assessment of a participant's ability to perform a set distance, duration or amount of work. They are widely reported to have fairly low variability (Laursen *et al.,* 2003; Jeukendrup *et al.*, 1996; Marion *et al.*, 2002). This tool is often utilised in such a way that the results can be applied to actual sporting settings (Coyle et al., 1991), leading to suggestions that they are the more appropriate exercise tests for athletes, as they are more race specific (Jeukendrup *et al.,* 1996). However, with a time-trial protocol the participants are required to pace themselves, which is often cited as a potentially confounding factor (Hinkson and Hopkins, 2005).

The choice of which test is most appropriate to use in a certain experiment is often dictated by the research question, and it can also be important in the investigators' subsequent interpretation of their findings. During this thesis both forms of exercise test were employed. The variability data for each exercise protocol are reported in the appropriate experimental chapters.

1.7 Summary
From the literature outlined above it can be seen that there are still a plethora of unanswered questions with regard to the role of the brain in fatigue during exercise.

Support for the probably overly simplistic Newsholme theory of central fatigue appears to be diminishing. However, before dismissing the theory completely there are still a couple of avenues which pose interesting opportunities for research. Watson *et al.* (2004) suggested, based upon their results and those of others, that the use of branched-chain amino acids to manipulate endurance exercise capacity may be an effective ergogenic aid in some individuals while others will not.

Furthermore, psychology research shows that there is more than one way of influencing tryptophan uptake into the brain. Acute tryptophan depletion is a technique which removes as much tryptophan as possible from the circulation, thereby reducing tryptophan uptake into the brain. This aim is shared by the technique of feeding branched-chain amino acids. Therefore, investigating the exercise capacity response to acute tryptophan depletion warrants investigation.

The availability of the serotonin precursor tryptophan is just one factor in the synthesis of serotonin. Once the serotonin has been produced it then fulfils many other roles within the central nervous system. The concentration of serotonin in the synaptic cleft is carefully regulated, with serotonin being transported out of and into neurons by transporters. The density and activity levels of these transporters may influence the rate of change of the concentration of serotonin in the synaptic clefts, which may in turn effect the actions of the serotonin. Therefore, the density and activity of these transmitters may be important in the aetiology of fatigue. Unfortunately it is impossible to study the neuronal serotonin transporters of athletes. However, peripheral models may offer and interesting insight into this aspect of fatigue. A study by Strachan and Maughan (1998) found that endurance trained individuals have more serotonin transporters on their blood platelets than sedentary people. This information poses many interesting questions, which warrant further investigation.

Serotonin is not the only central factor involved in fatigue. During heat exposure feelings of lethargy often increase, and it is well documented that

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endurance exercise performance and capacity are detrimentally affected. Skin temperature and core temperature have been shown to equally contribute to thermal comfort (Frank *et al.*, 1999). Applying menthol to the skin alters thermal sensation without altering skin temperature. The menthol acts upon peripheral thermoreceptors which originate in the hypothalamus. Applying menthol to the skin during heat exposure could alter the thermal comfort and thermal sensation of the skin. If this is the case then applying menthol to the skin during exercise in the heat may alter endurance exercise performance. Swilling menthol around the oral cavity has been shown to improve endurance exercise performance, and the advent of menthol infused clothing means that this is an emerging area of research which warrants further investigation.

1.8 Aims of the experimental chapters

1.8.1 Chapter Three

The aim of Chapter Three was to determine if inter-individual differences exist in the response to branched-chain amino acid supplementation on endurance cycling capacity in a warm environment.

1.8.2 Chapter Four

The aim of Chapter Four was to examine the responses to prolonged exhaustive cycling exercise in a warm environment when participants were either tryptophan depleted through amino acid supplementation or fed a control dose of amino acids.

1.8.3 Chapter Five

The aim of Chapter Five was to look for differences in platelet serotonin transporter density in former and current sprint and distance runners, and sedentary controls in order to discover if this is a state or a trait marker.

1.8.4 Chapter Six

The aim of Chapter Six was to elucidate the physiological and psychophysical responses to the application of a 1 % menthol solution to the skin whilst resting in the heat.

1.8.5 Chapter Seven

The aim of Chapter Seven was to determine if the manipulation of thermal sensation, without alterations in core or skin temperatures through the application of menthol to the skin, would alter endurance exercise performance in the heat.

CHAPTER TWO

General methods.

2.1 Introduction

This chapter outlines the methodologies used in the experimental research reported in subsequent chapters. The individual experimental protocols are detailed in the methods section of each chapter. With the exception of the sample collection undertaken in Chapter Five, all data collection and all sample analyses in this thesis were conducted in the exercise physiology laboratories at Loughborough University, UK.

2.2 Ethics approval

All experiments were approved by the Loughborough University Ethical Advisory Committee; Chapter Three reference number R06-P97, Chapter Four reference number R07-P121, Chapter Five reference number R08-P91, Chapter Six and Seven reference number R09-P92. The nature and the purpose of each study were clearly documented in the participant information sheets and were also verbally explained to all participants. Participants were informed of the procedures and demands of the study as well as any risks or discomforts they might experience.

Participants were always given opportunities to ask questions and only once they were satisfied with what was required were they accepted into the study. Each participant completed a health screen questionnaire to assess their suitability to take part. Participants then gave both verbal and written consent but also retained the right to withdraw from the study at any time and for any reason.

2.3 Participants

The participants recruited for all investigations were male. With the exception of Chapter Five, all participants were students or staff at Loughborough University or residents of the local community. Participants in Chapter Five were members of the wider community and were living in the United Kingdom. For all investigations participants were recruited mainly by word of mouth and through personal contacts, although posters and emails were also occasionally used. Participants were aged between 18 and 35 years for all investigations except Chapter Five, which due to the nature of the experiment

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had a maximum age for participation of 70 years. All participants were free from known illness which would have prevented them from participation in the testing, and were considered as healthy individuals.   Activity levels that covered a broad spectrum were required for Chapter Five, from being sedentary up to international competitors, but all other investigations used recreationally active individuals. Within each experimental chapter the activity level of the participants was kept as similar as possible. All participants in the investigations conducted in the heat were unaccustomed to exercise in a hot environment preceding the experiments.

2.4 Preliminary trials

2.4.1 Anthropometry

During the first visit to the laboratory in Chapters Three, Four and Seven nude body mass was measured to the nearest 0.02 kg using a digital floor weighing scale (AFW-120K or AFW-150K, Adam Equipment Co Ltd, Milton Keynes, UK).   Nude body mass was measured at the start of the second visit in Chapter Six using the equipment as previously stated. Height was measured to the nearest one centimetre using a wall mounted stadiometer (Seca 202, SECA Ltd. Birmingham, UK). Body mass and height were self-reported in Chapter Five.

2.4.2 Peak oxygen uptake testing

In Chapters Three, Four and Seven peak oxygen uptake ($\dot{V}O_2$peak) was determined for each participant at least 48 hours prior to the start of the familiarisation trials. A discontinuous incremental protocol was performed on an electromagnetically braked cycle ergometer (Gould Corival 300, Holland or Lode BV Corival V3, Groningen, The Netherlands) set in hyperbolic mode. This meant that participants could pedal at any cadence and the workload would remain constant.

Participants were given the opportunity to warm up if they wished to, though this was rarely done as the test always started at a low workload of 100 W for 4 minutes. They were then free to choose the progression of the increase in

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work load in increments from 15 to 100 W based on their performance in, and perception of, the previous stage. Each sub maximal exercise bout lasted four minutes, with approximately six minutes between bouts. During the final bout of exercise, if participants felt they could not complete the four minute bout they indicated when they were only able to continue for one more minute, so that a final gas sample could be taken.

The test was finished at the point of volitional exhaustion. The test was considered valid if one of the following two criteria were met: 1) heart rate was within 10 % of the age predicted maximum heart rate, 2) respiratory exchange ratio was above 1.15 (ACSM, 2000).   Based on the $\dot{V}O_2$ – work rate relationship, the required power output was then calculated for use during the subsequent trials.

2.4.3 Collection and analyses of expired air

Oxygen uptake was determined using the Douglas bag method (Astrand *et al.*, 2003C). Expired gas was collected into a Douglas bag through Falconia tubing attached to a two-way non-rebreathing valve, which was in turn attached to a mouth piece through which participants breathed. Participants also wore a nose clip to prevent any expired air being lost to the environment. Expired gas was collected for sixty seconds each sample, for the last minute of every stage in the $\dot{V}O_2$peak test and at predetermined times during Chapters Three, Four and Seven.

The expired gas was analysed for oxygen concentration by sampling through a paramagnetic transducer and carbon dioxide concentration was determined through an infra-red carbon dioxide analyser (Servomex 1440c, Crowborough, UK). The gas analysers were calibrated with gases of known concentration (British Oxygen Company, London, UK). The volume of gas expired was measured through a dry gas meter (Harvard dry gas meter, Harvard Apparatus Ltd, Kent, UK) and the temperature of the expired gas was recorded using an electronic temperature sensor at the dry gas meter inlet (Edale Instruments Ltd, Cambridge, UK).   All expired gas volumes were

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corrected to standard temperature and pressure for dry gas (STPD). Barometric pressure was measured using a standard mercury barometer. Oxygen consumption, carbon dioxide production and the respiratory exchange ratio, as well as estimations of the rate of carbohydrate and fat oxidation and energy expenditure, were calculated using the equations put forward by Frayn (1983) which are detailed in the Appendix A.

2.5 Familiarisation trials

Prior to starting trials in Chapters Three, Four and Seven, participants were given food and activity diaries to complete for the forty eight hours prior to the first familiarisation. They were then asked to reproduce this dietary and activity pattern prior to each subsequent familiarisation and experimental trial. This method of dietary standardisation is commonly used before intervention studies in an attempt to limit external influences on the exercise test due to its low cost and its simplicity for both participant and researcher (Jeacocke and Burke, 2010).

Participants were also asked to avoid alcohol and unaccustomed strenuous exercise for twenty four hours prior to each familiarisation and experimental trial. No restrictions were placed upon caffeine intake at any time. For Chapter Six participants were asked to replicate diet and activity patterns of the twenty four hours prior to trial one before trial two. During Chapter Four participants were given a list of suitable and unsuitable foods to consume in the twenty four hours prior to coming into the laboratory in order to minimise their dietary tryptophan intake.

During the exercise trials in Chapters Three, Four and Seven, participants undertook two familiarisation trials, after the $\dot{V}O_2peak$ test before the main data collection trials. The familiarisation trials offered the participant an opportunity to become accustomed to and comfortable with their surroundings prior to the collection of the data. Furthermore, familiarisation trials allow an investigator to fully understand a participant's response to the exercise test, ensuring that both the participant and the investigator enter the main trials

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fully prepared. The familiarisation trials followed exactly the same protocol as the main trials, other than the blood sampling protocol which was done only on the second familiarisation. Familiarisation trials were completed at the same time of day and on the same day of each week as the main trials. The familiarisation trials also allowed an opportunity to verify that the correct workload was set. Often workload was adjusted within twenty minutes of the start of the exercise bout in the first familiarisation in order to ensure that participants were working at the correct intensity.

During the exercise in Chapters Three and Four, exhaustion was defined as the point at which participants could not maintain a pedal cadence above 50 rpm despite verbal encouragement from the investigator. A cut-off rpm of 50 provided a 10 rpm cushion because below approximately 40 rpm the cycle ergometer used could not maintain the relationship between the workload and pedal cadence.

2.6 Experimental trials

Trials in Chapters Three, Four, Six and Seven were randomised in an attempt to eliminate any order effect. All were separated by at least seven days to allow for adequate recovery time, and in the exercise trials to minimise any training effect. Seven days has also been shown to be a sufficient time gap to prevent the development of heat acclimation (Barnett and Maughan, 1993). The time of day at which all familiarisation and main trials started was kept constant through the experiment to eliminate the effect of diurnal variation in core temperature (Reilly and Brooks, 1986). Due to the nature of the participants used in Chapter Five this was not possible and is discussed further in Chapter Five.

In Chapters Three, Four, Six and Seven all treatments and experimental hypotheses were blinded to the participants. The treatment was also blinded to the experimenter in Chapter Three and Chapter Four. At the start of each main trial a urine sample was collected. A small aliquot was retained for the analyses of urine osmolality. Participants were asked to empty their bladders as fully as possible and nude body mass was measured.

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All of the cycling exercise was done on electrically braked cycle ergometers (Chapters Three and Four Gould Corival 300, Groningen, Holland, Chapter Seven: Lode BV Corival. Groningen, Holland) operating in a hyperbolic manner to allow the workload to be independent of the pedal cadence. This allowed participants to exercise at a pedal cadence at which they felt comfortable, without affecting the work done. During exercise trials verbal encouragement was offered to the participants by the same investigator each time. During the exercise trials participants were provided with a small amount of water (100 – 150 ml) at fifteen minute intervals in an attempt to minimise the effects of hypohydration and also minimise the alteration in blood brain barrier permeability which may arise due to exercise in the heat (Watson *et al.,* 2005B).

At the end of the trials participants were promptly removed from the heated environment. All instrumentation was removed from the participants and final nude body mass was measured again to allow the estimation of body water loss during the trials. Body water losses were not corrected for respiratory water loss or metabolic water production since measurement of body water losses to this degree of accuracy were not deemed necessary in any of the following investigations, as this was not the focus of this thesis.

2.7 Experimental protocols

2.7.1 Time to exhaustion

When the participant was ready they started to pedal. The resistance was quickly set to the required power output (55 % of their $\dot{V}O_2$peak) and the clock was started. Participants were given no feedback of any kind throughout the protocol. Participants exercised until volitional exhaustion, which was defined as the inability to sustain a pedal cadence of 50 rpm or greater, despite verbal encouragement. Participants were given two warnings if pedal cadence dropped below 50 rpm. If they failed to respond, or it dropped below 50 rpm on a third occasion, the clock was stopped and the trial was deemed to be completed.

<u>2.7.2 Preloaded time trial</u>

When the participant was ready they started to pedal. The resistance was automatically applied to elicit the pre-determined workload over approximately six seconds, although for consistency the clock was always started when the resistance reached 100 W. During both the preload and the time-trial phases of the protocol participants had no feedback other than the duration for which they had been exercising.

The preload phase required participants to cycle for sixty minutes at 55 % of their $\dot{V}O_2$peak. After a short break of approximately five minutes, the twenty minute time-trial commenced. Participants were told to complete as much work as possible within the twenty minutes and were reminded that pedal cadence would not influence work done. An initial workload equivalent to 75 % of $\dot{V}O_2$peak was set but, immediately after the start of the time-trial, participants had control of their workload, which could be manually adjusted using the touch sensors on the cycle ergometer.

<u>2.8 Deep body and skin temperatures</u>

Rectal temperature (Chapters Three and Four used a 46TUC tele-thermometer and Chapters Six and Seven used a precision 4000A thermometer, both YSI, Ohio, USA) was monitored as an index of body core temperature using a rectal probe (YSI 400 series, Ohio, USA) inserted 10 cm beyond the rectal sphincter, held in place by a fixed knob in order to provide stable readings. Prior to the start of each study the rectal probes were calibrated using a water bath and a mercury in glass thermometer. Unfortunately, no firm link has been made between rectal temperature and brain temperature in humans although this has been established in animal studies (Fuller *et al.*, 1998).

Another criticism of this method is the suggestion that the measurement of rectal temperature may lag behind the true value of core temperature when rapid changes occur (Moran and Mendal, 2002). However, rectal temperature probably represents the best option for the measurement of core body

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temperature in these experiments, since tympanic temperature has been shown to both accurately reflect brain temperature (Mariak *et al.,* 1994), and be unrelated to brain temperature (Shiraki *et al.,* 1988) and can be painful when measured, while orally ingested temperature pills would be unreliable in view of the ingestion of drinks before and during the exercise in Chapters Three, Four and Seven.

Measurement of skin temperature in Chapters Three and Four used skin thermistors (YSI UK Ltd, Hampshire, UK) positioned on the right hand side of the body on the chest, triceps, thigh and calf and held in place with Transpore medical tape (3M, Loughborough, UK). Again, prior to the start of each of the experimental trials the skin thermistors were calibrated using a water bath and a mercury in glass thermometer. In Chapters Six and Seven, an infra-red laser site thermometer (thermometer LS, Micro-epsilon, Ortenburg, Germany) was used to determine skin temperature at the same four sites. For this measurement the emissivity was set to 0.97 for the measurement of skin surface temperature (Mitchell *et al.,* 1967). For Chapter Six each site was measured with a beam of 1 mm diameter and the mean temperature sited was reported. For Chapter Seven, due to the exercise the participants were performing during the measurements, a larger surface area reading was used and the maximum sited temperature was reported, in case clothing or peripheral temperatures falsely influenced the mean value. This instrument was also calibrated prior to the start of the experimental trials using calibrated skin thermistors. Matsukawa and colleagues (2000) have shown that a good level of association exists between analysis of skin temperature using an infra-red thermometer and a thermocouple system. Evans *et al.* (2010) also presented data showing that average weighted mean skin temperature, calculated using skin thermistors, correlated (R = 0.832, P<0.001) with values obtained when using the same type of infrared thermometer used in this thesis, over a range of skin temperatures. Furthermore, pilot data generated before the use of this instrument for data collection in the thesis also backs up these published findings.

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Weightings for the four skin temperature sites were applied using the equations of Ramanathan (1964) to compute mean skin temperature as 30 % chest temperature, 20 % thigh temperature, 30 % tricep temperature and 20 % calf temperature. This equation has been developed from the original formula proposed by Hardy and Dubois (1938) involving seven skin temperature sites. Although this is an imperfect method of calculation due to its inability to account for thermal sensitivity of different skin surface areas (Nadel *et al.,* 1973), it is the most commonly used equation and therefore its use allows comparisons to be made to the values observed in other studies.

Mean body heat content was calculated using the equation of Burton (1935) in which 65 % is contributed from the core temperature and 35 % is contributed from the mean weighted skin temperature, corrected for body mass. However, this equation has also come under criticism, as the specific heat of the body is related to body composition, which is impossible to standardise. Also, during exercise in the heat there is no clear ratio between the contribution of skin temperature and core temperature to body heat comfort, thereby impairing the analysis of mean body heat content (Wyndham, 1973).

2.9 Heart rate

Heart rate was measured using a Polar Electro heart rate monitoring system (Polar Electro Oy, Kempele, Finland). A transmitter positioned on the chest and held in place with an elastic strap. A Polar FS-1 wrist receiver was positioned near to the participant to note the heart rate whenever this information was required.

2.10 Subjective ratings

The ratings of perceived exertion scale (RPE; Chapters Three, Four and Seven; Appendix B) and the rating of thermal comfort scale (TC; Chapters Three and Four; Appendix C) were explained to all participants before the start of the familiarisation trials. Rating of perceived exertion was assessed using the Borg scale (1982) and rating of thermal comfort was assessed using a 21-point scale from -10 (cold impossible to bear) through 0 (neutral) up to +10 (heat impossible to bear) (Parsons, 2003C).

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Visual analogue scales were used to assess thermal sensation (Chapters Six and Seven; Appendix D) and thermal comfort (Chapter Six only; Appendix D). Participants had the scales fully explained to them prior to the start of the trials. The scales consisted of a 10 cm line with verbal anchors at either end ('Cold' to the left, 'Hot' to the right on the thermal sensation scales, and 'Too much cold' to the left and 'Too much hot' to the right on the thermal comfort scales; Parsons, 2003C). Participants were asked to mark the line at the point they felt best reflected their rating of thermal sensation or thermal comfort, with each millimetre representing a 1 % change. Chapter Six asked participants to consider the thermal sensation on their right forearm, their left forearm and their body overall, as well as their overall thermal comfort. Chapter Seven asked participants to consider the same three thermal sensation scales as in Chapter Six. Visual analogue scales were used in Chapters Six and Seven as these allow participants a wider choice of response options. With numbered scales when a participant becomes hotter between samples, the number stated may rise to indicate feeling hotter, but may not rise if the increased sensation is not deemed to be large enough to increase the rating by an entire category. A visual analogue scale eliminates such uncertainties.

2.11 Fluid provision

During the peak oxygen uptake tests participants were allowed to drink either water, squash or sports drinks *ad libitum*. During the exercise trials small amounts of the test drink (Chapter Three) or water (Chapters Four and Seven) were provided for the participants. In Chapter Three this acted to provide more branched-chain amino acids and, as in Chapters Four and Seven, also aimed to stave off severe hypohydration and avoid this being a major limiting factor to exercise capacity or performance. During Chapters Three and Four the fluids were kept in a water bath (23 °C) prior to consumption and were served every fifteen minutes during the exercise protocol. During Chapter Seven fluids were kept and served at room temperature (30 °C) immediately prior to, and every fifteen minutes during, exercise. These fluid temperatures were chosen purely due to logistical reasons and not physiological reasons.

2.12 Blood sampling

Participants were seated for fifteen minutes prior to any blood samples being drawn to stabilise the effects of postural change on blood volume. Blood samples were taken in every experiment with the exception of Chapter Six. In Chapter Five cannulation was not necessary and a single venepuncture sample was taken from an anticubital vein of each participant. A venepuncture was also used for the first sample taken from each participant in Chapter Four, as this was done the evening before the exercise trial. In Chapters Three and Four, one hand was immersed in warm (42-44 °C) water for ten minutes prior to cannulation while in Chapter Seven the participant was resting in a warm environment and therefore this was deemed unnecessary. An indwelling 21 g cannula needle (Surflo winged infusion set, Terumo. Tokyo, Japan) was inserted into a superficial forearm vein and a three way tap (Luer-Loc 360, BD Connecta. Heidelberg, Germany) was attached to the end to allow repeated blood sampling.

The indwelling cannula was kept patent by flushing with a small volume (2 – 3 ml) of heparinised saline (~25 Units / ml) after each sample. In Chapter Three each blood sample was a 10 ml volume, in Chapter Four a 7 ml volume, in Chapter Five a 40 ml volume and in Chapter Seven a 5 ml volume was taken. Immediately after sampling the blood was dispensed as appropriate and deproteinised where necessary.

2.13 Blood analyses

For the following analysis techniques, *Table 2.1* in Section 2.18 contains information on the coefficient of variation of the methods.

2.13.1 Haemoglobin (Hb)

This was done using the Cyanmethaemoglobin method. In duplicate, 10 µl of whole blood treated with an anticoagulant (K_2EDTA) was added to 2.5 ml of Drabkins reagent. This was left at room temperature, out of direct sunlight, for thirty minutes after which time the optical density was measured spectrophotometrically using an ultraviolet light at 540 nm. The values obtained from a blank sample and a known standard were used to formulate a

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straight line equation which allows the calculation of the haemoglobin in the samples. In the rare instances where the measured concentration was slightly greater than the known standard it was assumed that the linear relationship held true. The mean of each duplicated pair was reported. All haemoglobin analyses were done on the day the samples were collected.

Drabkins reagent contains potassium ferricyanide ($K_3Fe(CN)_6$) and potassium cyanide (KCN). Oxidation of the haem groups in haemoglobin, oxyhaemoglobin and carboxyhaemoglobin, by ferricyanide forms methaemoglobin, which then combines with the ionized form of cyanide to produce the red coloured, stable, cyanmethaemoglobin that is analysed.

2.13.2 Haematocrit (Hct)

Also called the packed cell volume, this was measured using whole blood treated with an anticoagulant (K_2EDTA). The blood was drawn up into a 50 µl capillary tube, sealed at one end and then centrifuged at approximately 12,000 g for five minutes. The packed cell volume is the percentage of the volume in the column occupied by the erythrocytes. These analyses were done in triplicate and the median value reported. All haematocrit analyses were done on the day the samples were obtained.

2.13.3 Blood volume calculations

From the values obtained for haemoglobin and haematocrit it is possible to calculate changes in blood, plasma and red cell volumes using the equations proposed by Dill and Costill (1974). These can be seen in Appendix E. The values reported throughout this thesis are as a percentage change relative to the first sample collected. This calculation is also listed in Appendix E.

2.13.4 Blood glucose

Using the GOD/PAP method, 100 µl of whole blood, treated with the anticoagulant K_2EDTA, was deproteinised in 1 ml of ice cold 0.3 N perchloric acid (PCA) and centrifuged for one minute and the resulting supernatant was used in the assay. 100 µl of the supernatant was added to 1 ml of glucose reaction mix and left to incubate at room temperature for thirty minutes.

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During this time the enzymatic oxidation of glucose in the presence of glucose oxidase, forms gluconate and hydrogen peroxide. The hydrogen peroxide reacts, under the catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye as an indicator of the presence of glucose. The absorbance was measured photometrically at a wavelength of 500 nm. These analyses were done using the Randox kit (Randox Laboratories Ltd, Crumlin, UK), performed in duplicate and the mean value is reported.

2.13.5 Blood lactate

This assay is based on the method as described by Maughan (1982). The assay required 100 µl of K_2EDTA treated whole blood to be deproteinised in 1 ml of ice cold 0.3 N PCA and centrifuged to obtain supernatant. This supernatant was then stored at -20 °C until analysis when it was defrosted and centrifuged again for one minute. The assay, done in duplicate and reporting the mean values, uses 20 µl of supernatant added to 200 µl of reaction mix and is left to incubate at room temperature for thirty minutes. The reaction mix contains nicotinamide-adenine dinucleotide (NAD), lactate dehydrogenase and hydrazine buffer. The hydrazine buffer solution contains hydrazine sulphate, hydrazine hydrate, Na_2EDTA and distilled water. The assay works on the principle that the lactate present in samples will be oxidised to form pyruvate, and the NAD^+ will be reduced to NADH and hydrogen ions in the presence of lactate dehydrogenase. This reaction is reversible, but by adding an alkaline reagent (hydrazine) that removes hydrogen ions the reaction will go to completion, which is when all the lactate is oxidised. It is the fluorescence of NADH that is measured at a wavelength of 340 nm. At the end of the incubation period 1 ml of distilled water is added to each sample to increase the volume. The fluorescence of the samples is read alongside a blank and three separate known concentration standards of 1.0, 3.0 and 5.0 mmol / L that provide a calibration curve.

2.13.6 Serum osmolality

Serum osmolality was measured by freezing point depression using a 300 mosmol / kg standard. To determine the osmolality of an aqueous solution,

the freezing point is compared to that of pure water. It is known that pure water has a freezing point of 0 °C, and a solution containing 1 osmol / kg sodium chloride has a freezing point of -1.858 °C. The method involves a monitored cooling of the sample solution until, at a temperature below the freezing point of water, the crystallisation of the solution is automatically initiated. At the same time a stainless steel needle is held well below 0 °C so that the water vapour in the air freezes as tiny ice crystals on the tip. The needle tip is then plunged into the super cooled solution. The initiation of crystallisation occurs and immediately after this the temperature of the solution begins to rise spontaneously as the heat of crystallisation is released during the freezing process. The rise in temperature of the solution is measured to an accuracy of 1.858×10^{-3} °C. The cryoscopic constant (K_f) for water is 1.858 K * kg / mol. Therefore, 1 mole of solute dissolved in a kilogram of water has a freezing point depression of 1.858 Kelvin.

Untreated blood samples were collected and dispensed into plain Z5 tubes and left to clot at room temperature for at least two hours. They were then spun for fifteen minutes at 1600 g in 4 °C. The resulting serum was extracted and kept refrigerated in Eppendorf tubes until analyses. Samples were analysed in duplicate and the mean value reported.

2.13.7 Serum free fatty acids

Done in duplicate this assay used 50 μl of serum obtained from whole blood left to clot at room temperature for at least two hours before being centrifuged at 1600 g for fifteen minutes. The resulting serum was extracted and stored in duplicate at -80 °C for analyses. This assay works on the principle that free fatty acids combine with coenzyme A and ATP, in the presence of acyl-CoA synthetase, to form acyl-Coenzyme A and other by-products. The acyl-Coenzyme A then reacts with oxygen in the presence of acyl-CoA oxidase to form 2,3-trans-enoyl-CoA and hydrogen peroxide. Two molecules of the hydrogen peroxide convert 3-methyl-N-ethyl-N(β-hydroxyethyl) aniline and 4-aminophenazone into a red dye (Quinoneimine-colour) and four water molecules in the presence of the enzyme peroxidase. The intensity of the dye is proportional to the free fatty acid concentration in the sample. It is

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measured spectrophotometrically at a wavelength of 500 nm. This assay was done on a Pentra (ABX Pentra 400, Horiba ABX, Diagnostics, UK), using the Wako NEFA C assay kit (Wako Chemicals GmbH, Germany).

2.13.8 Plasma amino acids

The plasma samples were stored at -80 °C due to the findings of Van Eijk *et al.* (1994), who found that although TRP levels were not influenced by storage at -20 °C or -70 °C, the branched-chain amino acids were more stable when stored at -70 °C. They also found no difference in the analyses between the samples being stored deproteinised or as plasma.

The amino acid concentrations of plasma collected in Chapters Three, Four and Five were analysed by high pressure liquid chromatography (HPLC) using a diode array detector set at 338 nm wave length within a Prominence HPLC System (Shimadzu Corporation, Kyoto, Japan). Whole blood (5 ml) was treated with the anticoagulant heparin. This was then centrifuged for fifteen minutes at 1600 g and the resulting plasma was extracted and stored at -80 °C until analyses. Upon defrosting, 500 µl of each plasma sample was deproteinised by adding 100 µl of 24 % 5-sulphosalicylic (2-hydroxy-5-sulfo-benzoic) acid, containing 2 mmol / L of norvaline and homoserine acting as an internal standard (Fekkes *et al.*, 1995).

After immediate vortex mixing and subsequent standing for fifteen minutes in a refrigerator at 4 °C, the precipitate was spun down for fifteen minutes at 18,400 g. Next, 400 µl of the resulting supernatant was mixed with 100 µl of 0.3 M lithium hydroxide in order to adjust the pH to 2.5. After this deproteinisation process the samples were refrigerated for a maximum of twelve hours before being prepared for analyses.

The analyses of the samples used a method developed by Henderson *et al.* (2000) for Agilent Technologies. The derivatisation procedure was performed by hand with the FMOC reagents omitted and with the fluorescence detector switched on, though not used for the analyses.

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The column used was 4.6 x 75 mm in length with 3.5 µm particle size for analysis with routine sensitivity and high throughput. A guard column was also used. Both were provide by Agilent Technologies (Cheshire, UK). Pre-formulated standards of 900, 225 and 90 pmol / µl were used, with the appropriate concentration of extended amino acid stock solution and internal standard stock solution added. Multiple preliminary runs were performed to ensure that pipetting error could be minimised for the derivatisation process and also to ensure there was no degradation in the amino acids within the samples, due to the derivatisation process being conducted well in advance of the sample being injected into the column for analyses.

Similarly to the plasma samples, plasma ultrafiltrate samples were also analysed. A 500 µl aliquot of the plasma yielded through centrifugation was then used to isolate free tryptophan by centrifugal ultra-filtration with a cellulose tri-acetate membrane to retain the molecules with a molecular weight of >10,000 (Whatman International Ltd, Kent, UK). The tube was capped and centrifuged for two hours at 1500 rpm in 4 °C and the supernatant was then stored at -80 °C until the time of analyses. Due to the small volume of the sample, and also the ultrafiltration process, no deproteinisation was required. The standards used for this analyses were 90, 22.5 and 9 pmol / µl, again with the appropriate concentrations of both stock solutions used.

For the interpretation of all chromatograms the area under the peak was calculated by the instrument software (Shimadzu LCsolution; Chromatography data system, Shimadzu corporation. Kyoto, Japan). Identification of the peaks was done by hand. No samples were run in duplicate due to the time and financial implications of this. Therefore the values reported are those generated from each individual sample. Where a result was generated which appeared to be exceptionally high or low the sample was re-run with fresh standards to check the value.

2.13.9 Platelet count and mean platelet volume

The mean platelet volume and the platelet count were determined using a haematology analyser (Ac.T 5diff, Beckman Coulter. High Wycombe, UK).

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Volume and count of platelets are done based on the coulter principle. This principle is based on the knowledge that cells will disrupt a flow of electricity due to their poor conductive properties.

2.13.10 Paroxetine binding to blood platelets

For the analyses of the platelets yielded from the blood samples collected in Chapter Five, liquid scintillation counting of tritiated Paroxetine was used as a peripheral model for investigating serotonin transporter density. The details of this method are presented in Chapter Five.

2.13.11 Sample protein content

The determination of the protein content of the samples used in the Paroxetine binding assay was done using the test-tube method of the Pierce BCA protein assay kit (ThermoScientific, Loughborough, UK). A 50 µl aliquot of the same sample used in the Paroxetine binding assay, or standards diluted as appropriate in the same incubation buffer as the samples for the Paroxetine binding assay, is added to 1 ml of working stock, which contains, among other things, bicinchoninic acid and cupric sulphate. The assay is then left to incubate at room temperature for two hours. The absorbance of all the standards and samples are then read spectrophotometrically at 562 nm within ten minutes of each other. The method combines the reduction reaction of CU^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of Cu^{1+} using a reagent containing bicinchoninic acid.

2.14 Urine collection and analyses

The urine samples collected from participants for each trial tended to be the second void of the day. The measurement of pre-trial urine osmolality aimed to ensure that participants were in a similar state of hydration prior to each trial. Samples (5 ml) were stored in the fridge and 50 µl samples were analysed in duplicate against a calibration curve generated from a blank of distilled water and a standard solution of 1200 mosmol sodium chloride per kilogram employing the same principles laid out in section 2.12.6. The mean value is reported.

2.15 Skin blood flow

Skin blood flow was measured qualitatively using a laser Doppler flow meter (Moor Instruments, Axminster, UK). A laser Doppler probe was positioned on both dorsal forearms of the participants at the same place on each arm on each occasion. From the beginning of the trials participants' forearms were placed on stools at heart level and they were instructed to keep their arms relaxed and as still as possible during the trial and particularly during the measurement period. Skin blood flow was measured continuously and ten second segments were used in the analysis. The laser Doppler flow meter was interfaced with a computer and data were displayed and analysed using data-acquisition software (moorSOFT, Moor Instruments, Axminster, UK).

Maintaining posture and the position of the Doppler probes is of critical importance to generate reproducible results and due to several methodological problems with the technique (including thickness of the skin), it is most appropriately employed to investigate relative changes in blood flow, as in this thesis, rather than to generate quantitative data (Swain and Grant, 1989).

2.16 Menthol solution and patch configuration

Chapters Six and Seven used patches containing menthol in an attempt to manipulate skin and overall thermal sensation. The patches used consisted of commercially available craft felt soaked in either a 1 % menthol solution (Menthol), made with 50 % methylated spirit and 50 % water, or the same solution with no menthol added (Control). To avoid possible evaporative cooling the felt patches were covered with non-porous adhesive tegaderm film (3M, Bracknell, UK). In Chapter Six the patches were positioned on either side of the upper back (12 x 10 cm), on the underside of each forearm (10 x 5 cm) and on the forehead (7 x 2.5 cm). In Chapter Seven the patches were positioned on either side of the spine on the upper and lower back (4 patches of 12 x 10 cm each), on the underside of both the right and left forearms (10 x 5 cm each) and on the forehead (7 x 2.5 cm).

Menthol is not soluble in water and therefore an ethanol based solution must be used. Other investigators have used ethanol as the vehicle for applying menthol to the skin (Tajino *et al*., 2007; Yosipovitch *et al*., 1996). However, a combination of methylated spirits and water were used in the present studies partly due to the cost implications of using ethanol, but mainly as the colour and smell of methylated spirits allowed the participants to be blinded as far as possible to the test solution being used. A 1 % menthol solution was chosen for the investigations as pilot tests revealed that thermal sensations did not intensify with higher concentrations of menthol, but feelings of discomfort, such as itching and pain, did increase.

2.17 Statistical analyses

The specific analysis performed on the data in each investigation is detailed in the appropriate chapter. Briefly, all data were tested for normality of distribution using the Shapiro-Wilk test and data were then investigated for skewness and kurtosis. For normally distributed data, a one-way or two-way (time x trial) repeated measures analysis of variance (ANOVA) was used to test for differences. Where appropriate, post hoc tests were done using paired t-tests with a Bonferroni correction for multiple comparisons. Where different groups of participants were used, a one-way independent ANOVA was performed with Bonferoni post hoc tests. Where data were not normally distributed, the Kruskal-Wallis non-parametric ANOVA was used and where appropriate pair-wise differences were investigated using the Wilcoxon matched-pair sign rank test. For any correlations either a Pearson's product-moment correlation coefficient was used of parametric data, or a one-tailed Spearman's correlation coefficient test was used for non-parametric data. Data are clearly presented as mean ± SD when parametric and median (range) when non-parametric. Statistical tests used the SPSS program, version 14.0 or version 17.0, and significance was set at $P < 0.05$, while a tendency was defined as $P < 0.09$.

2.18 Coefficient of variation of methods

The coefficient of variation for each method is shown in *Table 2.1.*

Table 2.1. The method, mean values and coefficient of variation for the analytical methods employed.

Measure	Method	Mean	CV (%)
Arm sensation	Visual	57 %	4.3
Overall sensation	analogue	59 %	3.9
Overall comfort	scale	58 %	5.0
Haemoglobin	Cyanmethaemoglobin	17.6 g / 100 ml	0.9
Haematocrit	Microcentrifugation	45.4 %	0.8
Glucose	GOD-PAP	4.9 mmol / L	1.8
Lactate	Fluorometric	1.2 mmol / L	5.0
Serum osmolality	Freezing point depression	290 mosmol / L	0.3
Free fatty acids	NEFA C	0.7 mmol / L	7.5
Tyrosine		64.6 µmol / L	0.7
Valine		254.5 µmol / L	2.7
Methionine		29.2 µmol / L	0.3
Tryptophan		49.6 µmol / L	1.9
Free-Tryptophan	HPLC	10.5 µmol / L	5.4
Phenylalanine		78.0 µmol / L	3.4
Isolecuine		153.3 µmol / L	0.9
Leucine		126.1 µmol / L	2.3
Platelet count	Coulter	240 x10^3 / µL	2.4
Platelet volume		9.1 fL	4.1
Paroxetine binding	Liquid Scintillation	942 fmol / mg protein	4.6
Protein assay	Pierce BCA	1680 µg / ml	1.4
Urine osmolality	Freezing point depression	571 mosmol / L	0.2

CHAPTER THREE

Inter-individual differences in the response to branched-chain amino acid supplementation during endurance cycling capacity tests in a warm environment.

3.1 Abstract

Branched-chain amino acid supplementation has been proposed to improve exercise capacity by delaying the onset of central fatigue. Several studies have failed to find an effect of supplementation, although a study by Watson *et al.,* (2004) suggested that the response to supplementation may vary between individuals. Therefore, the aim of this investigation was to determine if inter-individual differences exist in the response to branched-chain amino acid supplementation on endurance cycling capacity in a warm environment.

Eight males cycled to volitional exhaustion at 55 % $\dot{V}O_2$peak in an ambient temperature of 30.0 ± 0.6 °C and 47 ± 6 % relative humidity on four separate occasions. In two of the trials they consumed a drink containing 12 g / L of branched-chain amino acids (BCAA) and in the other two trials they consumed a taste matched control drink (CON). Participants were fed the appropriate drink for two hours before the start of exercise and then every fifteen minutes during exercise. Blood samples were taken before the drink was consumed, before the exercise commenced, every fifteen minutes during exercise and at the point of exhaustion. Core (Tc) and skin (Tsk) temperatures, heart rate (HR), rating of perceived exertion (RPE) and thermal comfort (TC) were recorded every ten minutes.

Plasma branched-chain amino acid concentration was higher ($P < 0.001$) and the free tryptophan to branched-chain amino acid ratio was lower ($P = 0.006$) in the branched-chain amino acid trials. However, the exercise time to exhaustion was not different between the two test drinks (CON: 105.8 ± 31.1 minutes, BCAA: 105.8 ± 31.3 minutes; $P = 0.993$). The variability of the exercise capacity for CON was 12.6 ± 13.4 %, while for BCAA it was 8.9 ± 9.4 %. There were no differences in Tc ($P = 0.675$), Tsk ($P = 0.361$), HR ($P = 0.292$), RPE ($P = 0.636$), TC ($P = 0.805$) or the respiratory exchange ratio ($P = 0.691$) between treatments. This experiment adds further weight to the argument that nutritional manipulation of the serotonergic system with branched-chain amino acids does not improve endurance exercise capacity.

3.2 Introduction

The synthesis of the neurotransmitter serotonin, which is widely accepted to influence feelings of lethargy, largely relies on the availability of the essential amino acid tryptophan. Tryptophan, or more specifically free tryptophan, entering the brain is thought to be directly responsible for determining the rate of serotonin synthesis (Fernstrom, 1977). Newsholme and colleagues (1987) hypothesised that during prolonged exercise, due to alterations in substrate utilisation, an increase in the availability and cerebral uptake of free tryptophan would occur, resulting in an increase in the synthesis of serotonin. This elevated level of serotonin could explain fatigue during prolonged exercise in the absence of any other peripheral factors.

As discussed in Chapter One, tryptophan transport across the blood brain barrier is open to competition from the large neutral amino acids including the branched-chain amino acids valine, leucine and isoleucine. During prolonged exercise there is a reduction in the concentration of circulating branched-chain amino acids (Blomstrand *et al.,* 1988), therefore allowing an increase in the uptake of tryptophan across the blood brain barrier. Theoretically, this will increase serotonin synthesis which may, at least in part, be responsible for the cessation of exercise.

It was suggested that if individuals were supplemented with branched-chain amino acids during exercise, the availability of tryptophan for transportation across the blood brain barrier would decrease, leading to a reduction in serotonin synthesis (Newsholme *et al.*, 1987). This theory of the development of central fatigue sparked much interest, partly due to the opportunity it offered to improve prolonged exercise capacity and performance by nutritional manipulation. Although there is support for the Newsholme theory of central fatigue in animal models, the evidence for the efficacy of nutritional manipulations influencing exercise capacity is conflicting in humans.

Micro-dialysis in rats showed that when valine (2 mg / 100 g) was injected by a venous catheter prior to exercise, there was no increase in the extracellular serotonin concentration in the hippocampus during subsequent exercise,

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compared to the steady increase during (123.7 ± 6.4 %) and after (133.9 ± 6.4 %) exercise that was seen when saline placebo was infused (Gomez-Merino *et al.*, 2001). Furthermore, there was a clear increase in exercise capacity in rats injected with 30 mg of branched-chain amino acids compared to those injected with saline (99 ± 9 minutes *vs.* 76 ± 4 minutes respectively; Calders *et al.*, 1997). This was again shown when rats receiving an injection of branched-chain amino acids (250 mg / kg) improved their run time to exhaustion by twofold compared to those receiving saline (Yamamoto and Newsholme, 2000). However, it is important to note that the rats used by Yamamoto and Newsholme had a genetically controlled lack of plasma albumin which may confound the interpretation of these results. Indeed, a study by Verger *et al.* (1994) in rats has found no difference in time to exhaustion between branched-chain amino acids and placebo supplements.

Human-based trials have generated even more contradictory results, and the interpretation and comparison of these is further confounded by the variety of exercise protocols used. These have included exercise to exhaustion tests (Blomstrand *et al.*, 1995; Blomstrand *et al.*, 1997; Galiano *et al.*, 1991; Struder *et al.*, 1998; Van Hall *et al.*, 1995; Watson *et al.*, 2004), time-trials (Hassmen *et al.*, 1994), incremental exercise tests (Varnier *et al.*, 1994), intermittent shuttle running tests (Davis *et a*l., 1999) and actual running races (Blomstrand *et al.*, 1991A). Details of these studies can be found in *Table 1.1*.

An early field-based experiment by Blomstrand *et al.,* (1991A) showed positive support for the theory. They tested experienced runners in a marathon or a cross country race and supplemented them with branched-chain amino acids and carbohydrate *vs.* carbohydrate alone (cross country running event) or branched-chain amino acids and water *vs.* water alone (marathon running event). Those who ran in a slower time (marathon time 3 hours 5 minutes to 3 hours 30 minutes) with branched-chain amino acid supplementation were found to run faster than those with a slower time who were given the placebo beverage. Cognitive function tests (Stroop Colour and Word Tests; Stroop, 1935) were also performed one to two hours before, and one to two hours after the race, and performance in the tests improved in

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those supplemented with branched-chain amino acids compared to those given the placebo beverages.

To further strengthen the case for the Newsholme theory, Mittleman *et al.,* (1998) found, in an apparently well-controlled laboratory based experiment, that branched-chain amino acid supplementation increased exercise time to exhaustion in 34 °C by around 16 minutes (153.1 ± 13.3 minutes on the branched-chain amino acid trial and 137.0 ± 12.2 minutes on the placebo trial).   However, other studies which also looked at exercise performance when acutely supplementing participants with branched-chain amino acids failed to find any ergogenic effect for exercise in the heat (Watson *et al.,* 2004) or exercise in temperate environmental conditions (Blomstrand *et al.,* 1995; Struder *et al.,* 1998; Van Hall *et al.,* 1995; Madsen *et al.,* 1996).

It has been suggested that hyperthermia may accelerate the onset of central fatigue (Bruck and Olschewski, 1987).   Indeed, it is now widely accepted that exercise performance (Tatterson *et al.,* 2000) and capacity (Galloway and Maughan, 1997) are both negatively affected in a warm environment. However, the exact mechanisms of this remain unclear. Work has shown that elevated core temperature alters brain activity (Nielsen *et al.,* 2001) and increases the perception of effort (Nybo and Nielsen, 2001C). By setting the exercise trials in the heat, the double stressor of heat and exercise was imposed, therefore increasing the chances of showing an effect of branched-chain amino acid supplementation, if such an effect exists.

The study by Watson *et al.* (2004), despite not finding a significant response to branched-chain amino acid supplementation, presented the idea that there may be inter-individual differences in the responses to branched-chain amino acid supplementation, where some individuals do experience an ergogenic effect, while others do not. Their data showed that, when glycogen depleted through prior exercise, there was a mean non-significant improvement of 7.1 ± 11.6 minutes when branched-chain amino acids were fed (111.0 ± 29.2 minutes on the branched-chain amino acid trial; 103.9 ± 26.9 minutes on the placebo trial).   Four of the eight participants showed an improvement in

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exercise capacity when consuming branched-chain amino acids, three showed no improvement and one individual exercised longer on the placebo trial. This led the authors to form the hypothesis that only some individuals may see an improvement in exercise capacity due to the ingestion of branched-chain amino acids, thereby giving an overall non-significant result when in fact branched-chain amino acid supplementation could still, potentially, improve exercise capacity in some individuals. However, the authors did not hypothesise any physiological mechanisms that might lead to a divergence in the effects seen between individuals,

Therefore, the aim of this investigation was to determine if inter-individual differences exist in the response to branched-chain amino acid supplementation during endurance cycling capacity in a warm environment.

3.3 Methods

3.3.1 Participants

Eight healthy, recreationally active males (Mean ± SD: age 25 ± 3 years; body mass 84.1 ± 8.9 kg; $\dot{V}O_2$peak 48.9 ± 10.0 ml / kg/ min; Median (range) height 1.82 m (1.78 - 2.01 m) were recruited to participate in the study.

3.3.2 Experimental protocol

Participants came to the laboratory on seven separate occasions. The initial visit was to determine the participants' $\dot{V}O_2$peak (detailed in Chapter Two). The following two visits to the laboratory were for familiarisation trials. Details of these and of pre-trial conditions can be found in Chapter Two. There were four experimental trials, all of which followed the same protocol, during which different drinks were provided on two occasions each to the participants, in a randomised, double blinded, cross-over design. A schematic of the protocol can be seen in *Figure 3.1*.

Figure 3.1. A schematic representing the protocol employed in the main trials of Chapter Three.
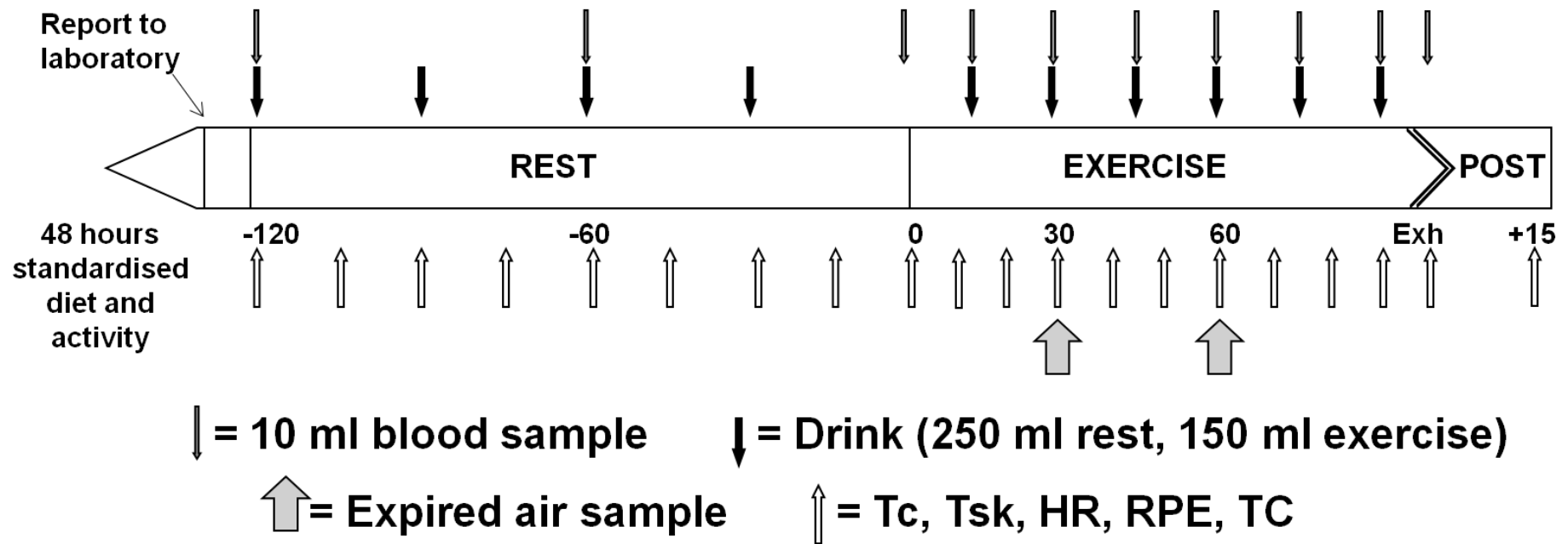
Participants reported to the laboratory at the same time each week between 0600 and 0900 hours. After arriving, participants emptied their bladder and a sample was retained (detailed in Chapter Two). Post-void nude body mass was recorded and the participants then positioned a rectal thermometer to allow the measurement of core body temperature. Skin thermistors were positioned on the chest, tricep, thigh and calf. A heart rate telemetry band was also worn around the chest to allow the continuous measurement of heart rate (see Chapter Two for details).

Participants were then seated in a comfortable environment (25.4 ± 1.4 °C) and one hand was immersed in warm (42 – 44 °C) water for ten minutes. At this point a cannula was inserted and baseline 10 ml blood sample was drawn (for details see Chapter Two), after which participants consumed 250 ml of either the flavoured control (Control; no added sugar lemon squash; Tesco Stores Ltd. Cheshunt, UK) or the branched-chain amino acid drink (BCAA; no added sugar lemon squash with 6 g / L leucine, 3 g / L isoleucine and 3 g / L valine; SHS International, Liverpool, UK). Participants drank a further 250 ml of the appropriate drink at thirty minute intervals throughout the two hour rest period. All drinks were served at 22 – 24 °C.   Resting blood samples were drawn at one and two hours. Heart rate, core temperature and skin temperature were recorded every fifteen minutes during the resting phase.

Following this period, the participants moved into a warm environment (30.0 ± 0.6 °C, 47 ± 6 % RH) and exercised on a cycle ergometer at a workload corresponding to 55 % $\dot{V}O_2$peak until volitional exhaustion (details given in Chapter Two). Blood samples were taken at fifteen minute intervals during exercise and participants consumed 150 ml of the appropriate beverage (Control or BCAA) immediately after each blood sample. Heart rate, core temperature, skin temperature, and ratings of perceived exertion and thermal comfort were recorded every ten minutes during exercise. Expired gas samples were collected every thirty minutes (see Chapter Two for details). A final blood sample and temperature readings were taken at the point of volitional exhaustion. Participants were then moved back into a comfortable

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ambient temperature. Final recordings of heart rate, core temperature and skin temperature were made fifteen minutes after the cessation of exercise. At this point all probes and the cannula were removed and the participant was reweighed to allow the calculation of body mass loss over the duration of the trial (see Chapter Two for details). They then showered and were provided with food and beverages before leaving the laboratory.

3.3.3 Blood handling and analyses

All blood samples (10 ml) were collected into dry syringes. Samples were analysed for the determination of blood lactate and glucose concentrations, haemoglobin and haematocrit values, plasma amino acid and plasma free tryptophan concentrations. Details of the techniques used can be found in Chapter Two.

3.3.4 Statistical analysis

Data are presented as mean ± standard deviation (SD) unless otherwise stated. Data were tested for normality of distribution using the Shapiro-Wilk test. Data were then investigated for skewness and kurtosis. Where data were not normally distributed, a Kruskal-Wallis ANOVA was performed and, if appropriate, pair-wise differences were investigated using the Wilcoxon matched-pair sign rank test. Statistical analysis for normally distributed data was done using a two way (time x trial) repeated measures analysis of variance test, followed, where appropriate, by a paired t-test with Bonferroni correction for multiple comparisons, in order to identify differences between trials. Where there was no difference between trials further analysis to investigate the effect of treatment was not undertaken. The absolute values and percentage change in exercise time were used to identify any statistically significant differences between trials for time to exhaustion. The difference in the control trials was compared to the difference in the branched-chain amino acid trials to investigate if there was greater variation in response on the treatment trial. This was done using paired samples t-tests and the Bonferroni correction for multiple comparisons where necessary. This was also performed for variables measured at a single time point. Statistical

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analysis was performed using the SPSS program, version 14.0, and significance was set at $P < 0.05$.

3.4 Results

3.4.1 Hydration status

Each participant started the trials in a similar state of hydration between weeks (Control 1, 448 ± 285 mosmol / L; Control 2, 612 ± 289 mosmol / L; BCAA 1, 433 ± 259 mosmol / L; BCAA 2, 585 ± 288 mosmol / L ;P = 0.472).

3.4.2 Time to exhaustion protocol

Due to the experimental design of this chapter it was possible to investigate the influence that the number of trials had on the reproducibility of the protocol. The co-efficient of variation after two trials (between the first and second familiarisations) was 21.7 ± 20.4 % (r = 0.613). The co-efficient of variation after three trials (between the second familiarisation and the first Control trial) was 11.0 ± 11.2 % (r = 0.934). This shows the importance of a first familiarisation when performing exercise time to exhaustion tests. The coefficient of variation after four trials (between the two Control trials) was 12.6 ± 13.4 % (r = 0.916), suggesting that there is no additional advantage in performing two familiarisation trials before the start of the main data collection.

3.4.3 Time to exhaustion data

There was no order effect of the trials (Trial 1, 107.8 ± 32.8 minutes; Trial 2, 105.1 ± 28.1 minutes; Trial 3, 104.4 ± 33.1 minutes; Trial 4, 105.8 ± 33.4 minutes; P = 0.817). There was no difference in the time to exhaustion between the Control trials (105.8 ± 31.1 minutes) and the BCAA trials (105.8 ± 31.3 minutes; P = 0.993; *Figure 3.2*) when the mean times to exhaustion were analysed as a complete group. When the data were analysed as the percentage change from the time achieved during the first Control trial there was also no difference (Control 1, 100 ± 0 %; Control 2, 101 ± 16 %; BCAA 1, 104 ± 13 %; BCAA 2, 98 ± 7 %; P = 0.973). When the difference between the two Control trials (11.5 ± 10.5 minutes) was compared to the difference between the two BCAA trials (8.5 ± 5.2 minutes) there was also no noteworthy difference (P = 0.498). Taken together these analyses show the branched-

chain amino acid supplementation protocol did not improve exercise capacity in a warm environment and did not show a greater variability in response than the Control trial.

When the data are interpreted on an individual, case by case level, only one individual appears as if he may have responded positively to the supplementation of branched-chain amino acids (*Figure 3.3*). This participant cycled for an average of 157.9 minutes on the Control trials (158.3 and 157.4 minutes) and 168.5 minutes on the BCAA trials (172.3 and 164.6 minutes). However, this participant had the lowest absolute work load, and the longest time to exhaustion compared to the overall group mean of 105 minutes. The variability in exercise capacity was 12.6 ± 13.4 % on the Control trials and 8.9 ± 9.4 % on the BCAA trials.
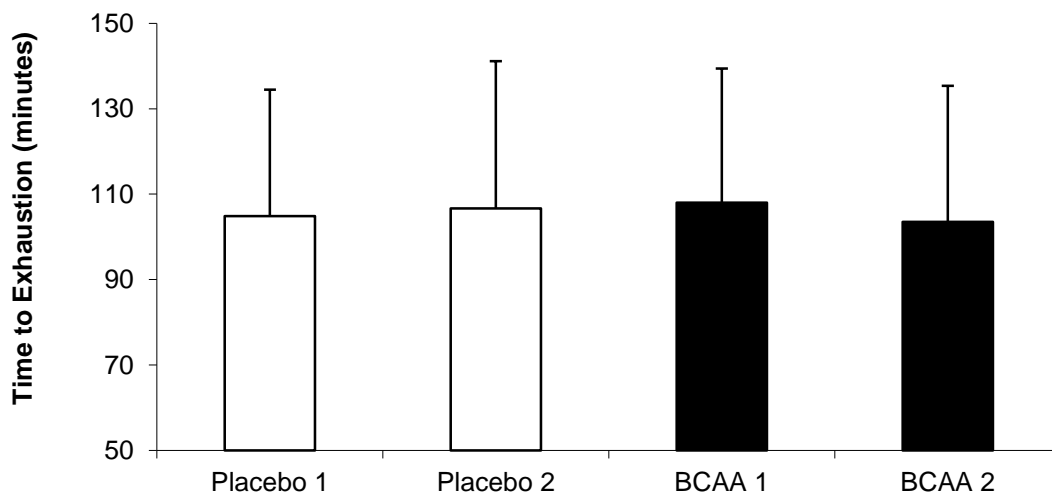
Figure 3.2. Mean group times to exhaustion for the Control and BCAA drinks. No difference was found between treatments or trials for cycling time to exhaustion.
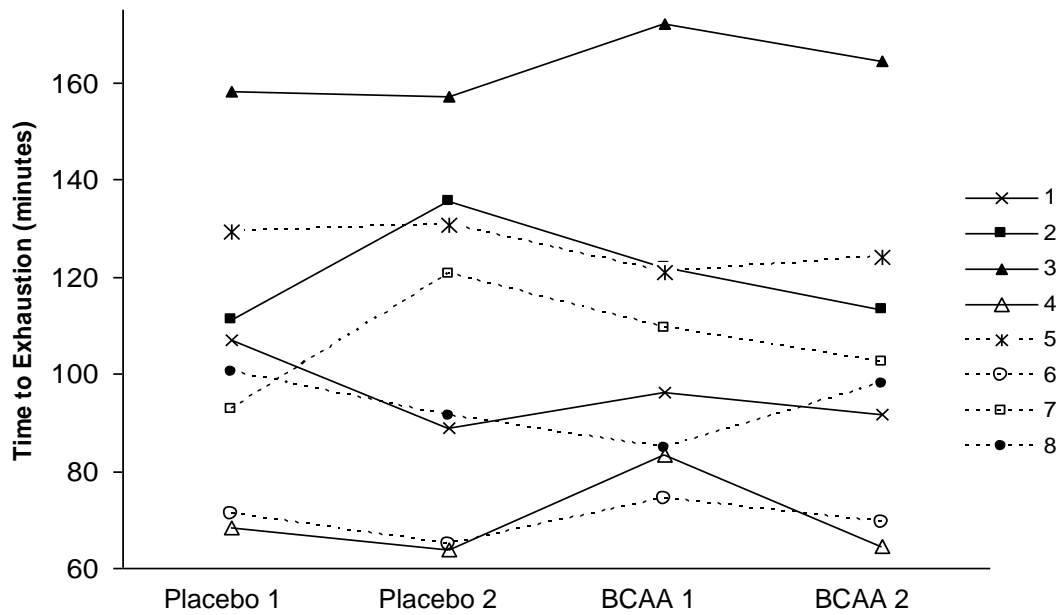
Figure 3.3. Individual cycling times to exhaustion on both of the Control and both of the BCAA trials.

3.4.4 Thermoregulatory measures

As expected, there was an effect of time on core temperature ($P < 0.001$). Core temperature increased at the onset of exercise, and it continued to rise steadily throughout the test, peaking at the point of fatigue (38.5 ± 0.4 °C). It then started to decrease during the subsequent fifteen minutes of monitored recovery. There was no effect of the treatments on core temperature ($P = 0.675$; *Figure 3.4*).

There was a significant effect of time ($P = 0.023$) on skin temperature but the trials did not differ ($P = 0.361$; *Figure 3.5*). Skin temperature followed a similar pattern to core temperature, rising during the exercise to a peak at the point of exhaustion, and once exercise had ceased skin temperature fell.

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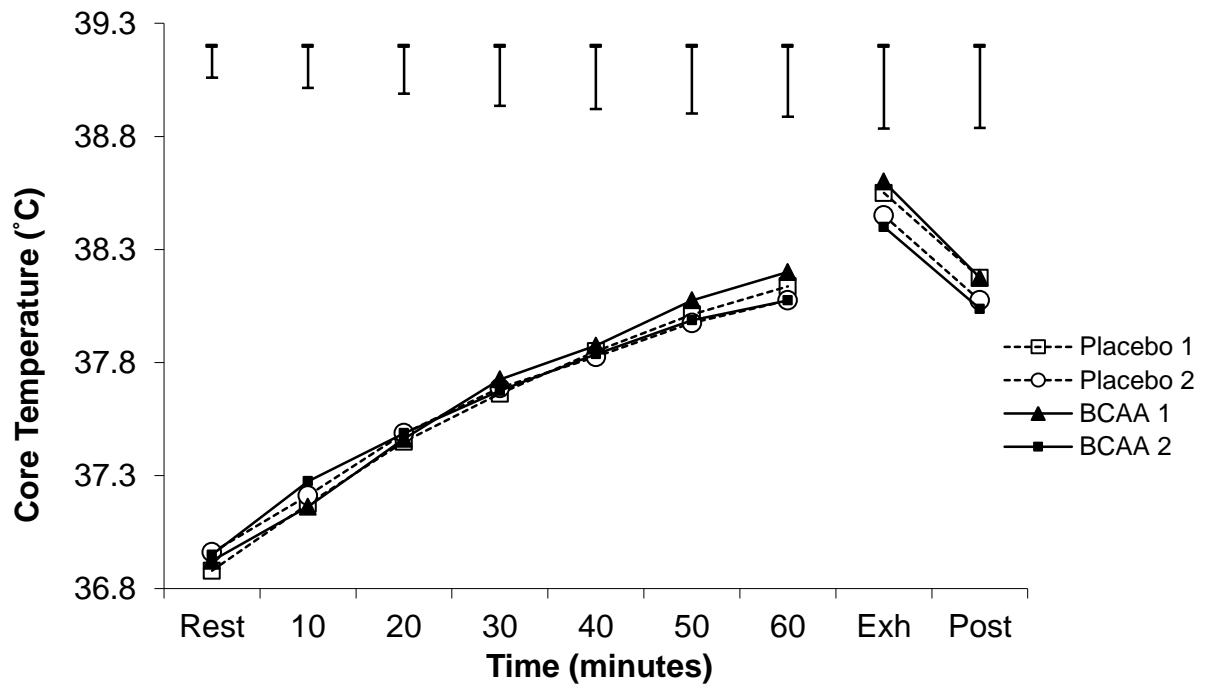
Figure 3.4. Mean core temperature over the duration of the experimental trials. There was a significant effect of time on core temperature, but there were no differences between any of the trials. The error bars above the data displays the variability in the data.
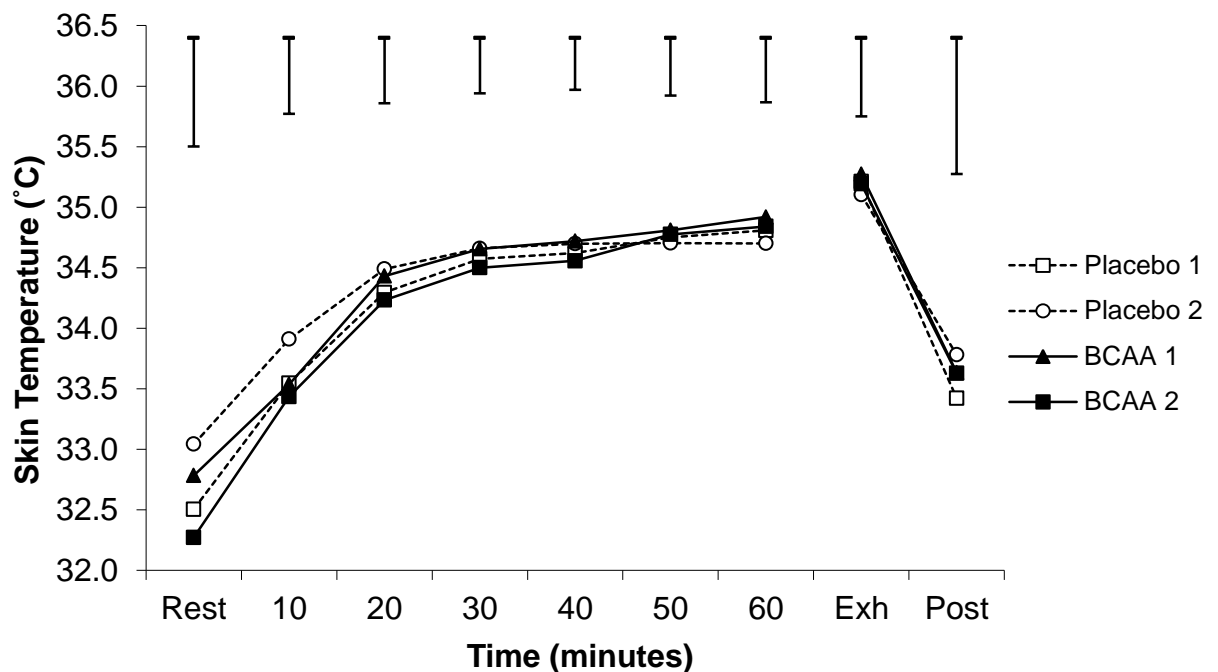
Figure 3.5. Mean of skin temperatures over the duration of the experimental trials. There were no statistically significant effects of time or trial. The error bars above the data displays the variability in the data.

Estimated body heat content showed a similar pattern to core and skin temperatures over the trials. Body heat content increased with exercise, peaking at the point of exhaustion and then falling during the initial recovery period. Body heat content was not affected by the different trials, but there was a significant effect of time ($P = 0.001$).

Mean body mass loss was 1.11 ± 1.24 kg on the BCAA trials and 1.12 ± 1.01 kg on the Control trials, despite receiving 150 ml of the required beverage every fifteen minutes during exercise. This was not significantly different between trials ($P = 0.969$).

3.4.5 Cardiovascular and metabolic measures

Heart rate increased by about 70 beats per minute during the initial ten minutes of exercise. It then increased steadily during the exercise session and peaked at around 170 beats per minute at the point of exhaustion ($P<0.001$). During the initial fifteen minutes of recovery heart rate fell rapidly to around 95 beats per minute. However, there was no difference between any of the trials ($P = 0.292$; *Figure 3.6*).

Ratings of perceived exertion increased slowly over the initial hour of exercise ($P < 0.001$), but again there was no effect of the trials ($P = 0.636$; *Figure 3.7*). The same pattern was apparent for the participants' ratings of thermal comfort, which increased steadily during the exercise ($P = 0.001$), but for which there was no effect of the different trials ($P = 0.805$; *Figure 3.8*).

There was no effect of time or trial on the use of carbohydrate (CHO) during exercise ($P = 0.104$; $P = 0.897$ respectively), nor on the use of fat during exercise ($P = 0.202$; $P = 0.843$ respectively). Energy expenditure (EE) did increase over time ($P = 0.018$), but did not vary between the trials ($P = 0.325$). Respiratory exchange ratio (RER) was not significantly different over time ($P = 0.150$) or between trials ($P = 0.691$; *Table 3.1*).
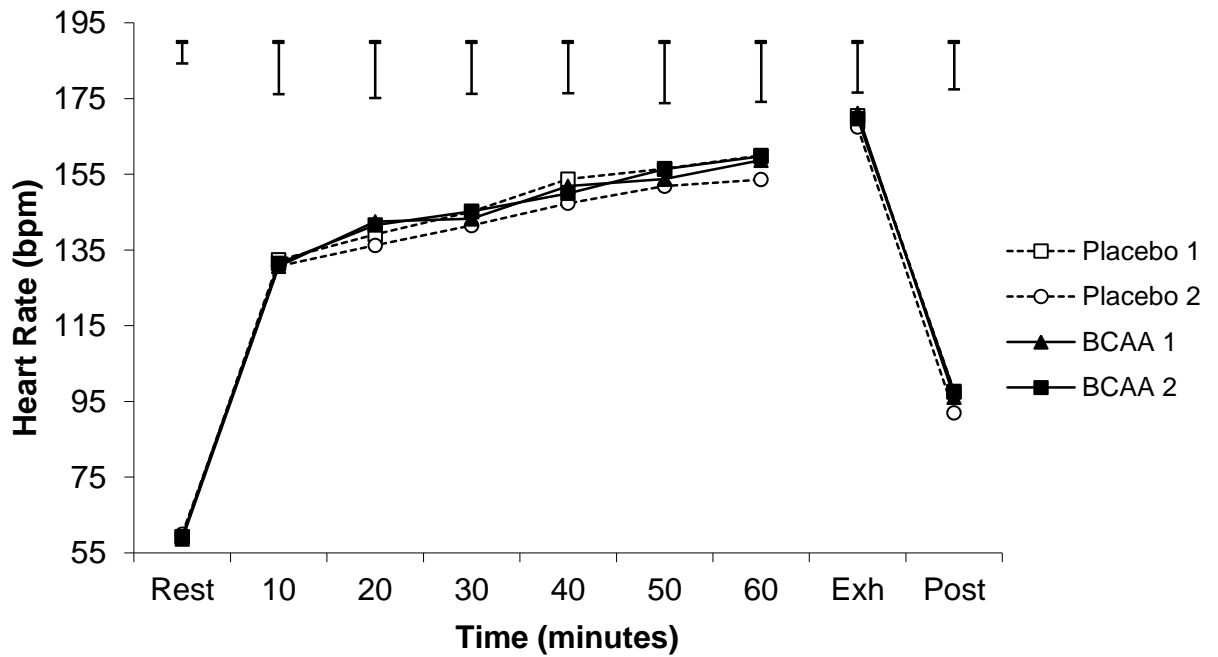
Figure 3.6. Mean heart rate over the duration of the experimental trials. The error bars above the data displays the variability in the data.
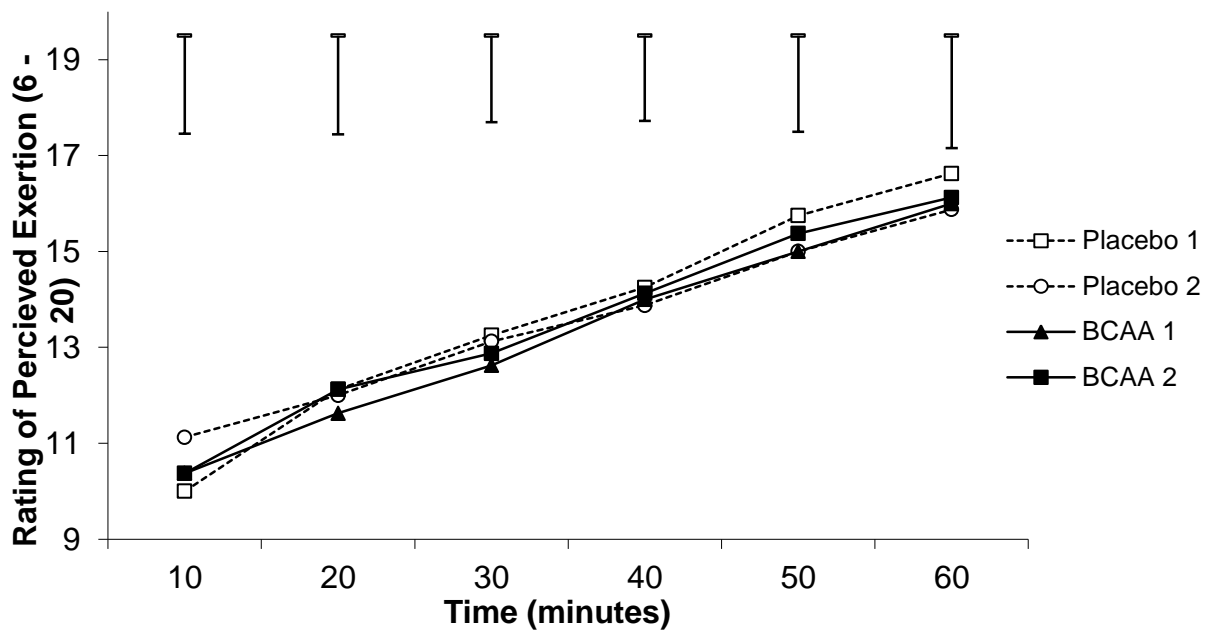
Figure 3.7. Ratings of perceived exertion during the exercise phase of the trials. There was no difference between trials, and a significant increase over time. The error bars above the data displays the variability in the data.
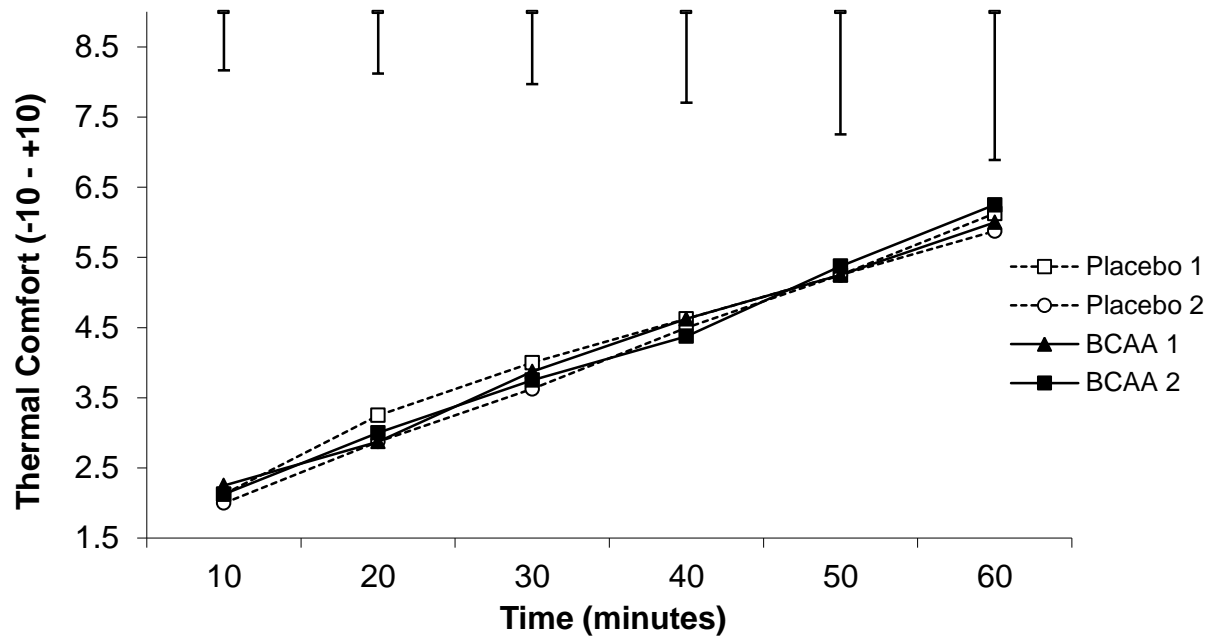
Figure 3.8. Ratings of thermal comfort during the exercise phase of the trials. This increased significantly during exercise, but was not different between trials. The error bars above the data displays the variability in the data.

Table 3.1. Carbohydrate and fat oxidation rates at two time points during the trials, the energy expenditure calculated from these and the RER value (see Chapter Two for details). * indicates a significant increase over time.

Time (minutes)	Control 1		Control 2		BCAA 1		BCAA 2	
	30	60	30	60	30	60	30	60
CHO oxidation rate (g / min) Mean ± SD	2.45 0.81	2.88 1.44	2.54 0.88	3.15 1.74	2.45 0.67	2.90 1.41	2.54 0.89	2.83 1.17
Fat oxidation rate (g / min) Median (range)	0.17 0-0.61	0.08 0-0.43	0.13 0-0.41	0.01 0-0.44	0.22 0-0.55	0.17 0-0.51	0.17 0-0.31	0.17 0-0.40
RER Mean ± SD	0.96 0.05	0.97 0.07	0.96 0.05	0.99 0.10	0.93 0.05	0.97 0.08	0.96 0.04	0.96 0.06
EE (kJ / min) Mean ± SD	45.5 12.4	48.3 13.4 *	45.7 10.6	49.4 13.3 *	47.6 10.9	50.2 12.7 *	45.7 12.3	50.0 13.1 *

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Table *3.2.* Blood glucose and lactate concentrations over the duration of the trials. *
indicates a significant increase over time.

	Trial	Rest	Pre 60	Pre 120	15	30	45	60	Exh
Blood Glucose (mmol / L) Mean SD	P1	5.30 0.52	5.37 0.54	5.29 0.48	5.22 0.57	5.61 0.52	5.64 0.56	5.49 0.68	5.56 0.88
	P2	5.00 0.48	4.87 0.57	4.91 0.47	5.04 0.51	5.00 0.61	4.96 0.57	4.96 0.64	5.37 1.10
	B1	5.36 0.22	4.95 0.31	4.86 0.44	5.02 0.35	5.06 0.52	5.20 0.53	5.18 0.62	5.29 0.68
	B2	5.28 0.42	4.92 0.53	4.94 0.54	5.15 0.56	5.24 0.57	5.32 0.64	5.33 0.73	5.68 0.66
Blood Lactate (mmol / L) Mean SD	P1*	0.74 0.36	0.75 0.33	0.81 0.32	2.03 1.00	2.22 1.05	2.17 1.15	2.47 1.91	2.87 2.37
	P2*	0.83 0.32	0.86 0.32	0.78 0.30	2.03 0.93	2.06 0.98	1.78 1.02	1.79 0.72	2.42 1.00
	B1*	0.62 0.33	0.75 0.22	0.82 0.26	2.05 0.88	1.98 0.83	1.96 0.76	2.03 0.97	2.32 1.34
	B2*	0.91 0.50	0.96 0.41	0.88 0.30	2.34 0.78	2.25 0.88	2.35 0.85	2.54 1.49	3.01 1.54
Plasma Volume Change (%) Mean SD	P1	0 0	0.2 1.9	-2.1 2.8	-12.5 3.2	-12.8 2.8	-13.4 2.6	-13.4 3.0	-15.0 2.7
	P2	0 0	0.9 2.0	-0.4 2.0	-10.2 4.2	-10.1 4.8	-10.6 5.1	-11.3 6.5	-13.1 6.8
	B1	0 0	1.9 3.2	0.9 2.8	-11.2 2.9	-11.5 3.5	-11.3 3.7	-11.6 3.5	-13.5 4.6
	B2	0 0	1.0 2.2	-0.6 2.7	-11.4 3.0	-11.1 4.4	-11.5 3.3	-10.8 7.0	-14.7 5.6

Plasma amino acid concentrations are displayed in *Figures 3.9A* and 3.9*B*, and the free tryptophan to branched-chain amino acid ratio is shown in *Figure 3.9C*. For the statistical analysis of the amino acids the values obtained on the two Control trials have been combined and the mean value reported, and the same has been done for the BCAA trials at each time point.   The concentration of all of the large neutral amino acids were significantly higher

(P < 0.001 tyrosine; P = 0.028 methionine; P = 0.012 tryptophan; P = 0.023 phenylalanine) during the Control trials than the BCAA trials (*Figure 3.9A*).

Valine, isoleucine and leucine concentrations were all significantly greater on the BCAA trial compared to the Control trial from one hour after the first bolus of drink was consumed. For each branched-chain amino acid, the plasma concentration was significantly elevated from baseline by sixty minutes after the first drink was consumed and remained elevated for the duration of the trials (*Figure 3.9B*).

Free tryptophan levels tended to differ over time (P = 0.055) but did not differ between trials (P = 0.166). However, the ratio between free tryptophan and the branched-chain amino acids was significantly (P = 0.006) higher on the Control trials than the BCAA trials. There was only a slight tendency for the values to differ over time (P = 0.069), though it can be seen that they were very similar between trials before the start of the supplementation protocol (*Figure 3.9C*).
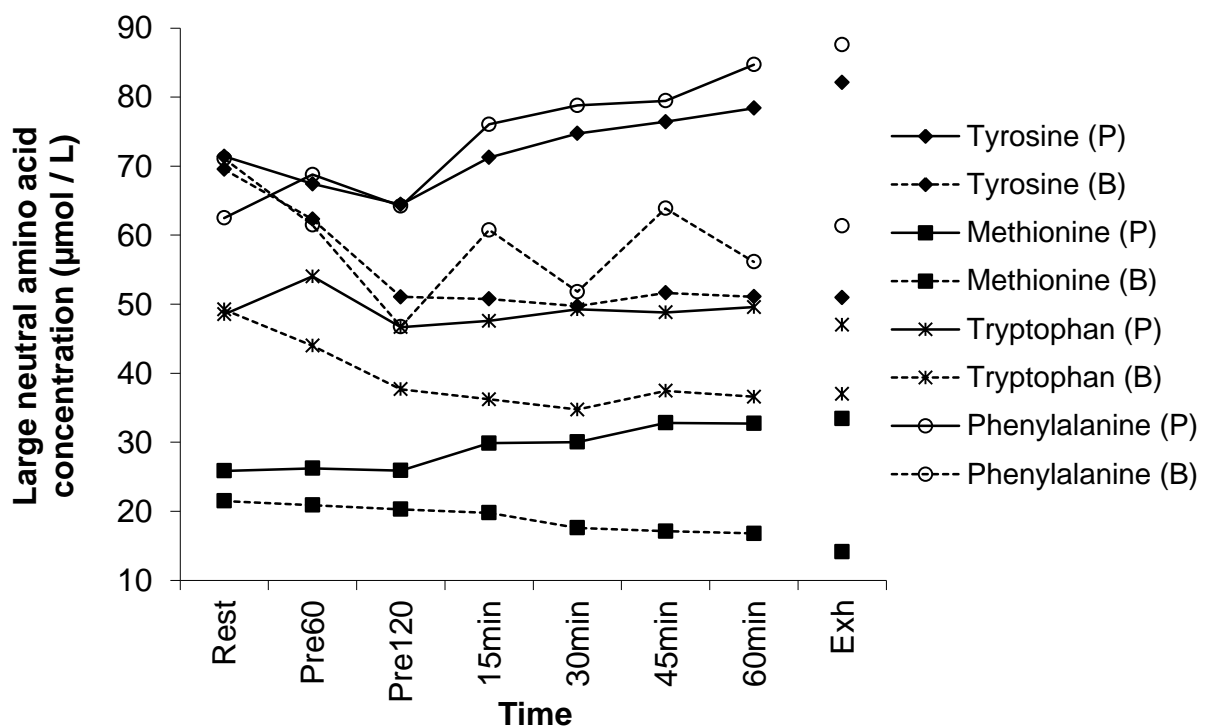
Figure 3.9A. Large neutral amino acid concentrations over the duration of the trials. (P) symbolises the Control trials and (B) symbolises the BCAA trials.
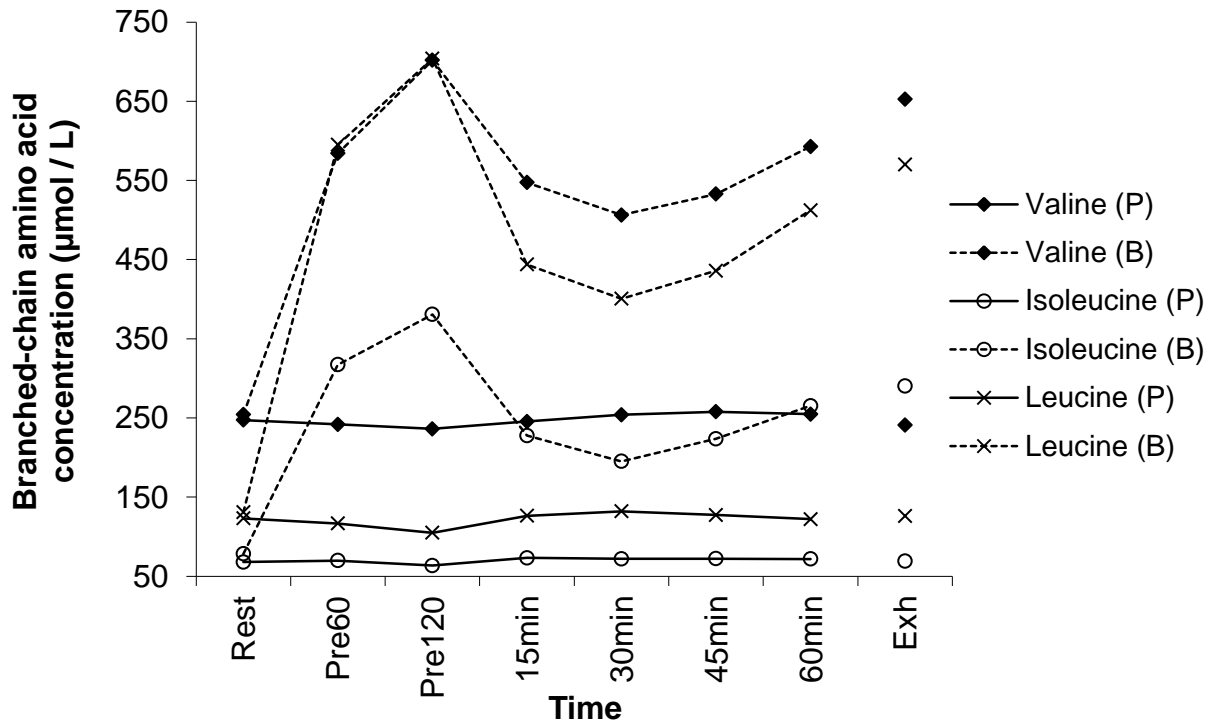
Figure 3.9B. Branched-chain amino acid concentrations over the duration of the trials. (P) symbolises the control trials and (B) symbolises the branched-chain amino acid trials.
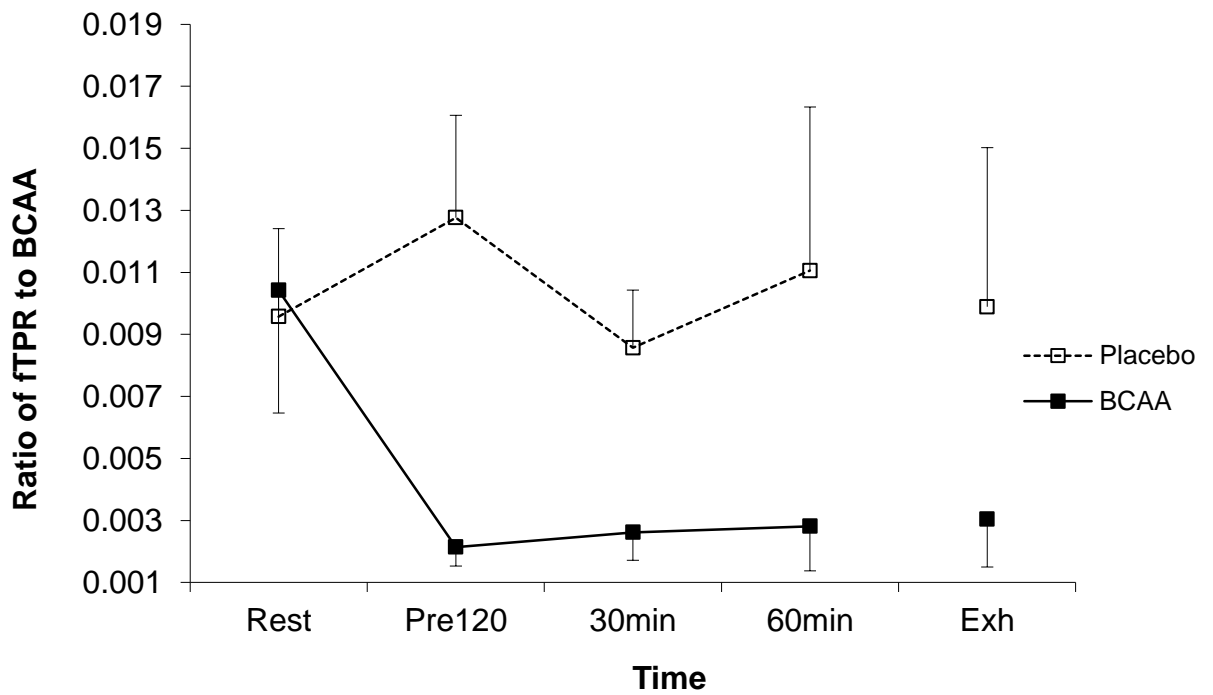
Figure 3.9C. The ratio between free tryptophan and the total branched-chain amino acids over the duration of the trials.

3.5 Discussion

The aim of this investigation was to determine whether there are inter-individual differences in the response to branched-chain amino acid supplementation on endurance exercise capacity in a warm environment. The data presented show there is no greater variability in the response to branched-chain amino acid supplementation compared to that seen when a control is administered, and that no individual appeared to perform markedly better with a BCAA drink than with the Control drink.

One participant did exercise for longer on both of the BCAA trials than on the Control trials (*Figure 3.3*). This individual exercised for longer than all other participants: approaching three hours. An effect of supplementation with branched-chain amino acids in exercise lasting over three hours was found by Blomstrand *et al.* (1991A). They found that branched-chain amino acid supplementation improved exercise performance in 'slower' (3 hours 5 minutes to 3 hours 30 minutes for a marathon) runners by around 3 %, equating to 5 - 6 minutes. The participant in this study increased his mean exercise time to exhaustion by 10.6 minutes (6.7 %) with the branched-chain amino acid supplementation. However, in the study by Blomstrand *et al.* (1991A) the supplementation protocol did not improve performance in faster (less than 3 hours 5 minutes) runners, the duration of exercise which is closer to the individual discussed here. Therefore, it is likely that these results do not originate from the same physiological mechanism.

However, it is important to note the differences between the participants used by Blomstrand and colleagues (1991A) and the individual who appeared to improve due to the present supplementation protocol. The participant in this study had an absolute $\dot{V}O_2$ of 2.7 L (32 ml / kg / min). It is highly unlikely that anyone with this level of fitness would be able to complete a marathon run within the time scale of the 'slow' runners (3 hours 5 minutes to 3 hours 30 minutes) in the study by Blomstrand and colleagues (1991A). Also, it is important to remember that studies undertaken in the field, like that of Blomstrand and colleagues, are open to many confounding and uncontrollable

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factors. These include weather conditions, the appropriate matching of participants when assigning to experimental groups, blinding of the participants to their test group, exercise intensity or dietary and fluid intakes before the event. This means that there may be no relevant effect of branched-chain amino acid supplementation on exercise over three hours in duration.

It seems highly improbable that the reason for fatigue in the present trials was a simple peripheral factor. Core temperatures at the point of exhaustion were not elevated close to the proposed 'critical' core temperature in humans of approximately 40 °C (Nielsen *et al.*, 1993; *Figure 3.4*), above which exercise may cease due to uncompensable thermoregulatory demands. Nor was the fatigue likely to have been caused by hypohydration since research has shown that performance decrements due to hypohydration occur at around a 2 % loss in body mass (Armstrong *et al.*, 1985). Most of the participants (6 of 8) in the present study were hypohydrated to a lesser extent (1.4 ± 0.6 %), meaning that this is an unlikely cause of fatigue. Blood glucose levels were also maintained over the duration of the trials. In the present study the heart rates of the participants at the end of exercise were not elevated to estimated maximum levels (195 ± 3 beats per minute), being 169 ± 13 beats per minute for the Control trials and 170 ± 15 beats per minute for the BCAA trials.

While the results of the present study may agree to some extent with the findings of Blomstrand *et al.* (1991A), they conflict with those of Mittleman and colleagues (1998), who performed an apparently well-controlled laboratory based study into the effect of supplementation of branched-chain amino acids on exercise (40 % $\dot{V}O_2$max) capacity in the heat (34.4 ± 1.8 °C). They found that exercise time to exhaustion was longer (153.1 ± 13.3 *vs.* 137.0 ± 12.2 minutes; 14 % improvement) when the participants were given drinks (5 ml / kg body mass) containing 5.88 g / L of branched-chain amino acids (54 % leucine; 19 % isoleucine; and 27 % valine) compared to when they were given the same volume of a placebo drink. Although the composition of the drinks between the Mittleman *et al.* (1998) study and the present study differed

slightly, this is unlikely to be a major contributing factor for the different findings. The total amount of branched-chain amino acids administered for the male participants of the Mittleman study was 15.8 ± 1.1 g. Mittleman and colleagues purported that the cause of the improved exercise capacity seen was due to the alteration in the free tryptophan to branched-chain amino acid ratio as a result of branched-chain amino acid supplementation. The present study administered between 19.2 g and 31.8 g dependent on exercise duration, and the ratio of free tryptophan to branched-chain amino acid was even further reduced. Therefore, an improvement in exercise capacity should have been even clearer in the present investigation, but this was not the case.

The ratio of tryptophan to branched-chain amino acids has been shown to be correlated with the brain tryptophan concentration (Leathwood, 1987). Therefore, this ratio is likely to be more important than the absolute values. By altering the free tryptophan to branched-chain amino acid ratio in the blood, there would be a reduced uptake of the serotonin precursor into the brain and therefore a reduction in the production of serotonin and consequently, theoretically, a delay in the onset of fatigue. Indeed, the values seen here for plasma branched-chain amino acid concentration or the free tryptophan to branched-chain amino acid ratio are representative of the trend seen in Mittleman *et al.* (1998), Blomstrand *et al.* (1991A), Watson *et al.* (2004), and Cheuvront *et al.* (2004).

A time delay is thought to exist between peripheral amino acid availability and the uptake of free tryptophan into the brain. Therefore, it is important that supplementation starts a sufficient length of time before exercise to allow for the increased availability of free tryptophan to be realised (Knott and Curzon, 1972). In this study the supplementation protocol was introduced two hours before exercise commenced and as a result the greatest difference in the free tryptophan to branched-chain amino acid ratio was seen at the onset of the exercise protocol. During the initial thirty minutes of exercise there was a drop in the concentration of amino acids in the plasma. It is possible that this initial reduction was due to the uptake of the branched-chain amino acids into the muscles at the onset of exercise for use as a fuel source.

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Although brain serotonin levels could not be directly measured here, it has been shown using micro-dialysis in rat brain that infusion with L-valine (2 mg / 100 g) prevented the exercise-induced serotonin release that was seen when a placebo (saline) was infused (Gomez-Merino *et al.,* 2001). The values reported in the above experiment reflect those seen in the current investigation, despite the different species used. Valine in this study was given equating to an average of 7 mg / 100 g. The valine plasma concentrations were similar before and after the valine administration between species (196 ± 14 µM / L in rats and 254 ± 17 µM / L in humans before valine was administered and 406 ± 15 µM / L in rats and 701 ± 111 µM / L in humans after valine was administered). Changes in tryptophan levels due to the valine supplementation (19 % reduction in rats and 22 % reduction in humans) were also similar.

Branched-chain amino acid supplementation led to a reduction in the plasma concentrations of all of the other large neutral amino acids in the present study. This is in line with the findings of Blomstrand *et al.* (1991A), although in their study the concentrations of tryptophan, tyrosine, phenylalanine, and methionine, were all reduced after branched-chain amino acid supplementation during a prolonged running race. However, it was only the reductions in tryptophan and methionine that were statistically significant. This shows that branched-chain amino acid supplementation, as well as increasing the competition for transport across the blood brain barrier also reduces the absolute plasma concentration of tryptophan which, along with the ratio of free tryptophan to branched-chain amino acids, has also been suggested to be important. Pardridge (1983) suggested that the large neutral amino acid transporter at the blood brain barrier may be able to disassociate tryptophan from albumin, thereby making bound tryptophan available for transport across the blood brain barrier.

The ratings of perceived exertion in this investigation were not different between trials (P = 0.636), though it might have been expected that they would have been lower on the branched-chain amino acid trials, as central serotonergic activity has been associated with reductions in arousal levels

and increases in the perception of tiredness and lethargy. Indeed, Blomstrand and colleagues found that during supplementation with branched-chain amino acids participants overall rating of perceived exertion was reduced by 7 % and their ratings of mental fatigue were reduced by 15 % during a sixty minute cycle at approximately 70 % $\dot{V}O_2$max (Blomstrand *et al.*, 1997). However, other investigations have also failed to find any differences in perceived exertion (Watson *et al.*, 2004; Mittleman *et al.*, 1998; Struder *et al.*, 1996).

Blomstrand's group also found that cognitive function (Stroop Colour and Word Test; Stroop, 1935) was improved one to two hours after a 30 km cross country running race compared to one to two hours before the race when supplemented with branched-chain amino acids, while those receiving a placebo drink did not improve performance (Blomstrand *et al.*, 1991A). Similar findings were also reported by the same investigators for the same cognitive tests using female football players tested before and after a match when consuming either a 6 % carbohydrate drink or the same drink with 7.5 g / L of branched-chain amino acids added (Blomstrand *et al.*, 1991B) However, another well controlled laboratory-based investigation found no effect of branched-chain amino acid supplementation on cognitive function, assessed by a battery of five tests, including those used in other studies investigating branched-chain amino acid administration, or exercise performance, assessed by a thirty minute pre-loaded time-trial performance in the heat (40 °C; Cheuvront *et al.*, 2004).

Performing exercise in the heat reduces exercise capacity, with 11 °C being optimum for exercise capacity at approximately 70 % $\dot{V}O_2$max (Galloway and Maughan, 1997). Exercise in a warm environment (32 °C) has also been shown to reduce mean power output of elite road cyclists by 6.5 % over a thirty minute time-trial, compared to when the same exercise was performed in an environment only 9 °C cooler (23 °C), despite no differences in rectal temperatures between the trials (Tatterson *et al.*, 2000).

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In the current study the exercise intensity was low (55 % $\dot{V}O_2$peak), thereby allowing the participants to exercise for a fairly long duration without core temperature becoming a limiting factor; core temperature at exhaustion was 38.5 ± 0.3 °C for the Control trials and 38.5 ± 0.4 °C for the BCAA trials. Participants' core temperature was not elevated close to the proposed 'critical' core temperature of approximately 39.7 ± 0.15 °C, above which exercise may cease due to uncompensated thermoregulatory demands, or as a protective mechanism within the body (Nielsen *et al.*, 1993). Elevated core temperature has been postulated to accelerate the onset of central fatigue through signals originating in the active muscles and/or the central nervous system, and the critical core temperature hypothesis has been supported by further research (Gonzalez-Alonso *et al.*, 1999B). However, there are many cases in the published literature where fatigue occurs at a core temperature well below this threshold (Watson *et al.*, 2004; Sawka *et al.*, 1992; Mittleman *et al.*, 1998).

There is a vast volume of literature on the peripheral physiological effects of varying environmental conditions in which exercise is performed, but the central mechanisms behind the reduction in performance are yet to be fully clarified. What is known, is that the increase in perceived effort seen during exercise in the heat, compared to the same exercise in a more temperate environment, is not related to the cerebral balance of tryptophan or dopamine measured by the arterial-venous differences in the blood, neither of which differed between exercise in an ambient environment, and the same exercise with heat exposure (Nybo *et al.*, 2003). There was no difference between the trials for the ratings of thermal comfort in the present study. Serotonin has been suggested to play a role in thermoregulation and therefore by altering the synthesis rate of serotonin, a difference in the thermal comfort scores reported between the two treatments might have been expected.

The intensity of the exercise performed in the present study was low and there was no difference between the trials for the RER values (P = 0.691), nor any effect of time (P = 0.150), showing that the supplemented branched-chain amino acids were not being metabolised as a fuel source. There is also no

evidence that carbohydrate supply was a limiting factor to exercise, as shown by the stable blood glucose concentrations seen throughout the exercise protocol. Indeed the supplementation of branched-chain amino acids did not alter the blood glucose ($P = 0.562$) or lactate ($P = 0.072$) levels between trials, a finding that is in line with other BCAA supplementation protocols (Watson *et al.*, 2004; Mittleman *et al.*, 1998; Blomstrand *et al.*, 1997).

3.6 Conclusion

The results of this study do not support the theory of inter-individual differences in the response to branched-chain amino acid supplementation proposed by Watson *et al.* (2004). Nor does it support previous findings that BCAA supplementation improves exercise capacity in the heat (Mittleman *et al.*, 1998), despite the large amount of branched-chain amino acids ingested, and the consequent reduction in the free tryptophan to branched-chain amino acid ratio. The BCAA drink did not cause any differences in the physiological responses to exercise compared to the Control drink. With the growing number of published studies showing no response to nutritional manipulations using branched-chain amino acids on the serotonergic system, it should be concluded that this is not a successful approach to improving exercise capacity or performance.

Although using branched-chain amino acids to manipulate tryptophan uptake into the brain does not seem to produce an improvement in exercise capacity, there is another nutritional manipulation that could reduce tryptophan uptake into the brain. By acutely depleting the body's circulating tryptophan concentration, this may reduce the uptake of tryptophan across the blood brain barrier and therefore prevent or reduce the production of serotonin. Based on the same theory as branched-chain amino acid supplementation, a reduced rate of serotonin production could improve exercise capacity. This principle provides the basis of the next chapter.

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CHAPTER FOUR

Effect of acute tryptophan depletion on
endurance cycling capacity in a warm
environment.

4.1 Abstract

Reducing the availability of tryptophan for transport across the blood brain barrier has been proposed to reduce the rate of production of serotonin, thereby improving endurance exercise capacity. Acute tryptophan depletion has never been tested as a method for improving endurance exercise capacity. The aim of this investigation was to assess the effect of acute tryptophan depletion on endurance cycling capacity in a warm environment.

Eight males cycled to volitional exhaustion at 55 % $\dot{V}O_2$peak in an ambient temperature of 30.1 ± 0.5 °C and 30 ± 7 % relative humidity, on two separate occasions. They did these tests seven hours after consuming either an amino acid load designed to deplete their circulating tryptophan concentration (TD), or a Control amino acid load (CON). Blood samples (7 ml) were taken before ingesting the amino acids, before the start of exercise, every fifteen minutes during exercise, and at the point of exhaustion. Heart rate (HR), core (Tc) and skin (Tsk) temperatures and ratings of perceived exertion (RPE) and thermal comfort (TC) were also monitored every ten minutes during the experiment. Gas samples were collected every thirty minutes.

Tryptophan (P = 0.003) and free tryptophan (P < 0.001) plasma concentrations, and the ratio of free tryptophan to branched-chain amino acids (P = 0.004) were all lower on the TD trials. However, there was no difference in the time to exhaustion between the two treatments (TD: 99.2 ± 24.4 minutes, CON: 108.4 ± 21.6 minutes; P = 0.088). There was also a tendency for HR and Tc to be higher on the TD trials (HR P = 0.053, Tc P = 0.069). There were no differences for any of the other parameters measured (Tsk, P = 0.597; RPE, P = 0.195; TC, P = 0.422; Blood [glucose], P = 0.666; Plasma [FFA], P = 0.695; Change in plasma volume, P = 0.211). It is possible the TD did reduce serotonin production, but that this actually increased HR and Tc, potentially causing the tendency for a reduction in endurance exercise capacity. The results of this study show that exercise capacity in a warm environment is not improved by acute tryptophan depletion.

4.2 Introduction

Exercise capacity is significantly reduced in a warm environment (Galloway and Maughan, 1997). In contrast to temperate conditions, during heat stress fatigue may be mediated through mechanisms residing within the central nervous system (CNS), with the development of hyperthermia thought to play a key role (Nybo and Nielsen, 2001B). Specifically, the neurotransmitter serotonin is thought to be of importance in the development of fatigue as it is involved in the control of arousal, sleepiness and mood (Blomstrand, 2001). Prolonged strenuous exercise increases serotonin synthesis and release which has led to suggestions that serotonin impairs exercise capacity (Newsholme *et al.*, 1987).

Serotonin, or 5-hydroxytryptamine (5-HT), is synthesised in the body using the essential amino acid precursor tryptophan. After the transportation of free tryptophan across the blood brain barrier, tryptophan is converted in the brain to serotonin through two enzymatic reactions. One of the enzymes, tryptophan hydroxylase, is only around 50 % saturated in normal circumstances, meaning that the main determinant of serotonin production is the availability of the substrate, i.e. tryptophan.

Tryptophan is found in protein rich foods such as meat, fish and dairy produce. The National Academy of Sciences (2005) has suggested that, based on RDA-level of protein intakes, males aged over eighteen years should be consuming 392 mg of tryptophan a day. Not all of the dietary tryptophan ingested crosses the blood brain barrier, as tryptophan in the brain usually ranges from 10 – 30 mg, but this is undoubtedly an important fate of tryptophan (Leathwood, 1987). Indeed, research is on-going about which protein sources offer the best tryptophan availability with a view to altering serotonin production and improving mood (Markus *et al.*, 2008). Completely removing tryptophan from the diet reduces plasma tryptophan by only around 20 %, and consequently has few behavioural consequences (Bell *et al.*, 2001). To induce greater falls in plasma tryptophan concentration, feeding an amino acid load with the particular omission of tryptophan achieves about an 80 % reduction in plasma free and total tryptophan levels five to seven hours

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after consumption: a technique known as rapid or acute tryptophan depletion (Delgado *et al.*, 1990). Such a reduction may therefore inhibit the synthesis and release of serotonin (Bell *et al.*, 2001).

Acute tryptophan depletion works by loading the system with amino acids causing protein synthesis to be stimulated in the liver (Bell *et al.*, 2001). This process requires tryptophan which, since it was not ingested, is utilised from the stores within the body. Administering the amino acid load also increases the systems' large neutral amino acid content, thereby further reducing the amount of tryptophan passing across the blood brain barrier, due to increased competition for the same energy requiring, active transport mechanism (Pardridge, 1983). The most successful brain tryptophan depletion protocols combine a low tryptophan diet with the ingestion of an amino acid load.

Acute tryptophan depletion is a popular, well documented technique and has been used extensively in clinical studies into depression (Neumeister *et al.*, 2004), anxiety (Bell *et al.*, 2001), aggression (McCloskey *et al.*, 2009), sleep patterns (Bhatti *et al.*, 1998), memory (Schmitt *et al.*, 2000), eating disorders (Weltzin *et al.*, 1995) and cognitive function (Gallagher *et al.*, 2003). Through the combined effects of a tryptophan free diet and acute tryptophan depletion, the lowered brain serotonin concentrations could, theoretically, lead to an improvement in exercise capacity based on the principals proposed in Newsholme's theory of central fatigue (1987).

Therefore, the aim of this investigation was to compare the response to prolonged exercise in a warm environment when participants were tryptophan depleted to when they were fed a control amino acid load.

4.3 Methods

4.3.1 Participants

Eight healthy, recreationally active males (Mean ± SD: age 24 ± 3 years; height 1.84 ± 0.07 m; mass 84.4 ± 9.9 kg; $\dot{V}O_2peak$ 52.8 ± 7.1 ml / kg / min) were recruited to participate in the study.

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4.3.2 Experimental protocol

The initial visit was to complete a discontinuous, incremental cycle ergometer test to volitional exhaustion in order to determine the participants' $\dot{V}O_2peak$ (details in Chapter Two). All subsequent visits to the laboratory were separated by at least one week and were performed at the same time of day. The following two visits to the laboratory were for familiarisation trials. Details of these and of the pre-trial conditions are in Chapter Two. There were then two experimental trials, both of which followed the same protocol, but during which slightly different amino acid loads were provided to the participants, in a randomised, double blinded, cross-over design. A schematic of the protocol used can be seen in *Figure 4.1*.

Each trial involved two visits to the laboratory. Participants were asked to attend the laboratory between 1700 and 1900 hours the day before the exercise trial, having consumed nothing but plain water for the previous four hours. Participants were seated in a comfortable room for fifteen minutes before a resting blood sample (7 ml) was taken by venepuncture. Participants were then given the amino acid load to take away and consume at 2330 hours that night. The amino acid load consisted of either 104.4 g of amino acids including tryptophan (CON: alanine (5.5 g), arginine (4.9 g), cysteine (2.7 g), glycine (3.2 g), histidine (3.2 g), isoleucine (8 g), leucine (13.5 g), lysine monohydrochloride (11 g), methionine (3 g), phenylalanine (5.7 g), proline (12.2 g), serine (6.9 g), threonine (6.5 g), tyrosine (6.9 g), valine (8.9 g) and tryptophan (2.3 g); SHS International ltd, UK), or the same amino acid load excluding tryptophan (TD). In order to make the amino acids consumable they were divided into a drink and capsules. The gelatine capsules (TAAB, UK) contained the isoleucine, tyrosine and methionine, while the drink contained all the remaining amino acids along with 8.25 g of intensive pineapple flavouring (SHS International ltd, UK) in 350 ml of tap water.

Figure 4.1. A schematic representing the protocol employed during the main trials in Chapter Four.

Consume amino acids
(1130 hours)

24 hours standardised
low tryptophan diet

Report to laboratory
(0500 hours)

EXERCISE

POST

24 hours
standardised
diet and
activity

Baseline blood sample
(1700 – 1900 hours)

Drink 500 ml water
(0430 hours)

0   30   60   Exh   +15

⇓ = 7 ml blood sample   ⬇ = Drink (100 ml)

⬆ = Expired air sample   ⇑ = Tc, Tsk, HR, RPE, TC
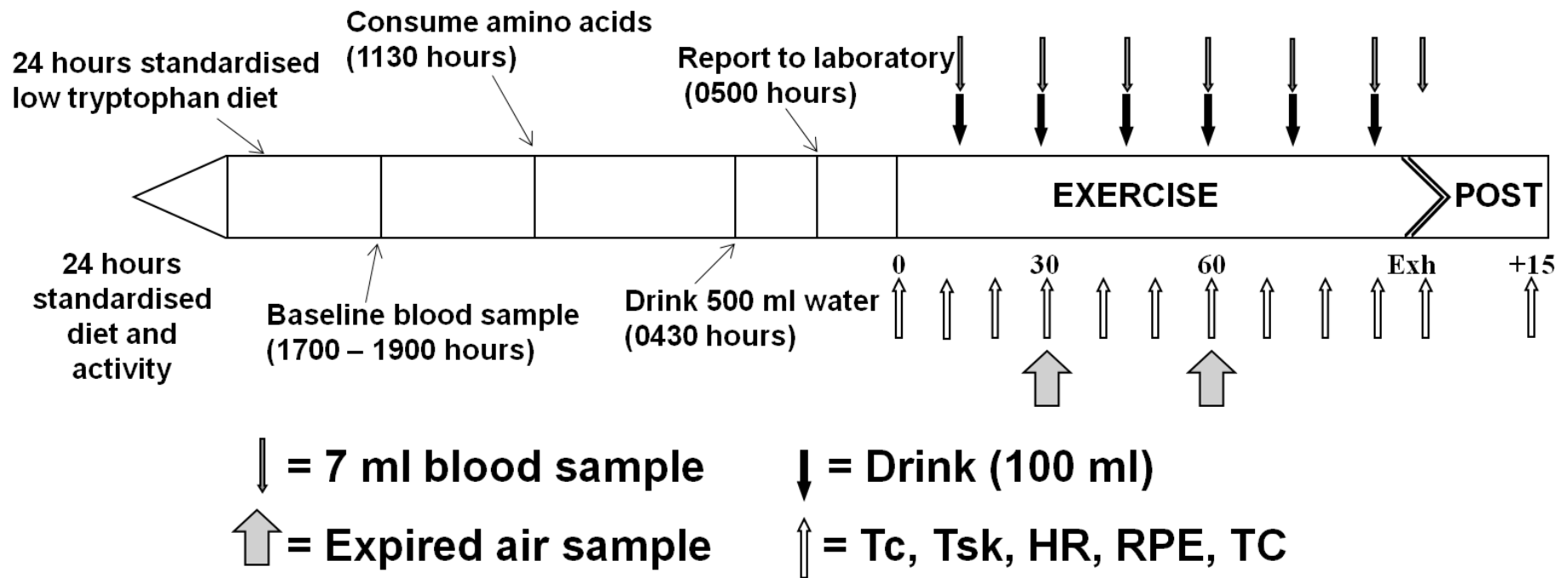
Participants reported to the laboratory at 0500 hours the following morning after an overnight fast and 500 ml of water at 0430 hours. After arriving, participants emptied their bladder and a sample was retained (details in Chapter Two). Participants' post-void, nude body mass was noted and they then positioned a rectal thermometer to allow the measurement of core body temperature. Skin thermistors were positioned on the chest, tricep, thigh and calf. A heart rate telemetry band was also worn (see Chapter Two for details).

Participants were then seated in a comfortable environment (22.9 ± 0.4 °C), and one hand was immersed in warm (42 - 44 °C) water for ten minutes. An indwelling cannula was inserted (detailed in Chapter Two) and a baseline blood sample (7 ml) was drawn. Baseline measures of heart rate and core and skin temperatures were taken before participants were moved into a warm environment (30.1 ± 0.5 °C, 30 ± 7 % relative humidity) and exercised on a cycle ergometer at a workload corresponding to 55 % $\dot{V}O_2$peak until volitional exhaustion (details in Chapter Two).

Blood samples (7 ml) were taken at fifteen minute intervals during exercise and participants consumed 100 ml of plain water immediately after each sample. Heart rate, core temperature, skin temperature and ratings of perceived exertion and thermal comfort were recorded every ten minutes during exercise; expired gas samples were collected every thirty minutes (see Chapter Two for details). A final blood sample and temperature readings were taken at the point of exhaustion. Participants were then moved back into a comfortable environment and their recovery was monitored. Final recordings of heart rate, core temperature and skin temperature were made fifteen minutes after the cessation of exercise. At this point, all probes and the cannula were removed and the participant was reweighed to allow the calculation of body mass loss over the trial (see Chapter Two for details). Participants then showered and were provided with food and drinks before leaving the laboratory.

4.3.3 Blood handling and analysis

All blood samples were collected into dry syringes. Samples were analysed for the determination of blood glucose and haemoglobin concentrations, haematocrit values and free fatty acid, free tryptophan and large neutral amino acids concentrations. Details of the techniques used can be found in Chapter Two.

4.3.4 Statistical analysis

Data are presented as mean ± standard deviation (SD) unless otherwise stated. Data were tested for normal distribution using the Shapiro-Wilk test. Data were then investigated for skewness and kurtosis. Where appropriate, statistical analysis was done by either a two-way repeated measures ANOVA with a Bonferroni correction or a paired sample t-test. Statistical analysis was done using the SPSS program, version 14.0, and significance was set at $P < 0.05$.

4.4 Results

4.4.1 Hydration status

There were no differences in hydration status between the two trials ($P = 0.427$). The mean urine osmolality for TD was 637 ± 308 mosmol / L and for CON it was 667 ± 369 mosmol / L. The body mass loss from the two trials was not different (1.43 ± 0.40 kg on TD and 1.53 ± 0.32 kg on CON; $P = 0.148$).

4.4.2 Time to exhaustion protocol

The co-efficient of variation between the first and second familiarisations was 14.4 ± 14.8 % (r = 0.781). The co-efficient of variation between the second familiarisation and the Control trial was 11.5 ± 12.4 % (r = 0.834). This backs up the findings of Chapter Three by again showing the importance of a first familiarisation when performing exercise time to exhaustion tests, in order to improve the reproducibility of the test.

<u>4.4.3 Time to exhaustion data</u>

There was no effect of trial order in the exercise times to exhaustion (Trial 1 102.0 ± 23.4 minutes, Trial 2 105.6 ± 23.6 minutes; P = 0.540). There was no effect of acute tryptophan depletion on exercise capacity as the mean time to exhaustion was 99.2 ± 24.4 minutes on the TD trial and 108.4 ± 21.6 minutes on the CON trial (P = 0.088; *Figure 4.2*). When the data are expressed as a percentage change from the second familiarisation trial there is still no effect of acute tryptophan depletion (P = 0.066), although there is a tendency for duration to be shorter on the TD trials (95.5 ± 7.4 %) compared to the CON (105.5 ± 10.7 %) trials relative to the second familiarisation.
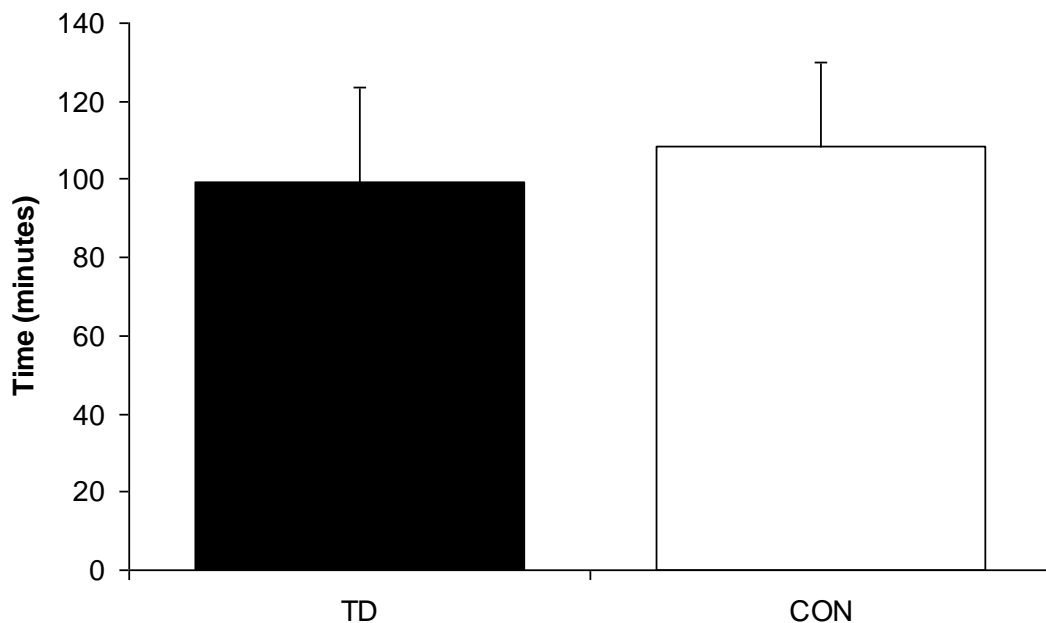
Figure 4.2. Mean cycling time to exhaustion (n = 8) for the TD and CON trials. There was no difference in the time to exhaustion between the two trials (P = 0.088).

<u>4.4.4 Thermoregulatory measures</u>

Core temperature increased during the exercise test (P < 0.001) from resting values of 37.0 ± 0.1 °C to 38.3 ± 0.3 °C on the TD trial and 36.9 ± 0.2 °C to 38.2 ± 0.2 °C on the CON trial at the point of exhaustion (*Figure 4.3*). The difference between the trials failed to reach statistical significance (P = 0.069). The core temperature then fell during the initial period of recovery on both trials.

As with core temperature, skin temperature increased during the exercise test (P < 0.001; *Figure 4.4*) but did not differ between trials (P = 0.597). Skin temperature increased from 32.5 ± 0.9 °C at rest to 35.0 ± 0.8 °C at the point of exhaustion on the TD trial, and from 32.3 ± 0.7 °C to 35.2 ± 0.9 °C on the CON trial. It then fell in the fifteen minute recovery period (to 33.2 ± 1.6 °C and 33.4 ± 1.2 °C for TD and CON respectively). Again, estimated body heat content followed a similar pattern to both core and skin temperatures, with no difference between the trials (P = 0.960), but a significant increase during the exercise bout (2471 ± 299 kJ and 2451 ± 283 kJ pre exercise increasing to 2587 ± 322 kJ and 2598 ± 318 kJ at the point of exhaustion for the TD and CON trials respectively; P < 0.001).
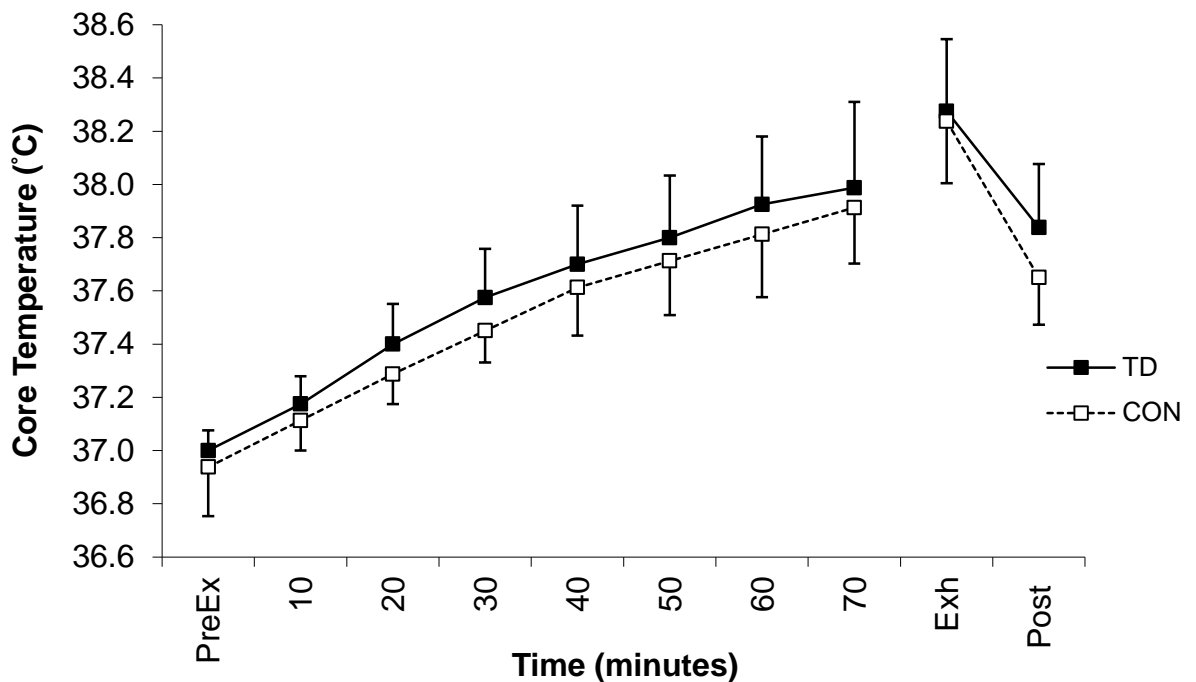
Figure 4.3. Core temperature over the duration of the TD and CON trials. There was a tendency for a core temperature to be higher on the TD trial than the CON trial.
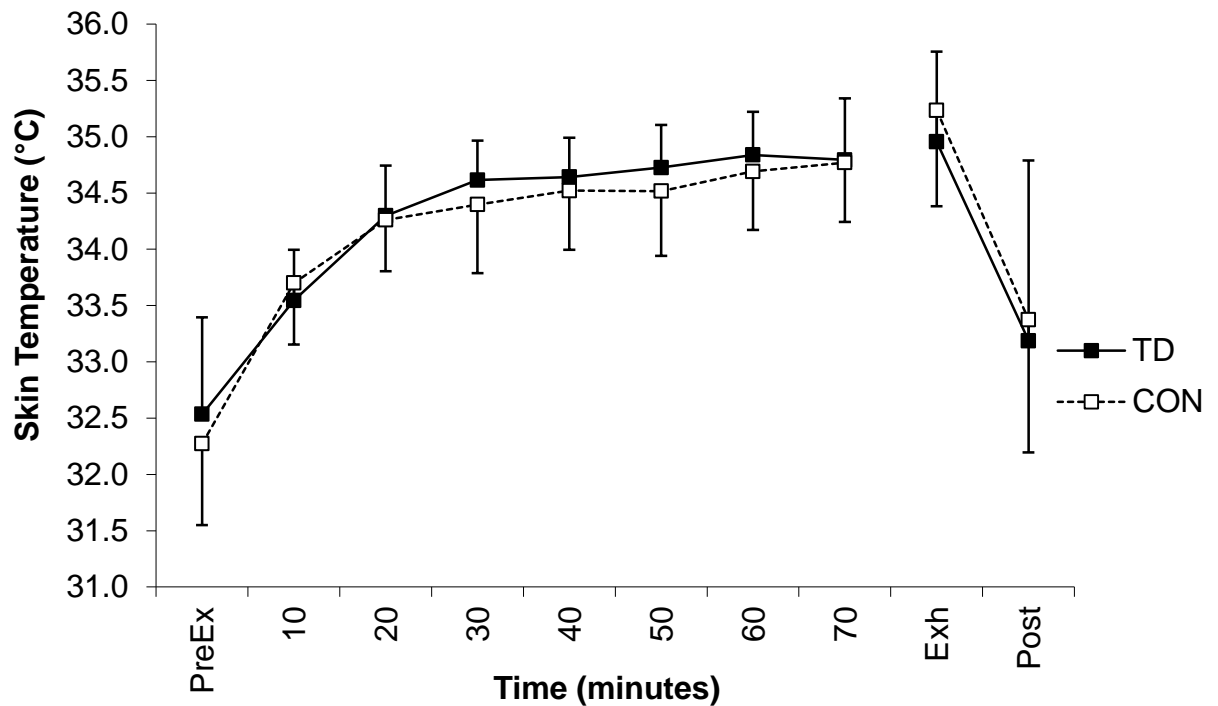
Figure 4.4. Skin temperature over the duration of the TD and CON trials.

4.4.5 Cardiovascular and metabolic measures

Rating of perceived exertion increased over time during the exercise (P < 0.001), but was not affected by the trials (P = 0.195; *Table 4.1*). Rating of thermal comfort also increased during the exercise bout (P < 0.001), but again, was not affected by the trials (P = 0.422; *Table 4.1*).

Table 4.1. Ratings of perceived exertion and thermal comfort over the exercise test.

		Time (minutes)						
		10	20	30	40	50	60	70
Perceived Exertion	TD	11 ± 2	12 ± 2	13 ± 2	14 ± 2	15 ± 2	17 ± 2	18 ± 2
	CON	11 ± 2	12 ± 2	13 ± 2	14 ± 2	14 ± 2	16 ± 2	16 ± 1
Thermal Comfort	TD	3 ± 1	4 ± 1	4 ± 1	5 ± 1	6 ± 1	6 ± 1	7 ± 2
	CON	3 ± 1	4 ± 1	5 ± 1	5 ± 1	6 ± 1	6 ± 1	6 ± 1

Heart rate tended to be higher on the TD trials (P = 0.053), but it did rise over the duration of the exercise (P < 0.001) to peak at the point of exhaustion (165 ± 11 and 161 ± 14 beats per minute on the TD and the CON trials respectively; *Figure 4.5*).
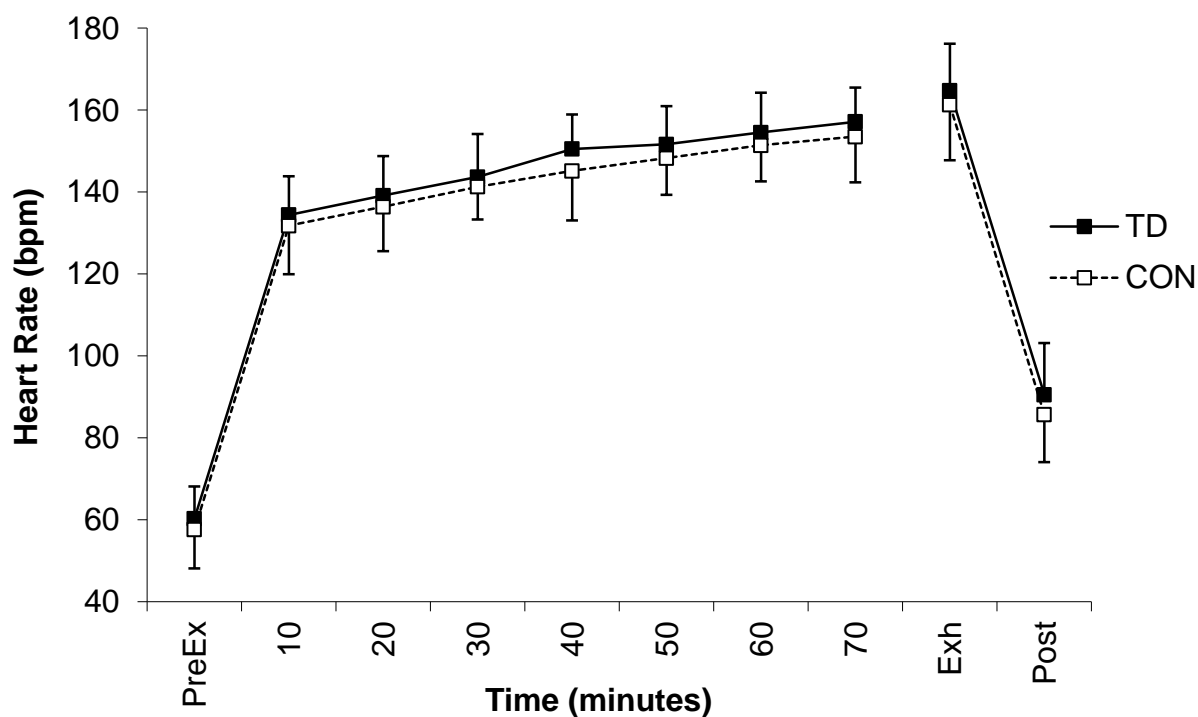
Figure 4.5. Heart rate over the duration of the TD and CON trials.

There was no effect of time (P = 0.840, P = 0.471) or trial (P = 0.636, P = 0.667) on the contributions of carbohydrate (CHO) or fat respectively during the exercise. Consequently, there was no effect of time (P = 0.172, P = 0.622) or trial (P = 0.881, P = 0.264) on energy expenditure (EE) or respiratory exchange ratio (RER) respectively (*Table 4.2*).

Plasma volume did change with time (P < 0.001). It increased initially between the pre-supplementation sample and the pre-exercise sample, however, it then fell during exercise and reached its lowest at the point of exhaustion. There was no difference between the trials (P = 0.211; *Figure 4.6*). Blood glucose changed significantly with time (P = 0.007), but again, there was no difference between the trials (P = 0.666; *Figure 4.7*). Free fatty

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acid concentration also changed significantly over time (P < 0.001) increasing through exercise, but again there was no effect of trial (P = 0.695; *Figure 4.8*).

Table 4.2. Contributions of different energy sources during the exercise performed and the respiratory exchange ratio and energy expenditure values.

	TD		CON	
	30 minutes	60 minutes	30 minutes	60 minutes
CHO oxidation rate (g / min)	2.42 ± 0.83	2.57 ± 1.31	2.42 ± 0.55	2.35 ± 0.87
Fat oxidation rate (g / min)	0.26 ± 0.29	0.30 ± 0.18	0.26 ± 0.14	0.33 ± 0.16
RER	0.93 ± 0.06	0.92 ± 0.06	0.93 ± 0.03	0.91 ± 0.05
EE (kJ / min)	48.4 ± 10.6	51.5 ± 12.8	49.0 ± 6.5	50.4 ± 9.2
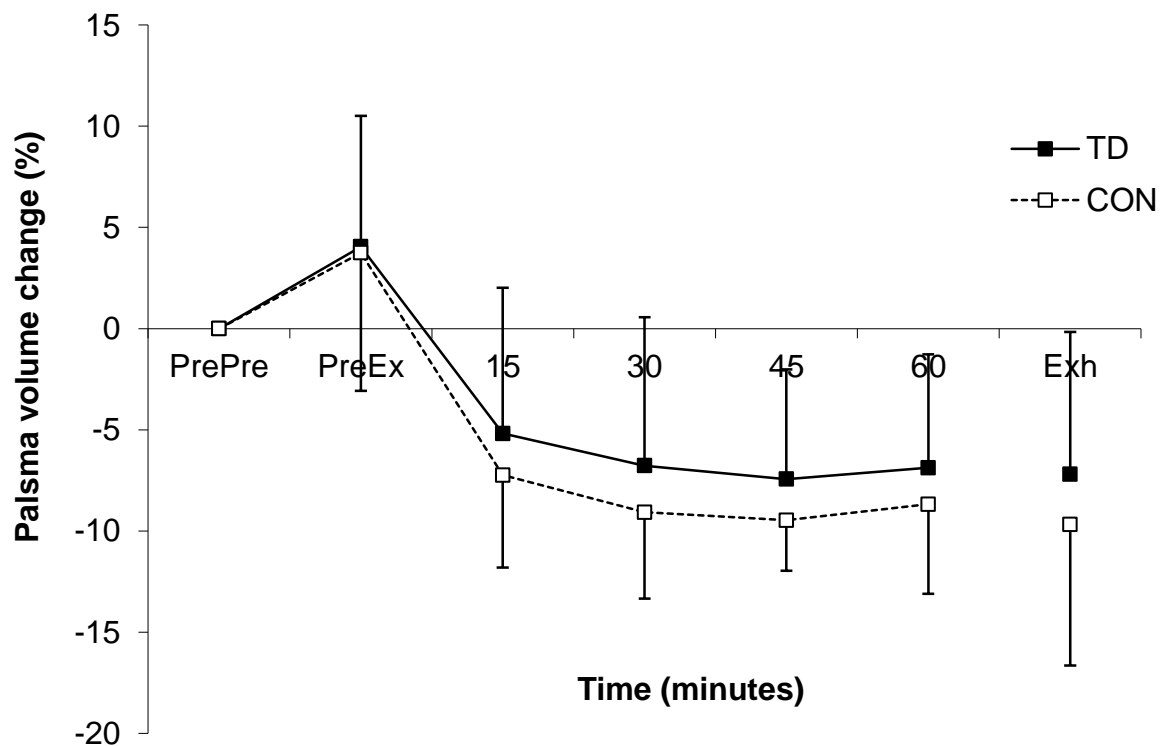
Figure 4.6. Plasma volume, presented as the percentage change from the initial blood sample, over the duration of the study. There was no difference between trials (P = 0.211).

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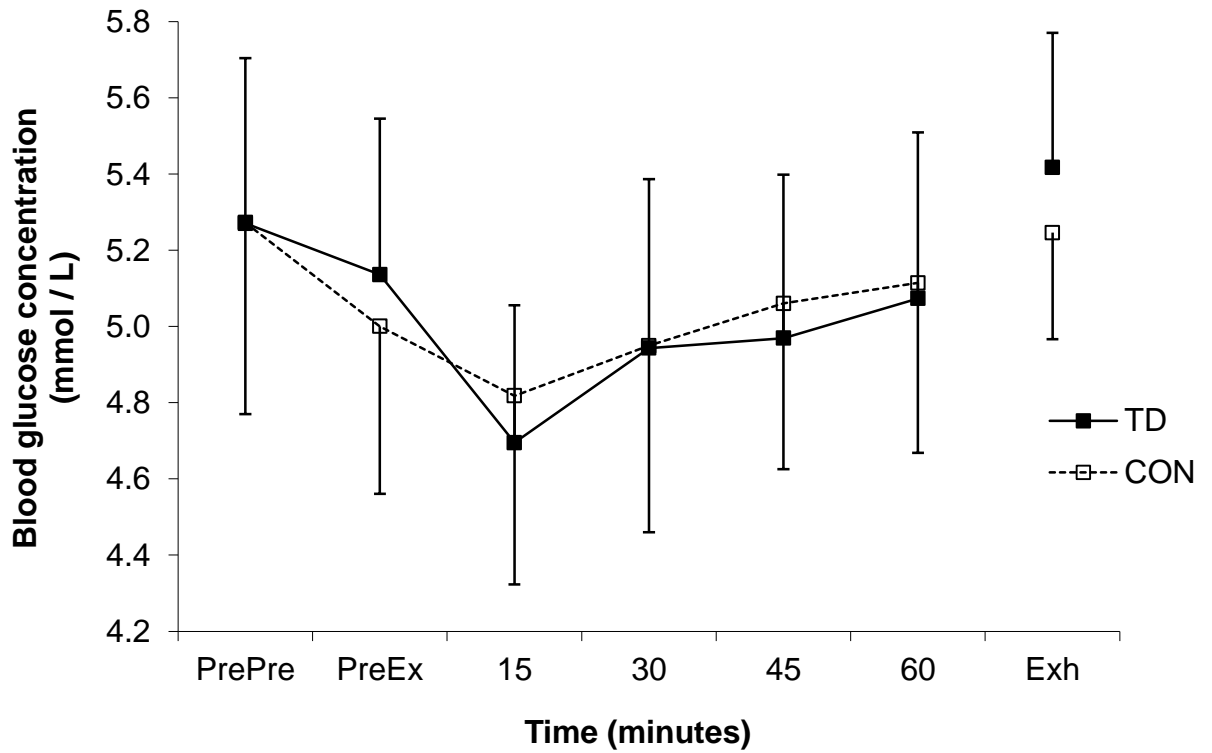
Figure 4.7. Blood glucose concentration varied over the duration of the TD and CON trials (P = 0.007), but was not different between trials (P = 0.666).
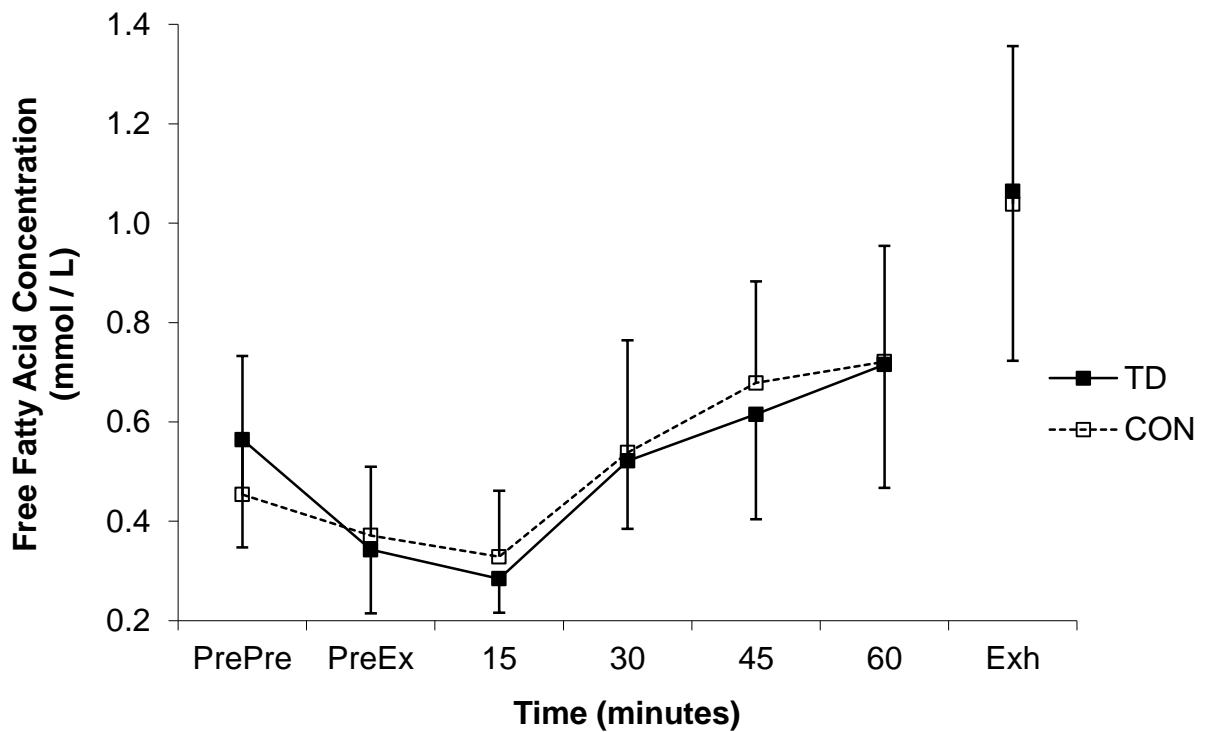
Figure 4.8. Free fatty acid concentration over the duration of the TD and CON trials. There was no difference between the trials (P = 0.695).

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Plasma branched-chain amino acid concentrations are shown in *Figure 4.9A* and plasma concentrations for the other large neutral amino acids are shown in *Figure 4.9B*. Other than leucine, all demonstrated significant changes over time ($P \leq 0.024$), with the majority rising either after drinking or during the exercise, none were different between the trials. *Figure 4.10A* shows plasma tryptophan and free tryptophan concentrations at the time points sampled. Tryptophan ($P = 0.003$) and free tryptophan ($P < 0.001$) concentrations are lower on the TD trials compared to the CON trials. *Figure 4.10B* shows the ratio of free tryptophan to branched-chain amino acids during the trials. The ratio was lower on the TD trials than the CON trials ($P = 0.004$) and there was also a significant effect of time on the ratio ($P = 0.048$), decreasing after consuming the amino acid load and then increasing during the exercise phase.
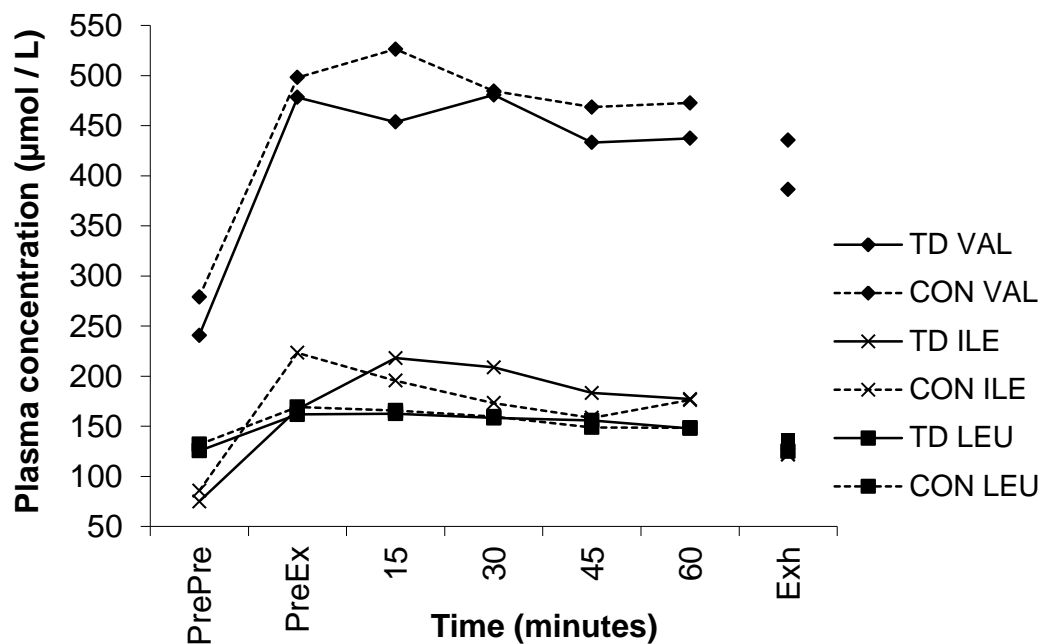
Figure 4.9A. Plasma branched-chain amino acid concentrations over the durations of the TD and CON trials. Valine ($P = 0.003$) and isoleucine ($p = 0.024$) were influenced by time over the duration of the trials but the treatment did not have any influence on any of the branched-chain amino acids.
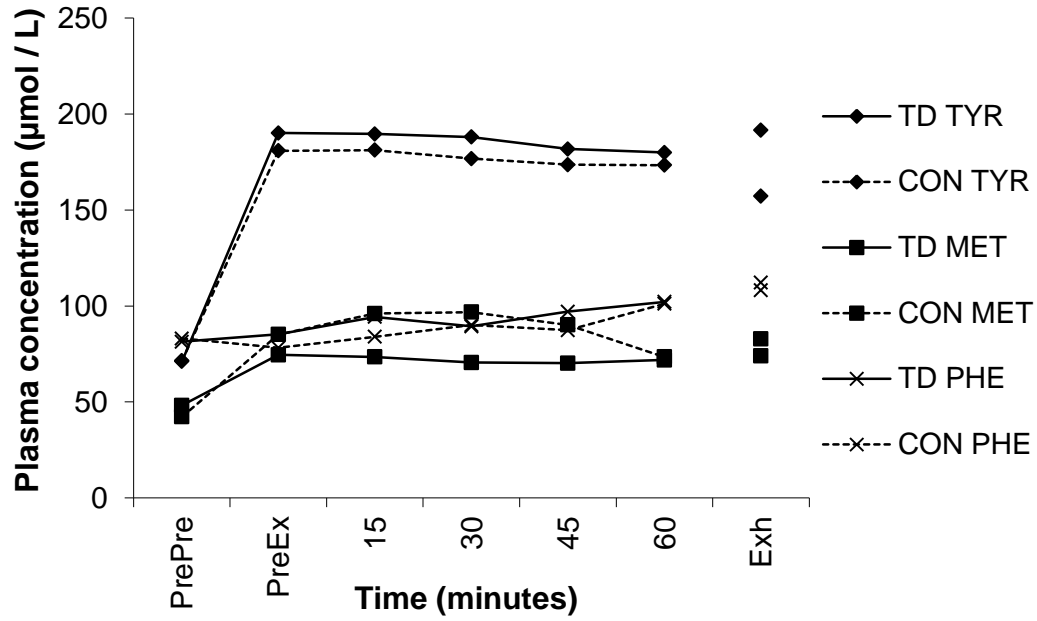
Figure 4.9B. Plasma large neutral amino acid concentrations over the durations of the TD and CON trials. Tyrosine (P = 0.001), methionine (P = 0.038) and phenylalanine (P = 0.004) were all affected by time during the trials but the treatment did not have any influence.
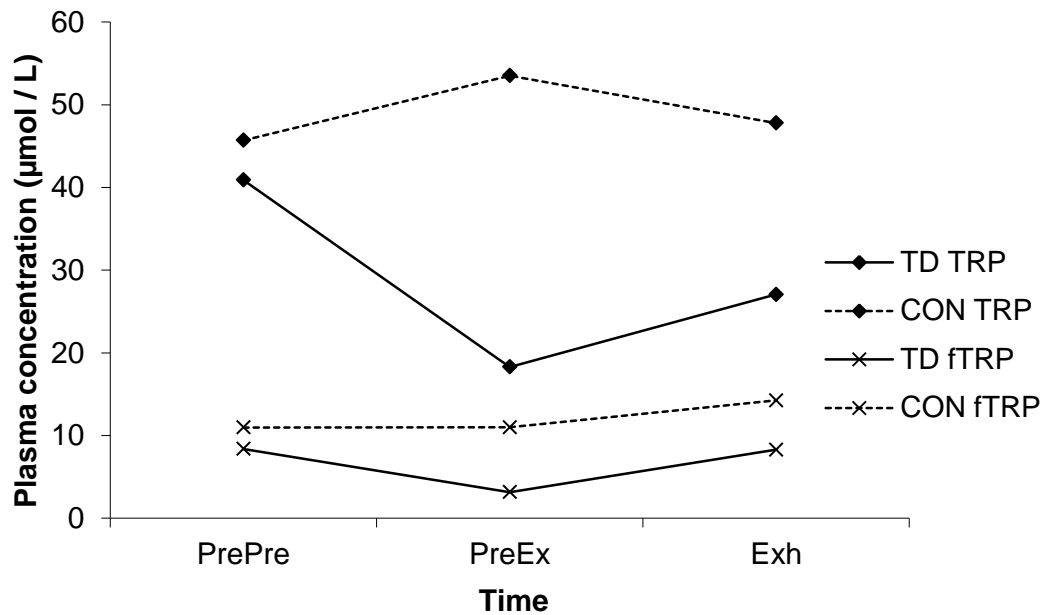
Figure 4.10A. Plasma tryptophan and plasma free tryptophan concentrations at the time points sampled over the TD and CON trials.

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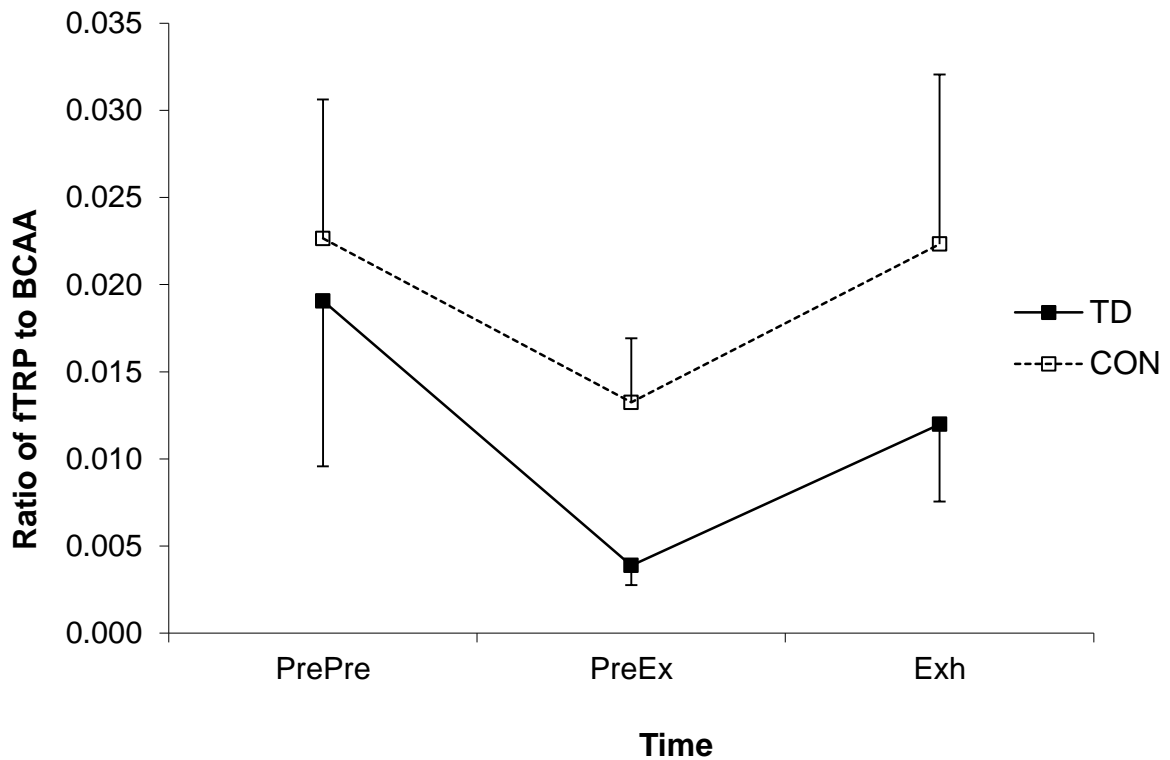
Figure 4.10B. The ratio of free tryptophan to branched-chain amino acids at the time points sampled over the TD and CON trials.

4.5 Discussion

The main finding of this investigation was that there was a tendency for exercise capacity to be impaired on the TD trials (99.2 ± 24.4 minutes) compared to the CON trials (108.4 ± 21.6 minutes; P = 0.088). This was also reflected when the data were expressed as a percentage change from the time to exhaustion generated in the second familiarisation (TD 95.5 ± 7.4 %, CON 105.5 ± 10.7 %; P=0.066). This is the opposite response to the one which may have been expected given the hypothesis for the mechanism of action of the tryptophan depletion protocol, however, potential explanations for this finding are presented below.

In a recent study, Van der Veen and colleagues (2008) found that acute tryptophan depletion, using a slightly smaller amino acid load to the one used in the present study, did not affect participants' mood levels or their performance in a response test designed by Miltner *et al.* (1997). The test was designed to provide participants with almost instantaneous positive or

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negative feedback based on their ability to estimate a one second interval. In the placebo trial, heart rate (continuously monitored by ECG) slowed when participants received negative feedback and this response was attenuated in the tryptophan depleted trial. The acute tryptophan depletion did not have any effect on heart rate compared to the placebo when positive feedback was given. This implies that phasic heart rate responses are sensitive to lowered serotonin and serotonin is known to play a critical role in the control of heart rate (Jordan, 2005). When Ramage and Fozard (1987) administered a 5-HT_{1A} receptor agonist, they found that this evoked a vagally mediated fall in heart rate in anaesthetised cats.

It is possible therefore, that the administration of an amino acid load designed to deplete the serotonin precursor tryptophan, may lead to a relative increase in heart rate. Put another way, the increases seen in heart rate during normal exercise, such as in the CON trial, may be slightly attenuated due to the presence of serotonin in greater concentrations than on trials where this is reduced by some means, such as through acute tryptophan depletion. This hypothesis is backed up by the trend throughout the TD trials in the present study for a higher heart rate than on the CON trials ($P = 0.053$). It should also be considered that serotonin is known to have vaso-active properties (Yildiz *et al.*, 1998) and therefore if a reduction in serotonin were to lead to vasodilation, heart rate could increase to maintain cardiac output.

The trend for an elevated heart rate on the TD trials may have subsequently led to the trend ($P = 0.069$) seen for a slightly elevated core temperature throughout the TD trials. The reduction in the time available for the diffusion of excess heat from the peripheral circulation to the skin, due to an increased heart rate, may have led to the trend for an elevated core temperature. However, the lack of any differences in skin temperature between the TD and CON trials means that this is unlikely to be the cause of the elevated core temperatures seen with tryptophan depletion.

If acute tryptophan depletion did cause a reduction in serotonin activation, then this could have led to the tendency for rectal temperatures to be higher,

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independent of the effect on heart rate. This was seen when a 5-HT_{2C} receptor antagonist (Pizotifen) was administered to healthy human participants, resulting in an elevated resting core temperature (Strachan *et al.*, 2005). However, the opposite was seen in rats where, by elevating the levels of 5-HT in the hypothalamus, synaptic 5-HT_2 receptors were activated, resulting in hyperthermic effects (Lin *et al.*, 1998). Of course, care must be taken in the interpretation of these results as the investigation was conducted in rats not humans and no exercise was involved Furthermore, the 5-hydroxytryptophan was directly perfused into the hypothalamus rather than tryptophan being ingested and transferred over the blood brain barrier for synthesis into 5-hydroxytryptophan.

It is also possible that the tendency for a higher heart rate and core temperature on the TD trials may have actually lead to the tendency for exercise capacity to be impaired on the TD trials (99.2 ± 24.4 minutes) compared to the CON trials (108.4 ± 21.6 minutes; P = 0.088). It is likely that any benefit which may have been seen due to the influences of the acute tryptophan depletion on serotonin production and metabolism are overridden by the trends for higher heart rates and core temperatures that acute tryptophan depletion appears to evoke. It is possible that any positive effect of acute tryptophan depletion on exercise capacity may be clearer in more temperate environments.

It is important to note that the core temperatures seen at the point of exhaustion in these trials (38.3 ± 0.3 °C on the TD trials and 38.2 ± 0.2 °C on the CON trials) are low in comparison to those reported in the wider literature on prolonged exercise in the heat. As discussed in Chapter One, a critical core temperature of 39.7 °C has been proposed, above which exercise cannot be sustained (Nielsen *et al.*, 1993). Although there are several reports of exhaustion during prolonged exercise in the heat occurring at core temperatures lower that the 'critical' level, [38.8 ± 0.3 °C Cheung and McLellan, (1998); 39.1 ± 0.3 °C Sawka *et al.*, (1992); 38.8 ± 0.1 °C Bridge *et al.*, (2003A); 38.7 ± 0.9 °C Hobson *et al.,* (2009); 38.9 ± 0.5 ° C Watson *et al.*, (2004)], the temperatures reported here are still very low. However, the pre-

exercise resting core temperatures are consistent with early morning core temperatures reported in the literature (Hobson *et al.*, 2009; Faria and Drummond, 1982) suggesting that the reason for this was not due to the instruments used or the methodology employed to measure core temperature.

In humans, the response to tryptophan depletion in the cerebrospinal fluid is delayed, with peak depletion occurring seven to ten hours after consuming an amino acid load (Carpenter *et al.*, 1998), compared to the maximum level of plasma tryptophan depletion, occurring five to seven hours post ingestion of an amino acid load (Delgardo *et al.*, 1990). Due to the timing of the present study, participants will have started exercise approximately six hours after consuming the amino acid load, which should have corresponded with the peak time for the depletion of plasma tryptophan. Data shown in *Figure 4.9A* supports this. For all participants the point of exhaustion will have been reached between seven and nine hours after consuming the amino acid load, which should have corresponded with the peak time of tryptophan depletion in the cerebrospinal fluid.

Laboratory tests on humans have shown that tryptophan depletion specifically reduces cerebrospinal fluid concentration of both tryptophan (by 80 – 90 %; Carpenter *et al.* 1998) and the serotonin metabolite 5-HIAA (24 – 40 %; Williams *et al.*, 1999). In animal studies acute tryptophan depletion leads to extremely rapid changes in plasma tryptophan and brain serotonin content and also influences behaviour through increased pain sensitivity and aggression, and reduced rapid eye movement sleep (Bell *et al.*, 2001). Therefore, if this translates into the human brain, the point of exhaustion should have coincided with the maximum level of tryptophan depletion in the cerebrospinal fluid, eliciting the highest chance of influencing the serotonergic system.

Tryptophan supplementation (1.2 g over approximately fifteen hours) has previously been shown to improve treadmill running time to exhaustion in humans running at 80 % $\dot{V}O_2$peak with a 5 % incline gradient, by, on average,

49 % (5 minutes 40 seconds on the placebo trial and 7 minutes 40 seconds on the supplementation trial; Segura and Ventura, 1988). However, the equivocal improvements in two of the participants (5 minutes to 18 minutes (260 %) and 2 minutes 30 seconds to 6 minutes 30 seconds (160 %)), coupled with the very short duration of the exercise test, despite fairly low running speeds (11.8 ± 2.0 km / hour), and the use of apparently active males (VO$_2$peak 53.9 ± 8.2 ml / kg / min), leaves the findings open to speculation (Segura and Ventura, 1988).

Furthermore, the protocol employed by Segura and Ventura (1988) was later repeated but with the addition of two familiarisation trials preceding the experimental trials. No difference in exercise capacity was seen between the tryptophan and the placebo trials (Stensrud *et al.*, 1992). Indeed, applying the Newsholme theory of central fatigue (1987), supplementing tryptophan would be expected to elicit a decrement in exercise capacity and this has been shown to be the case in horses (Farris *et al.*, 1998) and rats (Soares *et al.*, 2004; Soares *et al.*, 2007). However, in a well-controlled study in humans, the consumption of a 6 % sucrose solution containing 3 g / L of tryptophan (average total intake 3.9 g) by endurance trained males failed to limit cycle exercise capacity at 70 – 75 % of maximum power, despite greater than six-fold increases in plasma tryptophan concentrations (Van Hall *et al.*, 1995).

It can be seen that some of the participants may have started the exercise test slightly hypohydrated. However, none of the individual participants' urine osmolality's were more than 55 mosmol / L different between trials and the 500 ml of water they consumed before coming to the laboratory should have helped to minimise this. Therefore, it is unlikely that this will have influenced the results of the exercise capacity test.

An increase in brain tryptophan uptake during prolonged exercise is suggested to contribute to an elevation in central serotonin activity (Blomstrand *et al.*, 1989). This increase in serotonergic activity increases the perception of effort during exercise and therefore hastens the onset of fatigue (Blomstrand *et al.*, 1997). However, in this study acute tryptophan depletion

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did not cause a concurrent lowering of perceived exertion ratings during the exercise, as the RPE values seen were not different between trials (P = 0.195) and if anything actually tended to be higher on the TD trial towards the point of fatigue (RPE at 70 minutes 18 ± 2 for TD and 16 ± 1 for CON). However, it may be possible to explain this as there was a tendency for a shorter exercise duration on the TD trials, meaning that participants were nearer the point of exhaustion after seventy minutes of the TD trial than at the same time point on the CON trial.

Tryptophan depletion has been shown to increase aggression in animals (Bell *et al.*, 2001) and in humans (Bjork *et al.*, 1999). Bjork and colleagues (1999) conducted an experiment in which money was taken from participants who were either tryptophan depleted or food restricted as a control. Participants' aggression levels were monitored by recording the number of retaliatory responses the participants made ostensibly to reduce the earnings of other, fictitious, participants. Based on these findings, improvements in exercise capacity with acute tryptophan depletion may be more likely to be seen in directly competitive environments, where aggression is likely to play an influential role in overall performance, compared to a solitary lab based exercise time to exhaustion test as used here.

A tryptophan depletion study, conducted to look at the effects on executive function, showed an improvement on only one (Trail-Making Test) of four (The Wisconsin card sorting test; Verbal fluency; Stroop colour-word test) neurocognitive tests, when participants were acutely tryptophan depleted in the same way as in the present experiment (Gallagher *et al.*, 2003). However, since there was a learning effect on the tests and participants had not been familiarised with them, the true effects of tryptophan depletion on cognitive function cannot be seen. Gallagher and colleagues (2003) also found that the acute tryptophan depletion had no effect on subjective ratings of mood when measured on a battery of 16 visual analogue scales. However, women appear to be more susceptible to the mood lowering effects of tryptophan depletion compared to men, even when plasma tryptophan concentration is depleted to the same extent (Booij *et al.,* 2005). Therefore,

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gender differences may exist in serotonin metabolism meaning that acute tryptophan depletion, which causes no negative effects in males, does produce mood lowering in females (Nishizawa *et al.*, 1997).

4.6 Conclusion

There was no difference in exercise capacity when participants received a control amino acid load compared to a load designed to acutely deplete plasma tryptophan concentrations. Therefore, it seems likely that central fatigue is influenced by another factor, or, more likely, several other factors. The availability of tryptophan as a precursor to serotonin production is not the sole reason for fatigue during prolonged exercise in a warm environment. However, there seems little doubt that the neurotransmitter serotonin does have a role to play in the development of central fatigue. Therefore, the following chapter will investigate the transport of serotonin, rather than the synthesis of serotonin.

CHAPTER FIVE

Differences in platelet serotonin transporter
density in former and current sprint and
distance runners, and sedentary controls.

5.1 Abstract

Serotonin is thought to be of importance in the development of fatigue. Transporters remove serotonin from the synaptic cleft, actively lowering the concentration to a point at which maintaining the activation in the post-synaptic receptors becomes impossible. A reduction in the concentration of serotonin could be achieved by an increased number of serotonin transporters. Unfortunately, it is not possible to study the serotonergic neurons directly, but they share many similarities with blood platelets which represent a simple and accessible peripheral model. Therefore, the aim of this study was to investigate serotonin transporter density on the platelets of current and former athletes in sprint and endurance disciplines to discover if this is a state or a trait marker.

Thirty three healthy males [6 current international endurance runners (CE), 6 retired international endurance runners (RE), 9 current international sprinters (CS), 6 retired international sprinters (RS), and 6 sedentary controls (I)] all provided a resting 40 ml venous blood sample. From the blood samples the platelets were isolated, washed and the membranes were prepared. They were then incubated in eight different concentrations of [^{3}H]Paroxetine (10 – 500 pM) and specific binding was determined using Citalopram (1 µM). Membrane-bound radioactivity was analysed by liquid scintillation counting.

The maximum number of binding sites was significantly greater in the CE group (1090 ± 76 fmol / mg protein) than in any of the other groups (P ≤ 0.008). The maximum number of binding sites (Bmax) for the CS group (953 ± 68 fmol / mg protein) was also greater than that of the RS group (P = 0.042). There were no differences between the RE, RS and I groups for Bmax (892 ± 88, 866 ± 58, 885 ± 58 fmol / mg protein respectively). The data show that the CE group have a greater number of serotonin transporters on their platelet membranes. This suggests that training, particularly for endurance events, may increase the number of serotonin transporters on the serotonergic neuron of the brain, aiding serotonin reuptake, and possibly altering the perception of fatigue.

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5.2 Introduction

The brain appears to play a significant role in the development of fatigue during prolonged exercise. Specifically, the neurotransmitter serotonin is thought to be of importance in the development of fatigue as it has been shown to be responsible for, among other things, feelings of lethargy (Cooper et al., 2003A). Chapters Three and Four, coupled with the wider literature, suggest that reducing the synthesis of serotonin through the minimising the amount of tryptophan crossing the blood brain barrier does not influence exercise capacity. Unfortunately, the direct examination of the central serotonergic neurons is obviously restricted in humans. However, the blood platelets share several commonalities with the serotonergic neuron, the site of serotonin synthesis within the brain (Da Prada *et al.*, 1988). Therefore, it is possible to use the blood platelet as a simple, accessible peripheral model for the examination of the serotonergic neuron to further investigate the development of fatigue within individuals.

Platelets have sub-cellular organelles that contain ATP and they actively store serotonin and other biological monoamines in preparation for their release by exocytosis. There are similarities between platelets and the serotonergic neurones; they are both active transporters of serotonin and they are both active storage vesicles for serotonin. In addition, both contain monoamine oxidase B and both also have 5-HT$_2$ receptors (Da Prada *et al.*, 1988). Although there are many similarities between the blood platelets and the serotonergic neuron which can be exploited in further study of the central nervous system, there are also several differences. Platelets do not contain monoamine oxidase A and they also lack tyrosine hydroxylase, meaning that they are unable to synthesise serotonin. Platelets are also anucleate and therefore have no capacity for protein synthesis (Pletscher, 1988).

The half-life of a blood platelet is approximately five days, meaning that the entire original population is eliminated from the circulation within approximately three weeks. This implies that the chronic action of antidepressant drugs is that they act on the platelet synthesising cells (megakaryocytes), rather than the platelet receptors themselves (Lawrence,

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1991). Blood platelets vary in size and in age; younger platelets are larger and more dense and metabolically active, but the functional properties of the platelets are believed to be independent of size and density (Thompson *et al.,* 1983). Acute exercise increases blood platelet counts by the alpha-adrenergic stimulation of the spleen. The spleen contains approximately ⅓ of the exchangeable platelet population (Chamberlain *et al.*, 1990), and the age of these platelets is similar to that of the population circulating in the blood stream (Chamberlain *et al.*, 1990).

Serotonin transporters on the serotonergic pre-synaptic neuron remove serotonin from the synaptic cleft in order to rapidly terminate synaptic transmissions (Rudnick and Clark, 1993). The serotonin transporter removes the serotonin and therefore actively lowers the synaptic serotonin concentration levels to the point at which it becomes incapable of maintaining the activation in the post-synaptic receptors. A reduction in the concentration of serotonin could be achieved by an increased number of serotonin transporters, an increase in their activity levels, or a combination of these two factors. During exercise, if there is an increased activity level of the central serotonergic system, a reduction in the concentration of serotonin could delay or stop synaptic transmissions and therefore alter the perception of fatigue. Wilson and Maughan (1992) indirectly supported this theory by demonstrating that a serotonin reuptake inhibitor reduced prolonged exercise capacity.

There is previous research in healthy athletic populations investigating platelet serotonin transporter density (Strachan and Maughan, 1998). The investigators showed that the density of the serotonin reuptake transporter is greater on the platelet membranes of endurance trained participants than in sedentary controls. However, Struder *et al.*, (1999) found that acute endurance exercise reduced the density of platelet $5\text{-}HT_{2A}$ receptor $[^3H]$Ketanserin binding sites.

Due to the nature of investigations into the actions of the serotonergic neuron, the changes that may occur within the brain can only be hypothesised because all measures must be made peripherally. One of the few ways of

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directly assessing the brain is by using cadavers. Lawrence *et al.* (1997) looked at [³H]Paroxetine binding in ten brain regions in thirteen clinically depressed suicide victims who were being prescribed antidepressants and compared this to 'healthy' control cadavers. They found no difference in nine brain regions, and a lower number of binding sites in the putamen of the suicide victims. This corresponds well to previous research by the same group suggesting that there is no difference in the Bmax values, assessed using platelet membranes, of depressed and control participants (Lawrence *et al.*, 1993).

The non-tricyclic selective serotonin reuptake inhibitors (including Paroxetine and Citalopram) are over a thousand times more selective for serotonin-uptake inhibition than noradrenalin-uptake inhibition (Magnussen *et al.*, 1982). The binding proteins for the tritiated forms of Paroxetine and Citalopram have the same molecular weight (Plenge *et al.*, 1990), making them suitable for use when investigating binding to serotonin transporters.

The serotonin transporters in humans are encoded by a single gene on the chromosome 17q11.2, and are expressed in both brain and blood platelet cells. Genetic differences have been identified within healthy human participants for the expression and function of serotonin transporters in platelets. The insertion point of the serotonin transporter regions can be in either the long (l) or short (s) forms, and this will affect transporter expression and function. Greenberg *et al.* (1999) found that the long form allele was associated with a rapid initial rate of platelet serotonin uptake (Vmax). This is the inheritable index of platelet serotonin transporter function. They also found that the short form of the allele was dominant. Genotype had no effect on platelet [³H]Paroxetine binding (Bmax).

Therefore, the aim of this chapter was to look for differences in platelet serotonin transporter density in former and current sprint and distance runners, and sedentary controls in order to discover if this is a state or a trait marker. Within the same running discipline (endurance or sprint), current athletes were compared to retired athletes to investigate whether notable

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differences occur due to training. Comparing endurance and sprint running athletes allowed adaptations to specific types of training to be investigated. A sedentary control group provided a baseline 'norm' for comparison.

5.3 Methods

5.3.1 Participants

Thirty three healthy males were recruited to take part in this experiment. They were split into five experimental groups; current international level endurance (5 kilometres to marathon) runners (CE, n = 6), retired international level endurance runners (RE, n = 6), current international level sprint (60 meters to 400 meters) runners (CS, n = 9), retired international level sprint runners (RS, n = 6), and sedentary controls (I, n = 6). Details of the competitive levels of the participants are presented below in *Table 5.1*. Participant anthropometric information is presented in *Table 5.2*.

Table 5.1. Event and personal best time of athlete participants.

CE		RE		CS		RS	
Event	P.B.	Event	P.B.	Event	P.B.	Event	P.B.
Marathon	2:14:30	Marathon	2:10:12	100m	10.18	100m	10.17
Half	1:09:00	Marathon	2:10:48	100m	10.28	200m	20.31
Half	1:06:45	Half	1:05:31	100m	10.51	200m	21.32
10 mile	51:30	10 mile	49:46	100m H	13.53	200m	22.6
5,000m	13:25	5,000m	13:00	200m	20.87	400m	45.22
1,500m	3:45	5,000m	13:40	400m	45.84	400m H	52.5
				400m	46.21		
				400m H	50.2		
				400m H	50.4		

After giving signed informed consent, participants filled out a health screen questionnaire. Exclusion criteria for the study were any known haematological or metabolic disorders, or the consumption of any depression medication. Participants were then asked to complete a questionnaire on basic information which included details such as age, height and body mass

and also their sporting event, their current (and past for retired participants) training activities, highest sporting achievements and the number of years of training for top level competition. Due to restrictions in the availability for sampling of the elite athlete participants recruited to the study, diet and activity prior to the blood sample were not standardised.

5.3.2 Sample preparation

Whilst participants were seated, a single blood sample (40 ml) was taken from the antecubital vein (see Chapter Two for details). The blood sample was immediately mixed with 4 ml of the anticoagulant acid-citrate-dextrose. The mean platelet volume and the platelet count were determined using a haematology analyser as discussed in Chapter Two (Ac.T 5diff, Beckman Coulter. High Wycombe, UK).

Platelets and platelet-rich plasma (PRP) were isolated by centrifugation using a modified version of the original method for platelet isolation by Healy *et al.* (1990). Briefly, the blood samples were left overnight to separate and they were then gently centrifuged for ten minutes at 100 g. The platelet-rich plasma was removed and 500 µl was retained for the analysis of amino acid content in the plasma. The remaining platelet-rich plasma was then centrifuged at 2,500 g for fifteen minutes. Of the platelet-poor plasma (PPP) produced, 500 µl was retained for the analysis of amino acid content and a further 500 µl was centrifuged in an ultraflitrate tube (Whatman International Ltd, Kent, UK) for two hours at 400 g for the analysis of free tryptophan. The ultrafiltrate and the aliquots of plasma were all stored at -80 °C until analysis using HPLC, as detailed in Chapter Two.

The remaining platelet-poor plasma was removed, leaving a pellet of platelets. Very gently, a 3 ml volume of ice cold saline was added to wash the pellet. This was then removed and the wash step repeated once more before the pellet was flash frozen in liquid nitrogen and stored at -80 °C until the day of the assay. The freezing and subsequent thawing of membranes has been shown to have no effect on the Bmax values seen for [³H]Imipramine binding compared to the results obtained when the assay is performed when the

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membranes are fresh, suggesting no detrimental effect of freezing on the binding sites (Lawrence, 1991).

On the day of the assay the platelet pellet was allowed to defrost before 5 ml of ice cold wash buffer (50 mM Tris-HCl, 110 mM NaCl and 20 mM EDTA, pH 6.4) was added. This was then left on ice for five minutes before being homogenised (6 strokes at 800 rpm), then centrifuged at 18,400 g for ten minutes at 4 °C. The supernatant was then discarded and the wash process repeated. After this the platelets were re-suspended in 5 ml of ice cold lysing buffer (5 mM Tris-HCl, 5 mM EDTA, pH 7.4) and homogenised (12 strokes at 1,200 rpm). They were then left on ice for twenty minutes before being centrifuged at 42,000 g for ten minutes at 4 °C. All supernatant was discarded and the membranes were re-suspended in 5 ml of ice cold incubation buffer (50 mM Tris-HCl, 120 mM NaCl and 5 mM KCl, pH 7.5). They were then centrifuged at 42,000 g for ten minutes at 4 °C. All the supernatant was discarded and the membranes were re-suspended in 500 µl of ice cold incubation buffer. Of this, 100 µl was retained and stored at -80 °C for the analysis of the protein content of the sample (Pierce BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA; for details see Chapter Two).

5.3.3 Sample analysis

The assay performed was based on the original method established by Lawrence and colleagues (1993). The membranes were added to glass tubes (30 – 48 µg protein / tube) containing incubation buffer and eight concentrations (10 – 500 pM) of [^{3}H]Paroxetine (specific activity 22.9 Ci / mmol, PerkinElmer, Cambridge, UK) at a total volume of 2 ml per tube. The assay was incubated in a water bath at 23 °C for ninety minutes. Specific binding was defined as the radioactivity displaceable by an excess (1 µM) of a competitor drug (Citalopram hydrobromide, Tocris Bioscience. Bristol, UK).

The incubation period was stopped with the addition of 5 ml of ice cold incubation buffer. Membrane bound radioactivity was recovered by filtration under pressure (Brandel cell harvester, Alpha Biotech Ltd. London, UK)

through Whatman glass-fibre filters (Whatman International Ltd, Kent, UK). Filters were then washed with a further 15 ml of ice cold incubation buffer. Radioactivity was determined by standard liquid scintillation spectroscopy using 6 ml of scintillation cocktail (Gold Star multi-purpose liquid scintillation cocktail, Meridian, Surrey, UK) on a scintillation counter (LKB Wallac1219 scintillation counter, Turku, Finland) with a counting efficiency of 42 – 47 %.

5.3.4 Data analysis

Specific binding was determined from the measured values of non-specific binding subtracted from the total binding. The maximum number of binding sites (Bmax; fmol / mg protein) and the equilibrium dissociation constant (Kd; pM) were determined by non-linear regression analysis fitting to a one site binding model using GraphPad (Prism. San Diego, USA).

5.3.5 Statistical analysis

All analysis was performed on SPSS version 17.0 and statistical significance was set at $P < 0.05$. All data sets were investigated for normality of distribution using the Shapiro-Wilk test and for skewness and kurtosis. A one-way independent ANOVA was then used to investigate differences between groups for all of the measured parameters with the exception of Kd, for which the data was found to be non-parametric and therefore was analysed using a Kruskal-Wallis test. Where appropriate, Bonferroni post hoc tests were performed to compare the data between groups. For the correlation between age and Bmax a one-tailed Spearman's correlation coefficient was performed as the data were non-parametric. All data is presented as the mean ± standard deviation, unless otherwise stated.

5.4 Results

Within the five experimental groups there were differences in age (P = 0.005), body mass (P = 0.002) and BMI (P = 0.027) as shown in *Table 5.2*. As expected, current athletes (CE and CS) were younger than the retired athletes (RE and RS). The current endurance athletes (CE) were also significantly lighter than the current sprint athletes (CS) or the retired sprint athletes (RS). The height of the participants was not significantly different between groups,

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although there was a tendency for sprinters (CS and RS) to be taller (P=0.060).

Table 5.2. Participant Information. * symbolises a difference between the current and retired groups within the same distance discipline (P ≤ 0.005). + symbolises a difference between the retired and current groups in opposite distance disciplines (P ≤ 0.039). ^ symbolises a difference between the distance disciplines within the current groups (P = 0.033).

Group	Age (y)	Height (m)	Body Mass (kg)	BMI (kg / m^2)
CE (n = 6)	26 ± 4*	1.78 ± 0.04	63.8 ± 3.9	20.1 ± 0.8
RE (n = 6)	52 ± 11+	1.77 ± 0.08	74.0 ± 10.6	23.8 ± 4.5
CS (n = 9)	24 ± 4*	1.86 ± 0.06	75.0 ± 7.2^	22.3 ± 1.7
RS (n = 6)	45 ± 13+	1.84 ± 0.05	83.8 ± 5.7+	24.8 ± 1.6+
I (n = 6)	38 ± 16	1.78 ± 0.06	76.9 ± 11.2	24.3 ± 3.3

Figure 5.1 shows a scatter graph of the individual Bmax values for participants in each group. The maximum number of binding sites was significantly greater in the CE group than in any of the other groups (P ≤ 0.008). The Bmax for the CS group was also greater than that of the RS group (P = 0.042). There were no differences between the RE, RS and I groups. Table 5.3 also shows the mean values of Bmax for each group. Figure 5.2 demonstrates an example of saturation binding and the use of total binding and non-specific binding to calculate specific binding values. The equilibrium dissociation constant (Kd) data was not normally distributed in the CE, CS or RS groups. There were no differences in the Kd values between any of the groups (P = 0.182; Table 5.3).
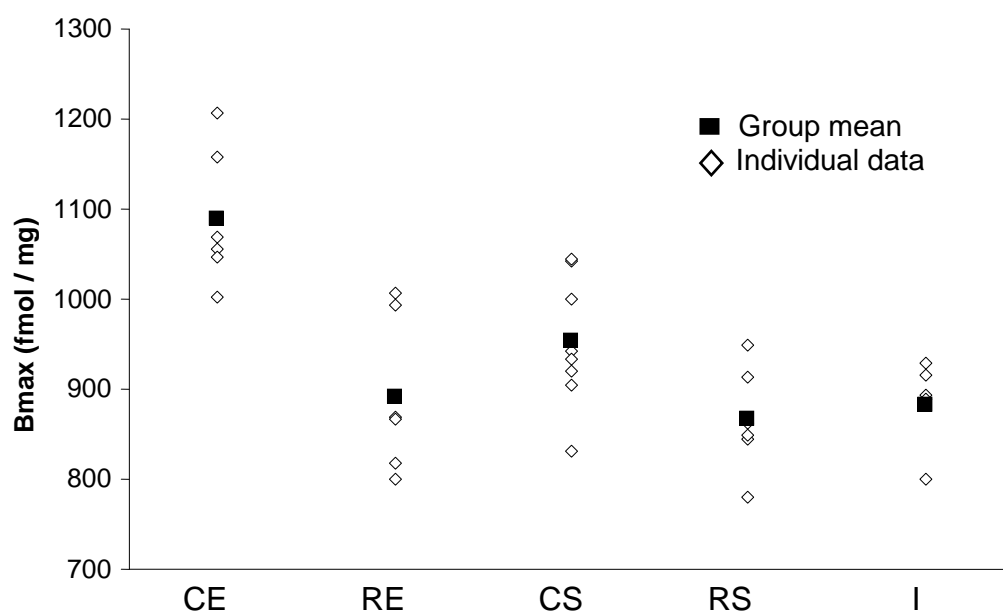
Figure 5.1. Maximum number of binding sites (fmol / mg protein) for each individual in each group, and the mean of each group.

Table 5.3. Average maximum binding capacity and average equilibrium dissociation constant for each group.

	CE	RE	CS	RS	I	TOTAL
Bmax fmol / mg protein Mean ± SD	1090 ± 76	892 ± 88	953 ± 68	866 ± 58	885 ± 58	936 ± 104
Kd pM Median (range)	63 (50–66)	62 (43–70)	61 (45–70)	73 (58–74)	55 (47–76)	62 (43–76)
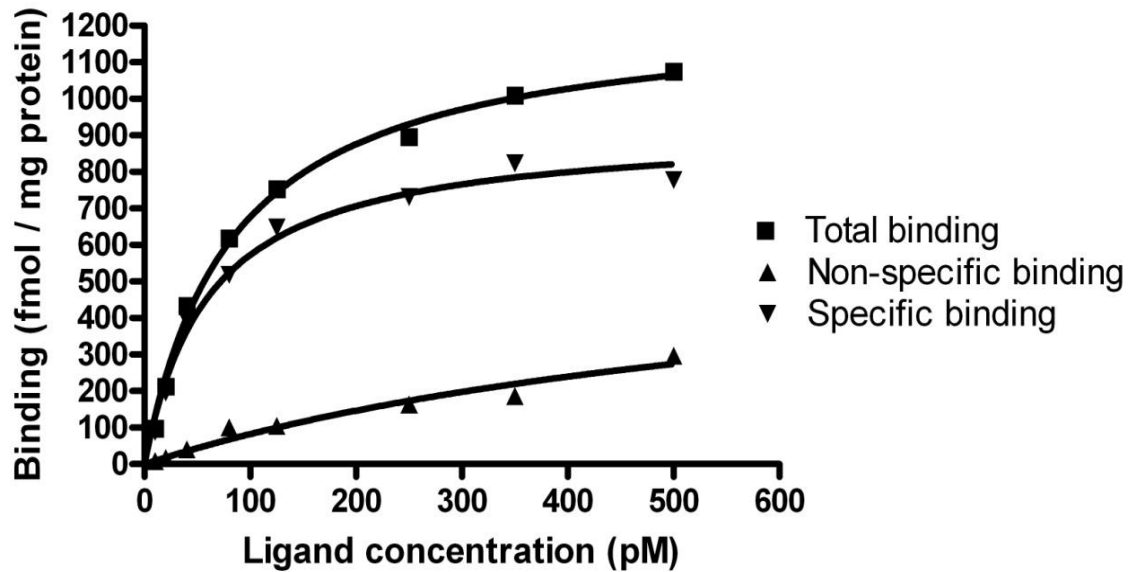
Figure 5.2. An example of the saturation binding of [³H]Paroxetine to platelet membranes in one participant (CE).

There were no differences in the platelet count or in the mean platelet volume between any of the groups. The analysis of the platelet-rich plasma amino acid concentration showed that the only difference between the groups was in the concentration of phenylalanine present. The concentration found in I (201 ± 35 µmol / L) was significantly greater than that found in either CE (108 ± 2 µmol / L; P = 0.031), RE (107 ± 13 µmol / L; P = 0.029) or RS (107 ± 36 µmol / L; P = 0.029), but was not different from the concentration in CS (151 ± 84 µmol / L).

There were no differences between any of the groups for the concentrations of the measured amino acids in the platelet-poor plasma. Furthermore, there were no differences between any of the groups for total branched-chain amino acid concentration in either the platelet-rich or the platelet-poor plasma (*Table 5.4*). The ratio of free tryptophan to branched-chain amino acids analysed using platelet-poor plasma was also not different between groups (*Figure 5.3*).

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Table 5.4. Branched-chain amino acid concentration in platelet-rich and platelet-poor plasma. There were no differences between any of the groups.

	CE	RE	CS	RS	I
PRP [BCAA] (µmol / L) Mean ± SD	431.9 ± 69.4	443.2 ± 74.1	496.9 ± 103.6	464.1 ± 73.6	480.8 ± 90.4
PPP [BCAA] (µmol / L) Mean ± SD	460.0 ± 63.0	481.6 ± 73.3	510.4 ± 109.3	468.1 ± 59.4	455.0 ± 50.8
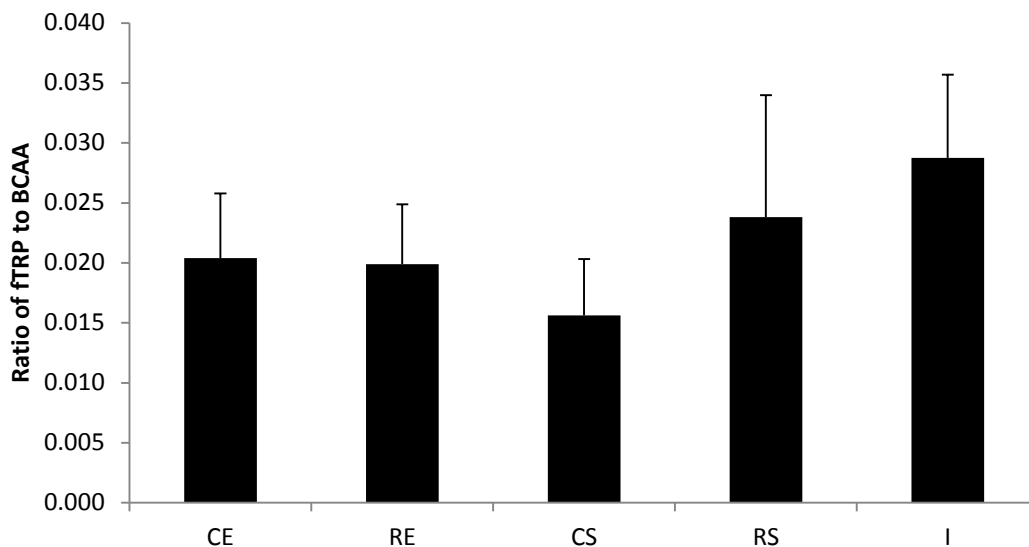
Figure 5.3. The ratio of free tryptophan to branched-chain amino acids in platelet-poor plasma. There were no differences between any of the groups.

5.5 Discussion

The main finding of this investigation was that those who follow an elite level endurance training program, have a greater number of serotonin transporters on their platelet membranes compared to those who used to train to a similar standard in a similar discipline, or in fact, those who have never done any previous training. The influence of endurance activity on the number of binding sites for serotonin is further supported by closer analysis of the data within the RE group. Two of the six retired runners recruited to this study still completed thirty to fifty minutes of endurance running every day and the Bmax

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value for these individuals were greater than the group average. In *Figure 5.1* they are the two individuals in the RE group with Bmax values of approximately 1000 fmol / mg protein compared to the group average of 892 ± 88 fmol / mg protein.

The current activity levels of the RE group as a whole were greater than those of the RS group. In the RS group, the participants were involved in infrequent recreational activity, whereas in the RE group all participants except one reported that they were recreationally active at least four times per week. Indeed the Bmax value for the RE group was higher, and the data are more widely distributed than in the RS group, although this was not a statistically significant difference. Despite the differences in current activity levels between the retired groups, it would appear that being merely recreationally active is not sufficient to generate adaptations in platelet serotonin transporter density.

Regulatory and adaptive mechanisms of controlling serotonin transportation remain largely undefined. It is also yet to be determined whether platelet serotonin transporter density is a state or a trait marker. A state marker was proposed by Rieder (1978) to be an aspect which can adapt under the influence of one or more factors. A trait marker is therefore an aspect which is fixed and cannot be influenced by other factors. Early studies into platelet binding and depression found that maximal [³H]Imipramine binding was significantly lower in those suffering (without treatment) from depression, compared to non-depressed controls (Briley *et al.*, 1980). This led to questions as to whether this lowered binding capacity was a state marker as a result of the depression, or a permanent phenomenon which may predispose certain individuals to suffering from depression, i.e. a trait marker. However, Lawrence *et al.* (1993) subsequently found that there was no difference in the number of platelet serotonin binding sites in depressed patients compared to healthy controls. The same group also found that the administration of antidepressant drugs, whilst altering clinical responses to treatments, did not alter the platelet binding parameters (Lawrence *et al.*, 1994).

Both of these studies suggest that platelet serotonin transporter density is a trait marker and that psychological state does not influence this parameter. However, contrary to this, Biegon and colleagues (1990) found that as patients recovered from depression (using the antidepressant drug Maprotiline) their serotonin receptor binding capacity (assessed using [^{3}H]Ketanserin) fell, compared to their pre-treatment values, suggesting that serotonin binding may be a state marker. Unfortunately, it is difficult to compare data between studies and determine whether platelet serotonin transporter density is a state or a trait marker due to the large variability in results from controls and drug-free depressed patients.

The drug used to determine binding is critical in ensuring that it displaces the radio-labelled drug from the specific binding sites but not from the non-specific sites. Imipramine has been shown to bind to two classes of sites on the human platelet membrane (Ieni *et al.*, 1984), one related to serotonin uptake and one unrelated to serotonin uptake. DMI displaces the [^{3}H]Imipramine from both sites, meaning that it generates inaccurate binding data. Indeed, Lawrence and colleagues (1993) showed that both [^{3}H]Paroxetine binding defined with Citalopram and [^{3}H]Imipramine binding defined with sodium dependence were 20 – 30 % lower than DMI defined [^{3}H]Imipramine binding in blood platelets. This suggests that DMI defined [^{3}H]Imipramine binding may not be a suitably specific test of serotonin transporter density, and therefore calls into question findings using this technique to assess serotonin transporter density. Indeed, the single binding site models for Bmax data has been shown in both animal (Marcusson and Eriksson, 1988) and human (Marcusson *et al.*, 1989) brain tissue to be the most appropriate method of data analysis for [^{3}H]Paroxetine binding and this model was therefore used in the present study.

Strachan (1996) suggested that his findings of elevated levels of serotonin transporters in platelets of endurance trained athletes compared to sedentary controls may represent further support for serotonin transporter density being a trait marker rather than a state marker. He states that the difference in serotonin transporter density between his population of endurance athletes

and sedentary controls may produce different chemical responses in the brain to exercise, possibly explaining why some individuals choose to exercise regularly, while others choose not to do so.

The data presented here would support the concept of serotonin transporter density being a state marker rather than a trait marker. The greater Bmax values of those who are currently international level endurance athletes compared to those who have always been sedentary suggests that serotonin transporter density is a parameter which increases in response to training. However, the lack of any differences in Bmax values between participants who used to be international level endurance athletes and participants who have always been sedentary suggests that the adaptations gained through elite level endurance training are lost once training has stopped.

The Kd values reported in this study (median of 62 pM (range: 43 – 76 pM) are slightly higher than those seen in the publications by Lawrence *et al.* (1993) who reported values of 34 ± 19 pM, Lawrence *et al.* (1994) who reported values of 45 ± 13 pM and Strachan and Maughan (1998) who reported values of 56 ± 28, all of which use the same technique as employed here. Increased Kd is associated with sub-optimal assay conditions such as the incubation temperature and duration (Bylund and Toews, 1993). However, the equilibrium dissociation constant values in the present study are by no means high enough to suggest that there were problems with the assay technique.

The Bmax values reported in this study are also slightly lower than those reported in the other literature employing the same techniques (936 ± 104 fmol / mg protein). Lawrence and colleagues found an average Bmax of 972 ± 246 fmol / mg protein and 1124 ± 286 fmol / mg protein (Lawrence *et al.*, 1993; Lawrence *et al.*, 1994) studying depressed patients and controls, while Strachan and Maughan (1998) found an average Bmax value of 1092 ± 232 fmol / mg protein in endurance athletes and sedentary controls. Further examination of the data presented by Strachan and Maughan (1998) shows that the endurance trained individuals had an average Bmax of 1237 ± 182

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fmol / mg protein and the sedentary controls had an average Bmax of 910 ± 119 fmol / mg protein. The current elite endurance trained participants in this study had a lower average Bmax value (1090 ± 76 fmol / mg protein), and the sedentary controls in this study also had a lower Bmax average (885 ± 45 fmol / mg protein) than those in the Strachan and Maughan (1998) study.

Platelets consist of approximately 60 % membrane protein and 40 % cytoplasmic protein (Barber and Jamieson, 1970). The results of the binding assay are expressed on the basis of membranous protein. Therefore, if during the preparatory stages of the assay there are some platelets left intact, which it has been suggested can occur (Friedl *et al.*, 1983), the amount of protein present in the samples will be artificially elevated. This would result in a lower level of binding when expressed relative to the protein present in the assay and it is impossible to say whether this is one of the reasons for the lower binding values seen in this study. It is interesting to note, however, that the variance of the data in this study is much smaller than in either of the papers by Lawrence and colleagues (1993; 1994) or that by Strachan and Maughan (1998). It is likely that this could be attributed to the relatively homogeneous population recruited to the present study.

The sedentary group (I) was included to act as a control group since before recruitment began it was expected that there would be a broad range of age and body mass between the athlete and retired athlete participants. The ages in the athlete groups spanned from 20 years to 69 years and the ages in the sedentary group spanned from 22 years to 61 years. [^{3}H]Imipramine binding in human platelet membranes has been shown to decrease with age (Langer *et al.*, 1980). Indeed, there was a significant correlation between age and Bmax values (P = 0.028, r = -0.458) in the participants, and therefore a large span of ages in the control group was essential.

The mass of the participants in the athlete groups spanned from 57 kg to 95 kg while in the sedentary group the mass spanned from 63 kg to 89 kg. The broad spectrum of values and the lack of differences between I and any of the

other groups for any parameters shows that this group provide a suitable control and comparison for the athlete groups.

In the present study there was no difference between groups in the number of platelets or the mean volume of the platelets in the samples. Blood platelets have been shown to vary depending upon their size and their age; younger platelets are generally larger, denser and more metabolically active (Corash *et al.*, 1977), although the relationship between age and size has since been called into question (Thompson *et al.*, 1983). Exercise has been shown to increase the platelet count by stimulating release of platelets from the store in the spleen, although the platelets released are believed to have similar properties to those in the circulation (Chamberlain *et al.*, 1990). Either way, in the current study there were no differences in the mean platelet volumes between groups and therefore this is unlikely to be a confounding factor in the Bmax data reported here.

All the participants in this study were Caucasian, with the exception of three participants in the CS group. If these participants are removed from the analysis there is a negligible change in the mean Bmax of the group (953 ± 68 fmol / mg protein (n=9) and 958 ± 53 fmol / mg protein (n=6)). Therefore, based on this small sample it would seem that ethnicity does not influence the maximum number of serotonin binding sites found on the blood platelet membrane. As far as the author is aware there is no other research available on this aspect of serotonin transport and therefore further investigation would be required to confirm this.

Due to a severely limited number of opportunities to collect samples from the elite athletes recruited to this study, it was impossible to take their blood samples with any standardisation. Samples were taken between 1000 and 2000 hours in the months of January, February or March. Exercise and diet were also not standardised prior to sampling. It has been suggested that in control participants and depressed patients, the binding of [3H]Paroxetine is significantly increased in the Spring months (March 21st – June 20th) than in the Summer, Autumn or Winter months (D'Hondt *et al.*, 1994). All of the

samples in the present study were taken during the Winter months and therefore it can be assumed that seasonal effects are not the cause of the differences seen in binding data.

Several of the athlete participants recruited to this study were sampled after training or racing. Prior strenuous exercise has been shown to result in platelet activation and hyperreactivity in sedentary individuals, but this finding is not reflected in physically active participants (Kestin *et al.*, 1993). Whether such heightened activation and reactivity would influence the binding of [^{3}H]Paroxetine to the platelet membrane remains to be seen. However, none of the sedentary participants, by their very nature, had taken part in any physical exercise prior to the sampling process and therefore this is unlikely to be a confounding factor in the present study.

Although the plasma amino acid concentrations showed no differences between groups for any of the amino acids analysed other than phenylalanine, this is possibly not a true reflection of the resting situation in these participants. Strachan and Maughan (1998) found that, at rest after an overnight fast, the plasma concentrations of tyrosine and tryptophan were lower in their endurance trained participants than in their sedentary participants. They hypothesised that these differences may be due to exercise induced increases in peripheral utilisation and metabolism of these amino acids (Chaouloff, *et al.*, 1985). The lack of a dietary standardisation prior to sampling in the current study means that it is impossible to determine if true resting differences do indeed exist between the groups of participants tested here.

A possible avenue for future research would be to attempt to elucidate the role of Vmax, the maximum rate of binding, within the blood platelet. As suggested before, a reduction in the concentration of serotonin in the synapse could be achieved by either an increased number of serotonin transporters, an increase in their activity levels, or a combination of these two factors. This study has investigated the role of the number of transporters present and therefore a logical progression would be to examine the activity levels of those

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transporters. An alternative route for future investigations would be to conduct a training study with the aim of elucidating the time course for increasing serotonin transporter density.

5.6 Conclusion

In conclusion, this study shows that those who are currently participating in an elite level endurance running training regimen have a greater number of serotonin transporter sites on the membranes of their blood platelets compared to those who have retired from elite endurance running competition. Current endurance athletes also have a greater number of serotonin transporters than those training for elite level sprint running events, those who used to train for elite level sprint running events and those who have never trained for any such activity. Athletes participating in elite level sprint running also have a greater number of serotonin binding sites on the membranes of their blood platelets than those who have retired from such training and competition.

Blood platelets have been shown to be a suitable peripheral model for the serotonergic neurones in the brain. Therefore, the data presented here suggest that training, particularly for endurance events, may increase the number of serotonin transporters on the serotonergic neuron of the brain, aiding serotonin reuptake, and possibly altering the perception of fatigue.

CHAPTER SIX

Responses to the application of 1 % menthol solution to the skin at rest in a warm environment.

6.1 Abstract

Menthol is a compound that activates cutaneous cold receptors without actually cooling the skin. The physiological and psychophysical effects of applying menthol to the skin while resting in a warm environment have not been investigated. Therefore, the aim of this investigation was to determine the response to the application of menthol while at rest in a warm environment.

Twenty four healthy male participants sat for thirty minutes in 32.5 ± 0.2 °C and 66 ± 3% relative humidity before patches soaked in a 1 % menthol solution (Menthol) of a control solution (Control) were applied to the back, forearms and forehead. They then remained seated for a further eighty minutes. During the trials, core, skin and arm temperatures, heart rate, perceived local (forearm) and overall thermal sensation, together with overall thermal comfort ratings, were taken every five minutes and skin blood flow was measured every ten minutes.

Thirteen participants perceived the Menthol patches to have a cooling effect, eight participants perceived a warming effect and three participants perceived no effect. Applying either the Menthol or the Control solution had no effect on arm temperature (P = 0.133), and there was no difference in the arm temperature between those who felt a warming effect and those who felt a cooling effect (P = 0.267). No differences were found between the two treatments or sensation groups for any of the physiological parameters measured. For local and overall thermal sensation and overall thermal comfort, there were no differences between the two treatments, although there were differences for these parameters between the sensation groups (P ≤ 0.004). These data suggest that while skin temperature receptors were activated by the application of menthol to the skin, no physiological changes occurred.

6.2 Introduction

Menthol is a plant extract that is widely used for many medicinal and cosmetic purposes due to the cooling sensation it elicits when applied to the skin or mucous membrane. Cool sensations can be caused by either the stimulation of cold receptors or the inhibition of warm receptors while conversely, warm sensations can be caused by either the stimulation of warm receptors or the inhibition of cold receptors (Eccles, 2000). Therefore, the application of menthol to the skin may result in a cooling or a warming sensation without a concomitant alteration in actual skin temperature.

Since the application of menthol to the skin does not elicit its effects through either evaporation from the skin nor any form of localised vasodilation or vasoconstriction, it was deemed, as far back as the nineteenth century by Goldsheider (1886) that the warming and cooling sensations were caused by stimulation of sensory nerve endings. It is now known that menthol exerts its effects on cold receptors by interfering with the movement of calcium across the cell membrane in thermoreceptors (Eccles, 1994). However, the mechanisms of the actions of menthol are more complex than a simple stimulation of cold receptors, since menthol also modulates feelings of warmth, and in high concentrations can cause irritation and local anaesthesia (Eccles, 1994).

Research on the dose-response characteristics of menthol is sparse and due to the different vehicles employed to apply it, comparisons between studies are difficult to draw. Equally sparse is information on the effects of menthol during exposure to different ambient and skin temperatures and, once again, the data are not easily comparable.

Existing research on topically applied menthol has predominantly been carried out in temperate environments of 18 - 23 °C (Yosipovitch *et al.*, 1996; Kozyreva and Tkachenko, 2008). It is probable however, that actual skin temperature plays a greater role in the perception of skin temperature after an application of menthol than the ambient temperature. Kozyreva and Tkachenko (2008) used participants with a forearm skin temperature of 32.3 ±

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0.11 °C (30.5 – 33.7 °C) to study skin sensitivity to the application of 1 % menthol. They found that there are individual differences in the sensitivity of skin receptors to menthol, with some people showing an increase in the number of functioning cold receptors, while others have a decrease and some people show no change. Indeed, this goes some way to explaining the findings of Yosipovitch and colleagues (1996) who showed that only around two thirds of people are sensitive to the cooling effects of menthol in temperate environments. However, they fail to state the skin temperature at which they found this.

Green (1992) maintained skin temperature of participants at 31 °C and found that the application of 5 % or 10 % menthol to the skin had an excitatory effect. Green (1992) also showed that at lower skin temperatures (21 °C) menthol solutions had a proportionally smaller effect on the perceived intensity of cooling than at 29 °C compared to a 0 % menthol control solution. However, he also showed that for all of the solutions, the lower the skin temperature the greater the intensity of the cooling stimulus. These findings were mirrored with higher skin temperatures, where the proportional intensity of the warming sensation of menthol was reduced as skin temperature increased from 33 °C to 41 °C, and, as skin temperature increased so did the perceived intensity of the sensation.

Watson *et al.* (1978) showed that the sensitivity of the skin to the application of menthol displayed a non-uniform response. In different body surface areas there are varying densities of thermoreceptors. Watson and colleagues (1978) showed that the eyes and the buccal region were most sensitive to the application of menthol, while the palms and soles were least sensitive. However, Nakamura *et al.* (2008) suggest that the preferential cooling of the head during heat exposure and maintaining the warmth of the core during cold exposure, could not be solely explained by the distribution of peripheral thermoreceptors, thereby implicating processing mechanisms within the central nervous system.

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The possibility that the central nervous system is involved in thermal comfort over and above simply receiving inputs from the peripheral thermoreceptors may have implications for heat exposure and exercise performance in warm environments. However, due to the lack of data on the effects of menthol on the skin in the heat, the aim of this study was to elucidate the physiological and psychophysical responses to the application of a 1 % menthol solution to the skin whilst resting in the heat. The results of this chapter will inform the study design of Chapter Seven.

6.3 Methods

6.3.1 Participants

Twenty four healthy male recreationally active and non-heat acclimatised volunteers participated in this study. The physical characteristics of the participants were (mean ± standard deviation): age 25 ± 5 y, height 1.80 ± 0.09 m and body mass 79.7 ± 9.9 kg.

6.3.2 Experimental protocol

Participants undertook one preliminary trial and two experimental trials. The experimental trials were performed at least seven days apart and at the same time of day, in a single-blinded, randomised, counter-balanced, cross over design.

The preliminary trial was completed at least twenty four hours before the first main trial. It involved participants sitting in a comfortable environment and having a patch soaked in the 1 % menthol solution (see Chapter Two for details) applied to the skin of their right forearm. This remained in place for twenty minutes. The preliminary test was a precautionary one, undertaken simply to ensure that there was no adverse reaction to the patches or the menthol solution used in the main trials. No other measures were taken during this time.

Briefly, the experimental trials involved a period of resting heat exposure, during which patches containing a 1 % menthol solution or a 0 % control solution were applied to the skin of the forehead, the upper back and the

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forearms (see Chapter Two for details). The physiological and psychophysical responses to the heat exposure were monitored before and after the application of the patches. On one occasion all of the patches applied contained the 0 % control solution (Control). On the other occasion all of the patches contained the 1 % menthol solution (Menthol) except the one which was applied to the left forearm. The patch that did not contain the menthol solution (applied to the left forearm) instead contained the control solution, to allow the direct comparison of the two solutions on the same day. Participants were blinded to the different solutions used in the forearm patches during the Menthol trial, as well as to the trial order.

A schematic of the protocol employed is presented in *Figure 6.1*. Details of the pre-trial conditions and analytical techniques used are given in Chapter Two. Participants reported to the laboratory in the morning at either 0600 or 0900 hours. After arriving, participants emptied their bladder and a sample was retained (see Chapter Two for details). Post-void, nude body mass was noted and the participants then positioned a rectal thermometer to allow the measurement of core body temperature. A heart rate telemetry band was positioned and skin temperature was measured at four sites (chest, triceps, thigh and calf) for the calculation of weighted mean skin temperature using an infrared laser thermometer (see Chapter Two for details). Left and right forearm temperatures were also measured slightly distally of where the patches were to be placed. Participants wore only shorts and shoes for the trials. Finally, before entering the chamber, participants were asked to complete the subjective feelings questionnaire made up of four visual analogue scales (see Appendix D). The first two scales referred to the thermal sensation of the right and left forearms (local thermal sensation). The third scale related to the overall thermal sensation of the body and the final scale referred to the overall thermal comfort of the body.
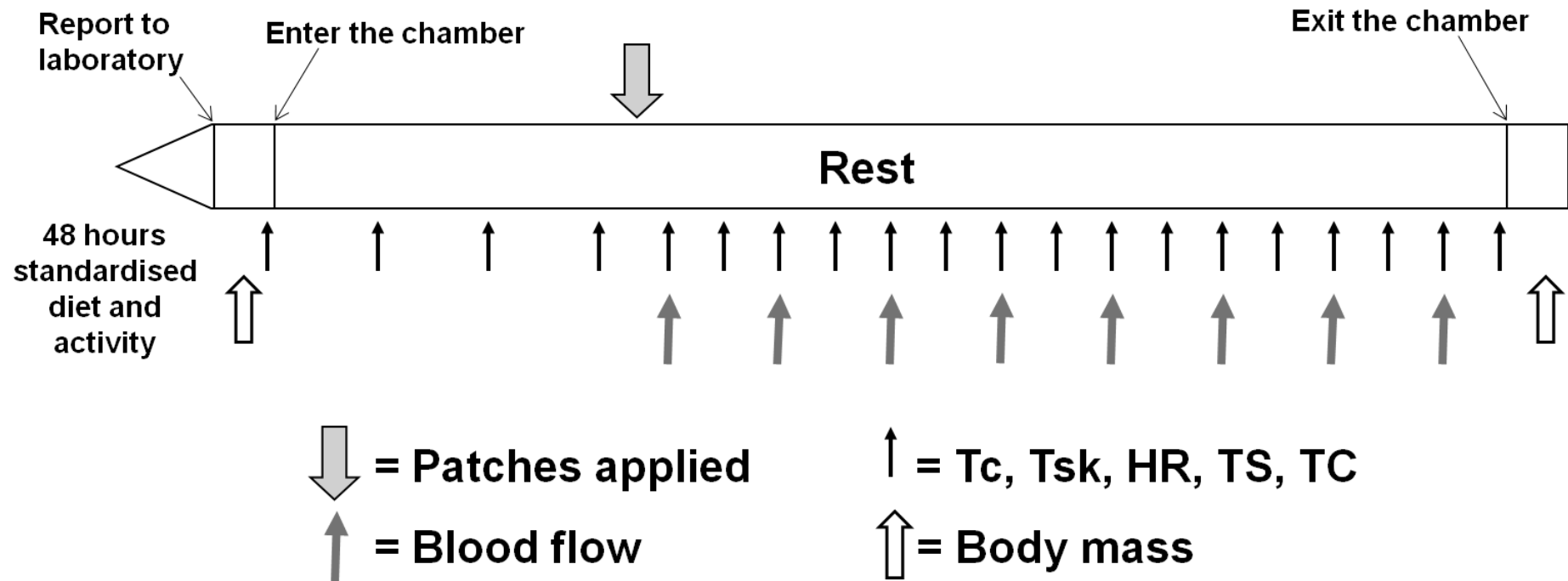
Upon entering the climatic chamber, maintained at 32.5 ± 0.2 °C, 66 ± 3 % relative humidity and 0.7 ± 0.1 m / s wind speed, participants were seated. Participants sat for thirty minutes and measures of core temperature, skin temperature, heart rate and subjective feelings were taken every ten minutes. After thirty minutes skin blood flow on each forearm was also measured (see Chapter Two for details). After this, the patches containing the solutions appropriate to the trial being completed were applied to the skin. Participants remained seated in the chamber for a further eighty minutes. Every five minutes after the patches had been positioned measures of core temperature, skin temperature, heart rate and subjective feelings were taken. Skin blood flow was taken every ten minutes from minute five (see Chapter Two for details). At the end of the test period participants left the heat chamber, removed the patches, heart rate monitor and the rectal probe and were again weighed nude. They were then given food and beverages and were allowed to leave the laboratory.

6.3.3 Statistical analysis

All data sets were investigated for normality of distribution using the Shapiro-Wilk test, and for skewness and kurtosis. Where the data were analysed within participants, i.e. between the two conditions, paired t-tests or repeated measures ANOVA were used. Where the data were analysed between individuals, i.e. between those who perceived a warming effect and those who perceived a cooling effect, independent samples t-tests were used, or a repeated measures ANOVA mixed design where appropriate. Correlations were analysed using a Pearson's product-moment correlation coefficient. In this part of the analysis those who perceived no effect of the treatment were omitted from the investigation. All analysis was performed on SPSS version 17.0 and statistical significance was set at $P < 0.05$.

6.4 Results

6.4.1 Menthol *vs.* Control

The data from all participants was included in the following analysis (n = 24). Urine osmolality was not different between the two treatments (P = 0.784), showing that participants were in the same hydration state on both trials (667

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± 244 mosmol / L for the Menthol trial and 680 ± 248 mosmol / L for the Control trial).

The main finding of this aspect of the study was there appeared to be no physiological effects of applying menthol to the skin. Forearm skin temperature was not affected by the Menthol compared to the Control (P = 0.133), although it did increase significantly (P < 0.001) upon entering the chamber from 31.1 ± 0.7 °C to 34.6 ± 0.5 °C by Pre20; it then fell very slowly over the trial to 34.2 ± 0.5 °C at the end of the trial. For the mean skin temperature there was no difference between the two treatments (P = 0.097), but it did change over time (P = 0.004) from 30.4 ± 0.6 °C to 33.9 ± 0.5 °C during the initial thirty minutes in the heat chamber; it then slowly fell to 33.4 ± 0.4 °C by the end of the trials. Core temperature also did not differ with treatment (P = 0.172) but did show an effect of time (P = 0.001), rising upon entering the chamber (from 37.00 ± 0.31 °C to 37.04 ± 0.30 °C), falling before the application of the patches (37.02 ± 0.29 °C) and then rising again very slowly to the end of the trials (37.11 ± 0.26 °C; *Figure 6.2*).
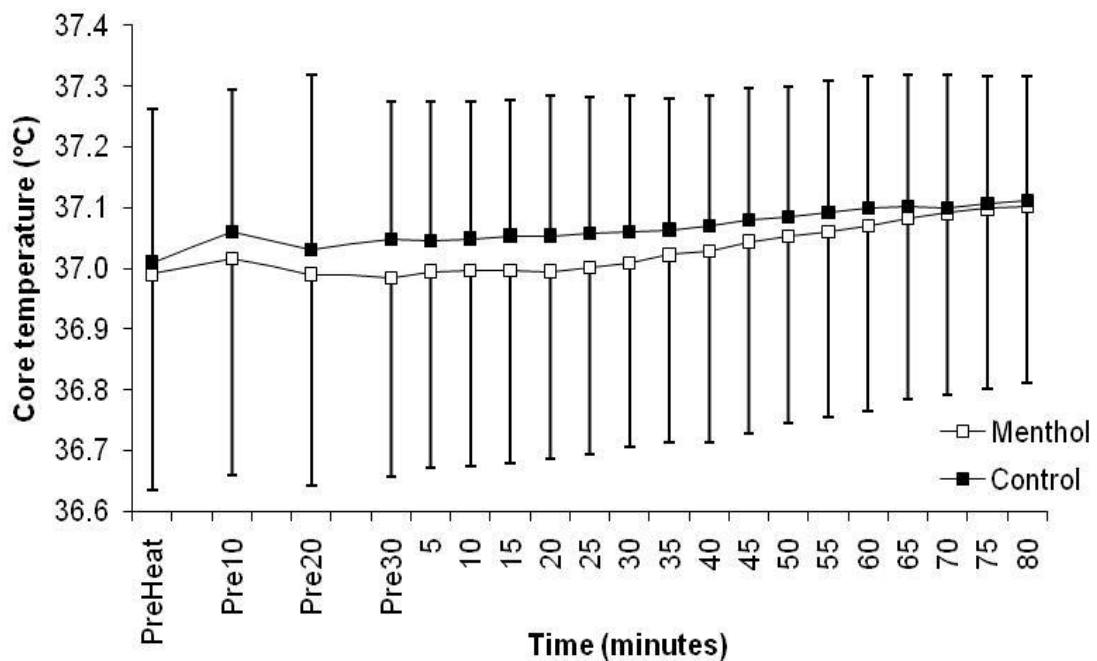
Figure 6.2. Core temperature over the duration of the Menthol and Control trials.

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Body mass loss was not different between the two treatments (0.23 ± 0.11 kg and 0.26 ± 0.11 kg for the Menthol and Control trials respectively; P = 0.175). Heart rate did not differ between treatments (P = 0.219) nor over time (P = 0.415; *Figure 6.3*). The percentage changes in skin blood flow between the Menthol trial and the Control trial were also not different between treatments (P = 0.448), nor over time (P = 0.059).
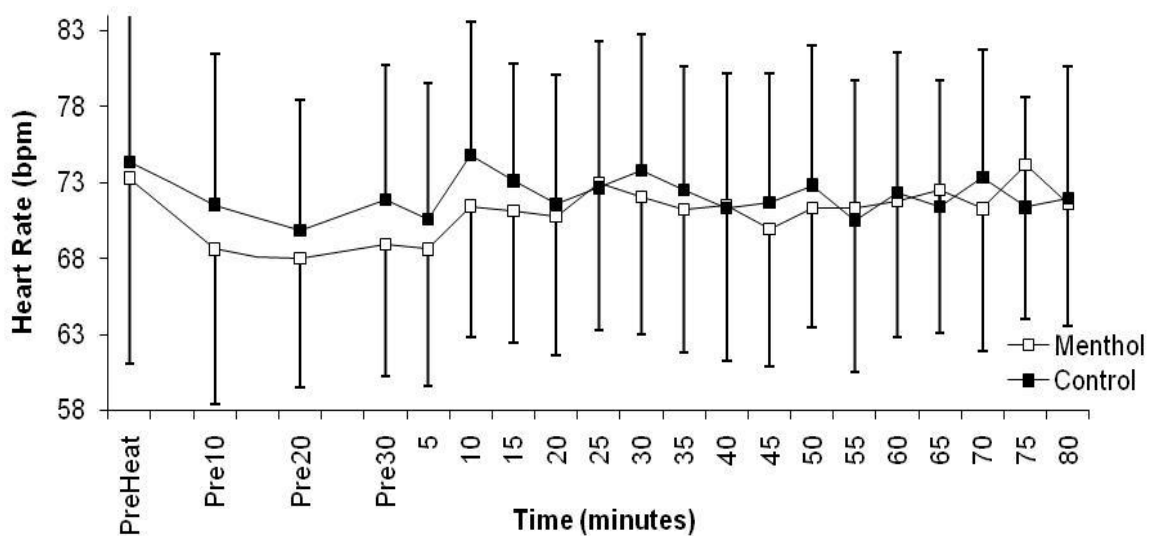
Figure 6.3. Heart rate over the duration of the Menthol and Control trials.

When comparing the Menthol and the Control trials there was no difference in the temperature sensation of the arms (P = 0.789), nor was there an influence of time (P = 0.107). Overall thermal sensation was not different between treatments (P = 0.068), but was affected by time, increasing during the first ten minutes of heat exposure in the chamber (P < 0.001; *Figure 6.4*). Overall thermal comfort followed the same pattern; it was not different between treatments (P = 0.231) but there was a shift towards being too hot within the first ten minutes of heat exposure (P < 0.001).
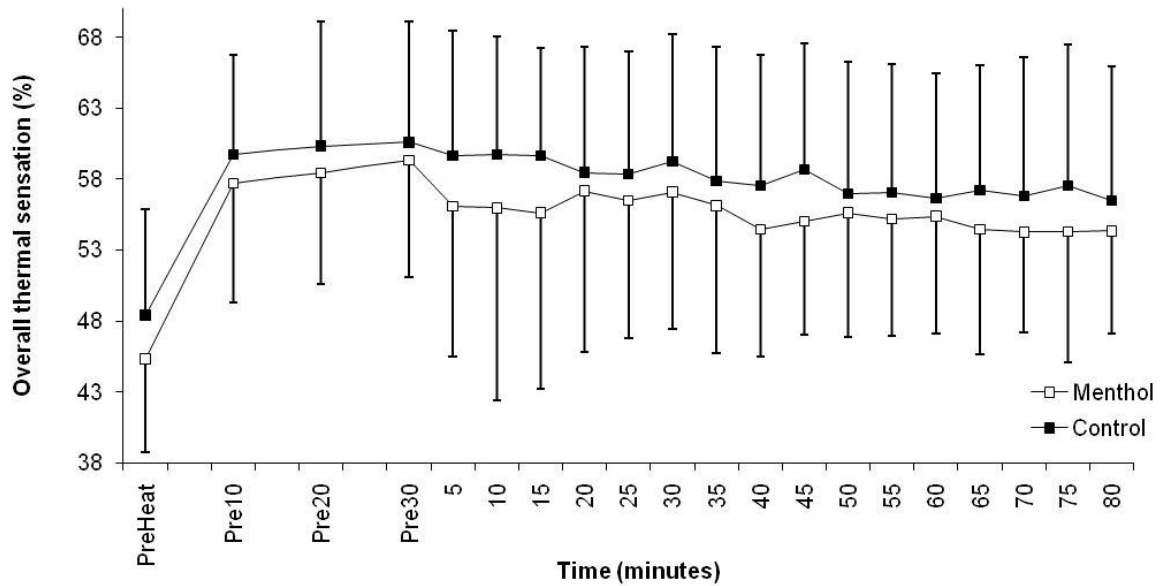
Figure 6.4. Overall thermal sensation over the duration of the Menthol and Control trials.

6.4.2 Warming *vs.* cooling

The greatest difference for any individual at any time point between the arm thermal sensation scores from the start of the trial to immediately before the patches were applied was 2 %. Therefore, to interpret thermal sensation data in a conservative manner it was deemed that a difference of 5 % or more was a clear indication of a variation in perception and not merely a careless error in reporting.

From the data gathered on the Menthol trial, participants were retrospectively grouped according to their perception on the thermal effects of the menthol. If their right arm (1 % menthol solution) thermal sensation score was 5 % or more below their left arm (control solution) thermal sensation score, at the same time point within ten minutes of the patch application, they were classified as perceiving a cooling effect. Equally, if their right arm thermal sensation score was 5 % or more above that of their left arm at the same time point within ten minutes of the patch application, they were classified as perceiving a warming effect. Of the twenty four participants recruited to the study; thirteen perceived that the patches elicited a cooling effect; eight felt that the patches warmed the skin; and three did not perceive any difference

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compared to the control patch. All subsequent analysis has omitted the participants who perceived no effect of the menthol (n = 3). The details of the participants in the warming and cooling groups are presented in *Table 6.1*.

Table 6.1. Participant information grouped by local thermal sensation ten minutes after the application of menthol patches, and the difference between the groups.

	Body mass (kg)	Height (m)	Age (y)
Cooling	77.6 ± 7.0	1.77 ± 0.07	24 ± 3
Warming	83.3 ± 11.4	1.84 ± 0.11	26 ± 6
P value	0.156	0.115	0.623

Figure 6.5 shows the difference in the local thermal sensation between the right arm (menthol patch) and the left arm (control patch) over the duration of the trial, divided into those who felt a cooling sensation (n = 13) and those who felt a warming sensation (n=8). There was a significant difference between the groups over the trial (P < 0.001). Fifteen minutes after the menthol was applied the cooling sensation group perceived their right arm (1 % menthol solution) to be 12 ± 7 % cooler than their left (control solution), while the warming sensation group perceived their right arm to be 16 ± 10 % warmer than their left, although this was not significantly affected by time (P = 0.076).

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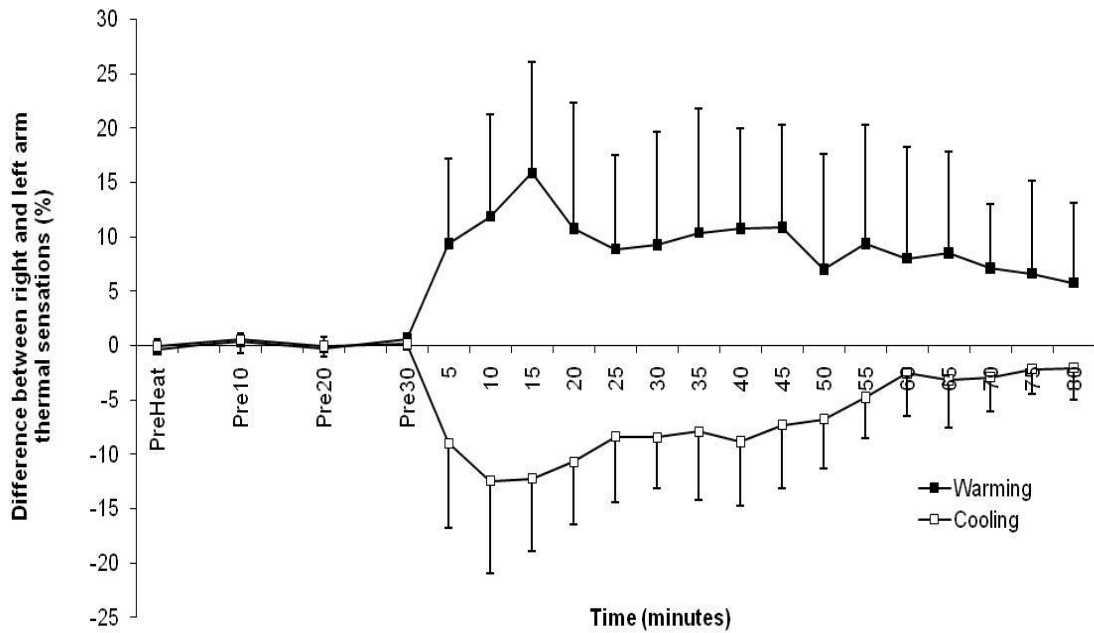
Figure 6.5. The difference in thermal sensation scores on the Menthol trial between the right arm (1 % menthol solution) and the left arm (control solution), leading to the grouping of participants into a group who perceive a warming sensation (n = 8) and a group who perceived a cooling sensation (n = 13).

The local thermal sensation scores also reflected the overall thermal sensation scores (P = 0.004; *Figure 6.6*), and this was likewise significantly influenced by time (P < 0.001). This difference between the groups was greatest ten minutes after the application of the menthol, where the cooling group had a mean score of 49 ± 10 % and the warming group had a score of 67 ± 13 %. Thermal comfort data mirrored that of thermal sensation, with a significant difference between those who felt a local warming effect and those who felt a local cooling effect (P = 0.004). Again, this also demonstrated a significant effect of time (P < 0.001) with the greatest difference between the two groups occurring after ten minutes (52 ± 9 % and 67 ± 11 %, cooling and warming respectively; *Figure 6.7*).

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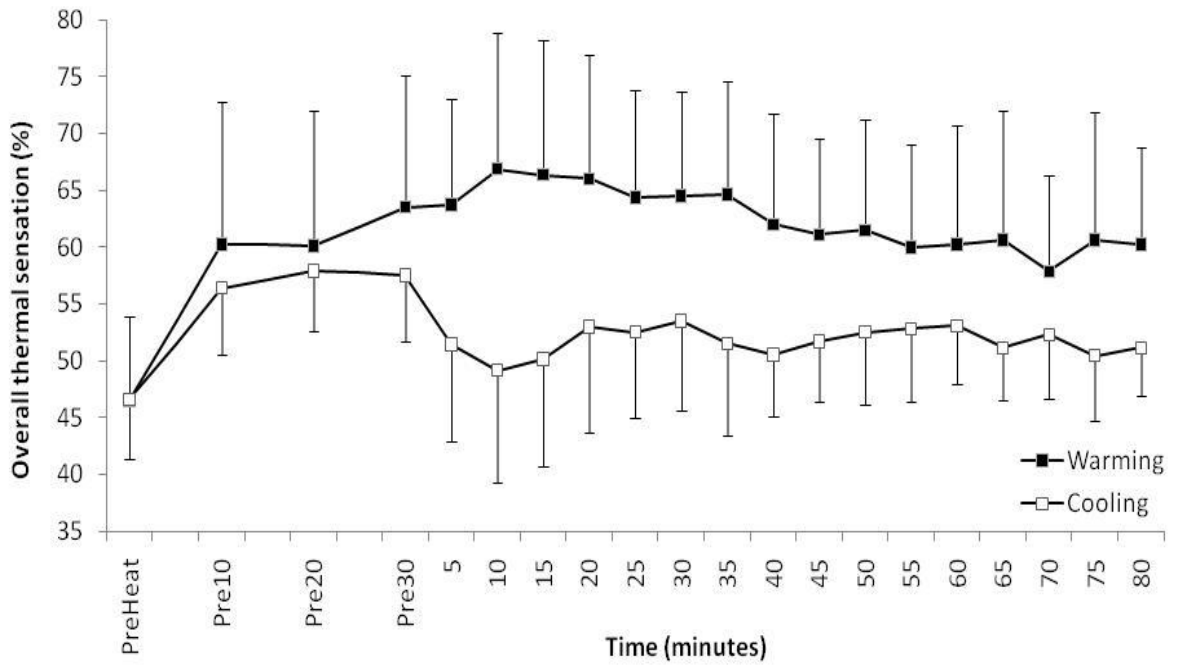
Figure 6.6. Overall thermal sensation of participants grouped according to local thermal sensation ratings.
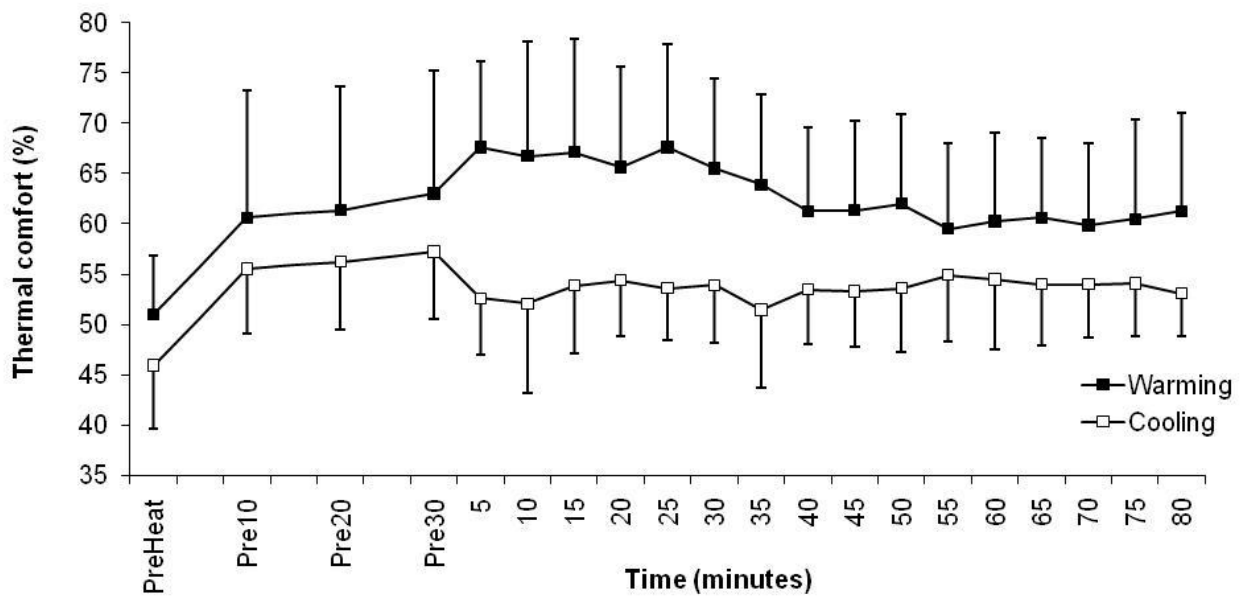
Figure 6.7. Overall thermal comfort of participants grouped according to local thermal sensation ratings.

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There was no difference in body mass index between those who perceived the patches as warming (25.2 ± 2.9 kg / m^2) and those who perceived them as cooling (24.4 ± 3.7 kg / m^2; P = 0.607). Similarly, the body mass loss did not differ between the warming group (0.28 ± 0.12 kg) and the cooling group (0.22 ± 0.11 kg; P = 0.299). Percentage change in blood flow did not alter over time (P = 0.462) nor was it affected by the local thermal sensation of the participants (P = 0.072). Heart rate showed a significant effect of time (P = 0.045; *Figure 6.8*) but did not differ with sensation (P = 0.223).
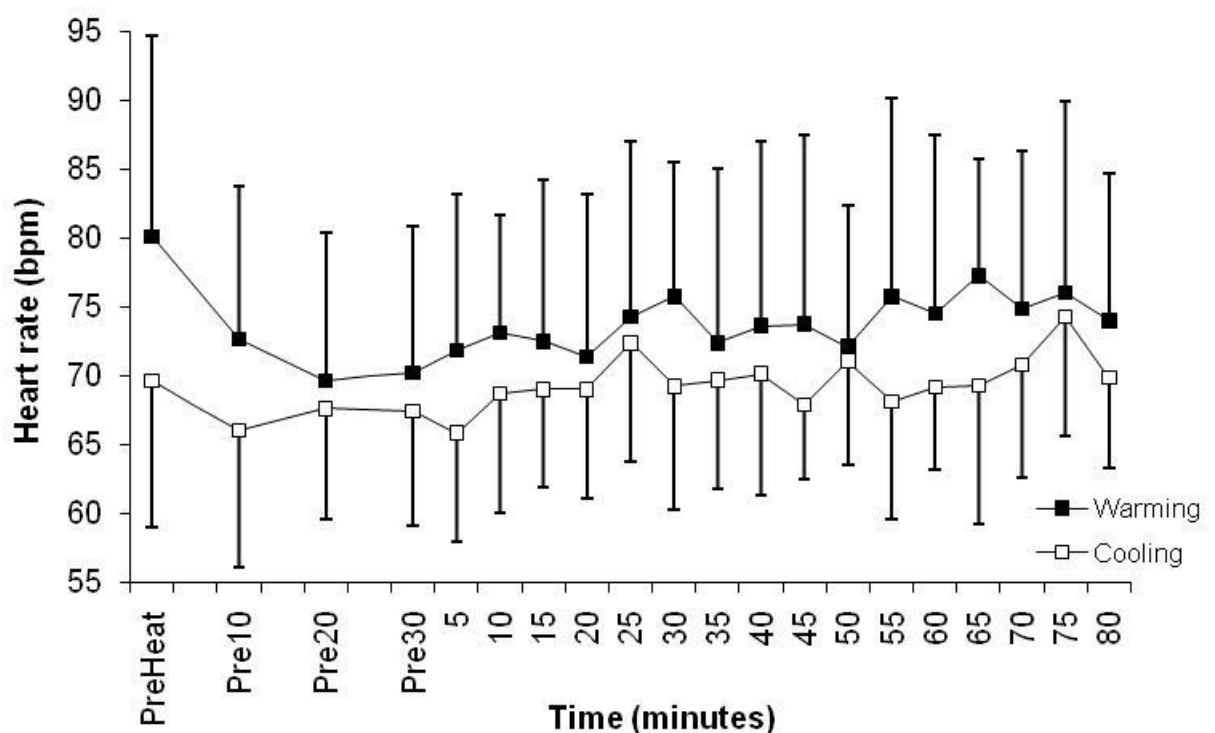
Figure 6.8. Heart rate of participants over the Menthol trials, split into groups depending upon their perceived local thermal sensation.

There was no difference in arm temperature between the two groups (P = 0.267). There was a significant effect of time as arm temperature increased for the initial twenty minutes of heat exposure from 31.1 ± 0.9 °C to 34.5 ± 0.7 °C in the cooling group, and 31.2 ± 0.6 °C to 34.7 ± 0.4 °C in the warming group (*Figure 6.9*). Core temperature and mean skin temperature also changed significantly over time (P < 0.001 for both); both increased upon entering the heat chamber. Skin temperature then fell slowly over the trials;

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core temperature increased very slightly over the trials. There was no difference between the groups for either core temperature ($P = 0.486$) or skin temperature ($P = 0.983$).
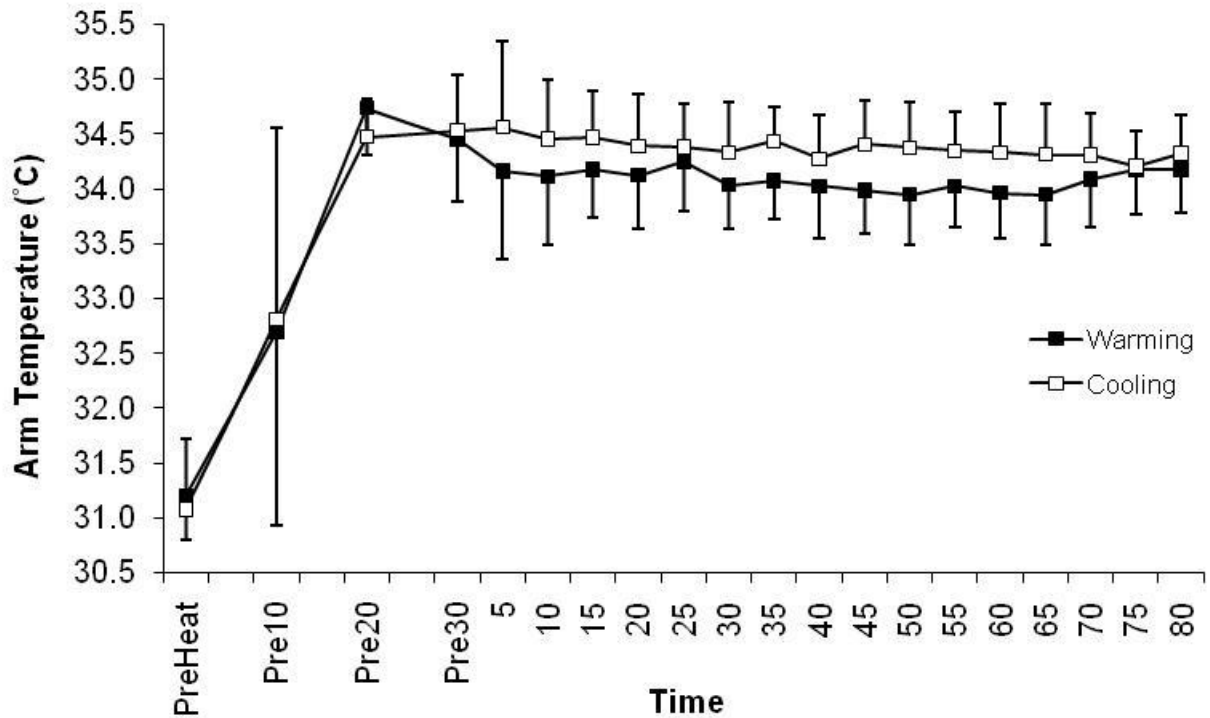
Figure 6.9. Arm temperature of participants over the Menthol trials, grouped depending upon their perceived local thermal sensation.

6.4.3 Sensation *vs.* treatment

The two treatments (Menthol and Control) have been compared to each other. The participants, having been split into two groups, depending upon their local perception of thermal sensation in response to the application of menthol, have also been compared to each other. The final comparisons to be drawn are those within the same participant between the two treatments. As before, those who perceived no difference in local thermal sensation between the Menthol and the Control patches have been omitted from this analysis.

For those who perceived a warming effect ($n = 8$) and for those who perceived a cooling effect ($n = 13$), there was no difference between the two treatments for body mass loss (0.22 ± 0.11 kg Menthol and 0.23 ± 0.10 kg Control in the

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cooling group, P = 0.598; 0.28 ± 0.12 kg Menthol and 0.31 ± 0.11 kg Control in the warming group, P = 0.423). Heart rate was not different between trials (P = 0.219, P = 0.753) nor over time (P = 0.062, P = 0.13) for cooling and warming groups respectively. There were also no changes in skin blood flow between trials (P = 0.959, P = 0.148) nor over time (P = 0.26, P = 0.136) for the cooling or the warming groups respectively.

The core temperature of those in the cooling group showed no difference between treatments (P = 0.237) and no change over time (P = 0.100). Those in the warming group showed no difference between treatments (P = 0.440) but there was an increase in core temperature over time (P = 0.010). Mean skin temperature did not differ between trials for the cooling (P = 0.395) or the warming group (P = 0.619), but both groups showed an increase over time (P < 0.001). The same pattern was seen for arm temperatures, where there was an increase over time for both groups (P < 0.001), but no difference between the treatments (P = 0.236 for the cooling group and P = 0.299 for the warming group).

For the local thermal sensation, the cooling group showed a significant difference between the treatments (39 ± 10 % Menthol treatment and 53 ± 8 % Control treatment at fifteen minutes; P < 0.001), with a significant influence of time (P = 0.002). The same was seen for the warming group, the local thermal sensation between the two treatments was different (74 ± 14 % Menthol treatment and 59 ± 7 % Control treatment at fifteen minutes; P = 0.015) and there was also an effect of time (P = 0.001; *Figure 6.10*). Overall thermal sensation in the warming group did change with time (P = 0.001), but did not differ between the two treatments (P = 0.887). However, in the cooling group both time (P = 0.02) and treatment (P = 0.02) affected overall thermal sensation (*Figure 6.11*). The same pattern for overall thermal sensation is mirrored in overall thermal comfort measures (*Figure 6.12*). In the warming group there is no effect of the treatment (P = 0.256), but there was an effect of time (P = 0.003), whereas in the cooling group the data were significantly affected by the treatment (P = 0.007) and by the time (P = 0.035).
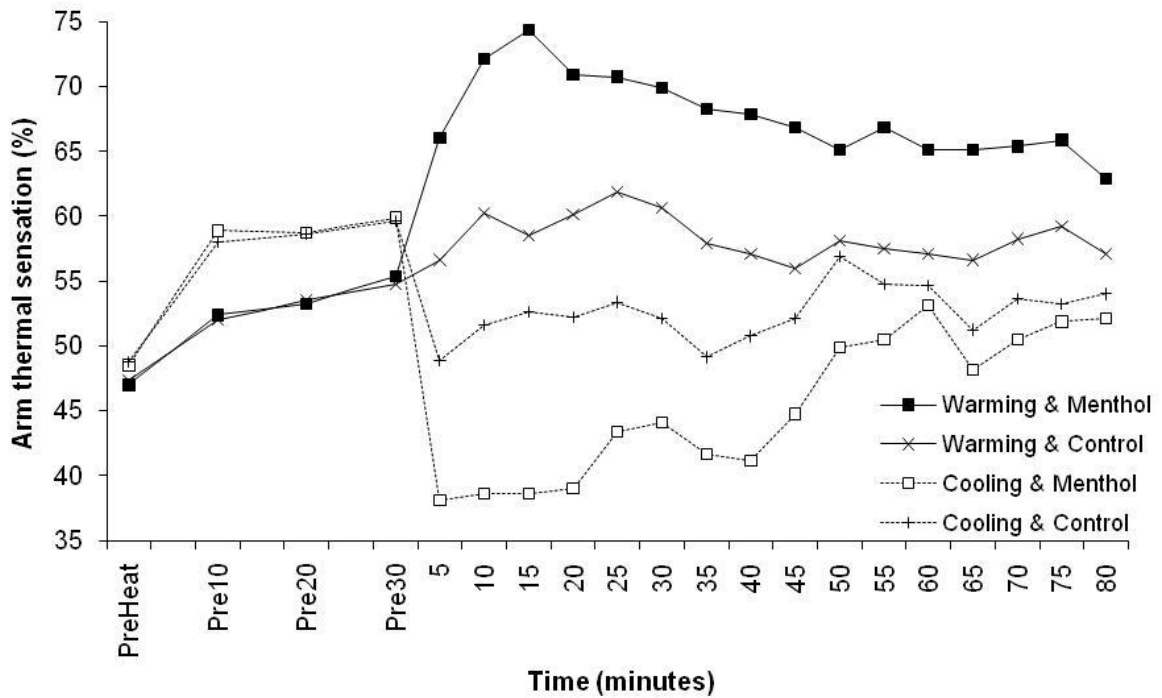
Figure 6.10. Arm thermal sensation scores over the duration of the trials with participants split into warming (n = 8) and cooling (n = 13) sensation groups and the data from their Menthol trial compared to their Control trial.
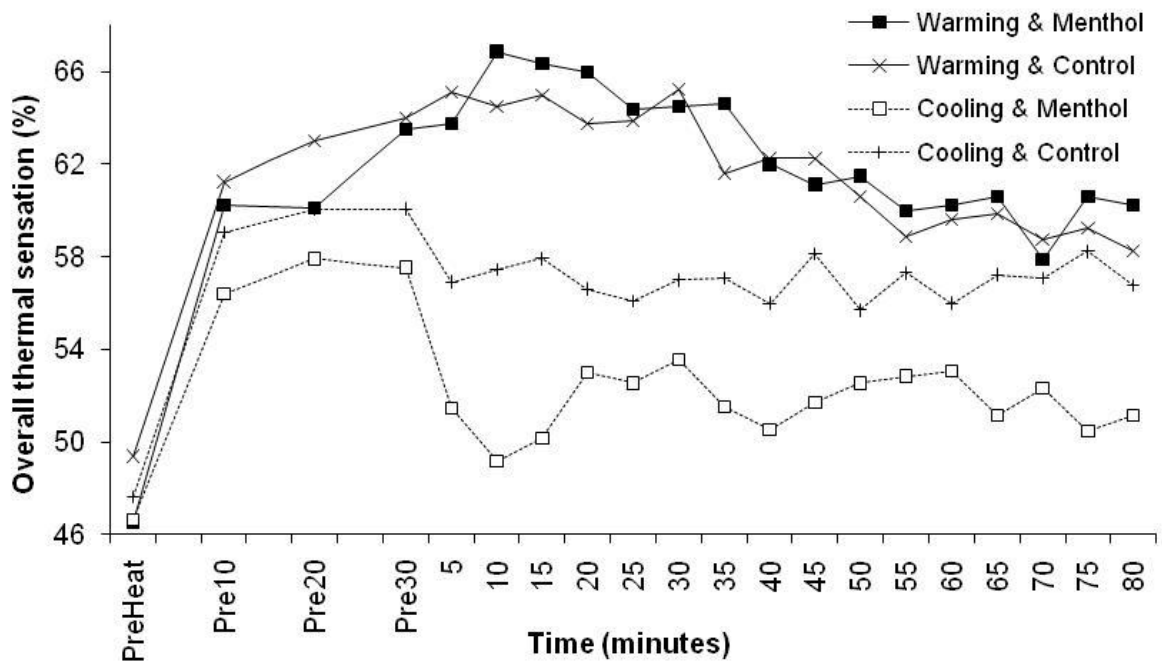
Figure 6.11. Overall thermal sensation scores over the duration of the trials with participants split into warming (n = 8) and cooling (n = 13) sensation groups and the data from their Menthol trial compared to their Control trial.

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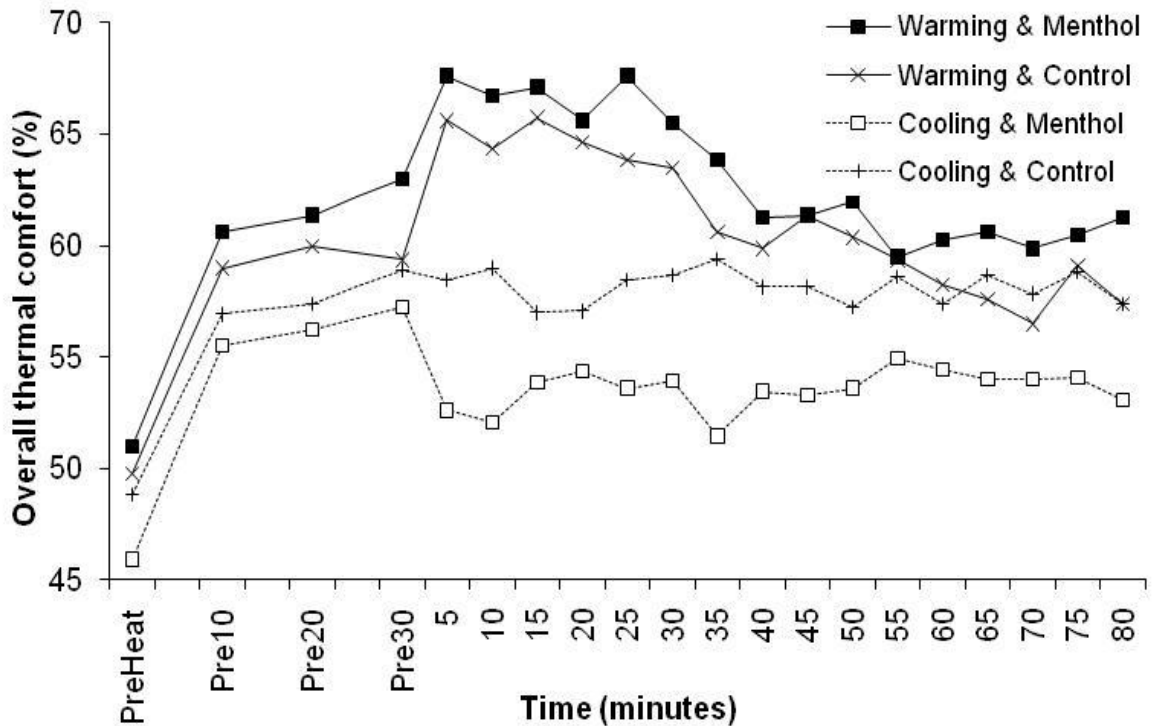
Figure 6.12. Overall thermal comfort scores over the duration of the trials with participants split into warming (n = 8) and cooling (n = 13) sensation groups and the data from their Menthol trial compared to their Control trial.

6.5 Discussion

Arm skin temperature was not different between the Menthol and Control trials, nor was it different between the right arm (1 % menthol solution) and the left arm (control solution) in the Menthol trial and finally, it was also not different between those who perceived a warming sensation compared to those who perceived a cooling sensation on the Menthol trial. Likewise, mean skin temperature did not show any differences between the Menthol and the Control trials, nor between the warming and cooling groups and the same can also be said for the skin blood flow data. Therefore, this shows that there was no effect of the menthol on actual skin temperature or skin blood flow, and that the alteration in perceived thermal sensation occurred centrally as a result of the stimulation of thermoreceptors. This supports the data seen in other investigations using human participants (Yosipovitch *et al.*, 1996; Kozyreva and Tkachenko, 2008), but it conflicts with findings in animal studies.

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Tajino and colleagues (2008) found that when a 10 % menthol solution was applied to the skin on the whole trunk of mice, after an initial fifteen minute lowering of core temperature probably due to the evaporation of the vehicle from the skin, menthol caused an increase in core temperature of 1.3 °C in the subsequent fifteen minutes compared to when the vehicle only was applied in the same way. This elevated core temperature then persisted for over ninety minutes (Tajino *et al.*, 2008). The authors suggest that the menthol, through activating the skin thermoreceptors, evoked a heat gain response, possibly through peripheral vasoconstriction and shivering-like autonomic heat gain responses, which have been seen before as a result of menthol application to mouse skin (Tajino *et al.*, 2007), resulting in the increase in core temperature seen in the mice.

It seems that for those who felt a cooling sensation of the menthol, it was a stronger sensation than for those who experienced a warming sensation. Significant differences were seen in the cooling group between the Menthol and Control trials in overall thermal sensation and overall thermal comfort, which were not present in the warming group (*Figure 6.11* and *Figure* 6.*12*). Menthol is most commonly known for its cooling sensation properties and therefore it is possible that participants were predisposed to assume a cooling sensation upon application to the skin. By mixing the menthol solution in methylated spirits there was no difference in colour between the test liquids. Methylated spirits also helped to mask the smell of the menthol and therefore acted as a suitable vehicle for the menthol, while also allowing for the trials to be blinded, thereby minimising any confounding factor due to the possible expectation of a cooling sensation.

In a study by Green (1992) skin temperature was manipulated and the response to applying either a 0 % vehicle only or a 5 % or 10 % menthol solution was investigated. At skin temperatures of 33 °C, similar to those in the present study, 75 % of trials on the 5 % menthol application saw the sensation of warmth reported, while the 10 % menthol solution elicited a response of close to 90 % reporting a sensation of warmth. However, it should be noted that the control substance also elicited a rate of 75 % of trials

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eliciting a warm sensation, therefore suggesting that the menthol had little or no effect at 33 °C compared to the vehicle alone. The perceived intensity of this warming sensation was slightly greater on both of the menthol trials than on the 0 % control trial during the initial five and ten minutes of exposure. Over a large range of skin temperatures the pattern emerged that at lower skin temperatures, menthol elicited a large cooling effect (at 29 °C ~75 % *vs.* ~40 % of trials reported cooling for menthol and control trials respectively). When skin temperature was increased a warming sensation was elicited from the menthol application (at 39 °C ~55 % *vs.* ~20 % of trials reported warming for menthol and control trials respectively).

However, in the present study there was only a weak correlation between arm temperature and overall thermal sensation in the cooling (r = 0.31) and warming (r = 0.45) groups. There was no correlation between skin temperature and overall thermal sensation, skin temperature and arm sensation, or arm temperature and arm sensation, in either the warming or the cooling sensation groups. Of course, the present study did not have the range of skin temperatures seen in the Green (1992) study.

Previous research has shown that around one third of people are not sensitive to the application of menthol to the skin (Yosipovitch *et al.*, 1996). In the present study, three participants of the twenty four recruited (12.5 %) did not perceive any effect of the menthol solution. It is possible that by coincidence, more of the participants recruited to the Yosipovitch study were immune to the effects of the menthol than the general population, or that by the same coincidence, fewer of the participants recruited to the present study were immune to the effects of menthol. As the Yosipovitch study only recruited eighteen participants, and this study only recruited twenty four participants, to gain a true perspective on the prevalence of skin sensitivity to menthol in the general population, much greater numbers would need to be recruited.

It is also possible that the ambient temperature or the skin temperature may alter individual sensitivity to menthol. There could be a specific, possibly individualised, temperature at which the thermal sensation of menthol

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changes from a cooling sensation to a warming sensation, passing through no perceivable temperature sensation. This hypothesis might also explain the difference in the levels of sensitivity between the Yosipovitch trial and the present study. It might be expected that the findings of the two studies would be reversed since the menthol applied here was only a 1 % solution, while the solution Yosipovitch and colleagues (1996) applied contained 10 % menthol and consequently the stronger concentration of menthol might reasonably be expected to increase the number of people able to feel its effect.

There also appears to be a difference between individuals in the rate of response to heat exposure in mean skin temperature and arm skin temperature. The standard deviation bars at time point Pre10 on *Figure 6.9* demonstrate that after ten minutes of heat exposure some participants' arm temperature had not yet responded to the heat exposure, while other participants' arm temperature had already reached the elevated arm temperature seen in all participants after twenty minutes of heat exposure. No such lag was seen in participants' perception of thermal sensation (*Figure 6.4*). This suggests that the rate of initiation of physiological responses to increased environmental temperature varies between individuals, but that this is not reflected by equally variable rates in the perception of environmental conditions.

A 1 % menthol solution was used here, since in a pilot study looking at different concentrations of menthol that could be used, feelings of burning, itching and discomfort increased with increasing concentration, but perception of cooling or warming did not intensify. Indeed, Green (1992) found that doubling the concentration of menthol (5 % to 10 %) used in his study at skin temperatures ranging from 21 °C to 35 °C, resulted in a greater incidence of burning sensation but did not change the intensity of the cooling sensation. He suggests that this could be due either to a near saturation point of the effect of menthol or that the psychophysical methods employed in the study were not sensitive enough to detect such a change.

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In the present study the menthol patches were covered with a non-porous adhesive dressing to prevent the effects of evaporative heat loss. However, the action of menthol is to activate the skin thermoreceptors and this activation remains even once the menthol is removed, meaning that after application to the skin, the menthol makes other substances such as water or air which pass over the body feel cool (Eccles, 2000). Therefore, it is possible that if the patches had been removed after a time and the measures continued, the findings of the present study may have been altered.

Certain parts of the skin are more sensitive to temperature changes than others. By isolating a participants' head and exposing it to different temperatures, McCaffrey *et al.* (1975) showed that sweat rates could be altered. When sitting from the neck down in 40 °C, the participants' head was exposed to either heating (70 °C) or cooling (29 °C) for thirty minutes. Naturally, with heating head skin temperature increased quickly, followed by a slower rise in core temperature, and sweat rate increased rapidly and continued to increase, even after skin temperature had stabilised. When the participants' head was cooled, sweating rates fell and core temperature remained unchanged. Indeed people preferentially cool the face when in a warm environment (Nakamura *et al.*, 2008) and therefore, by applying menthol solution to the forehead, a stronger thermal sensation may be expected than if applying the menthol to only other skin surface areas.

The hypothalamus co-ordinates the thermoregulation of the body. Skin temperature receptors terminate in the pre-optic area of the hypothalamus and core temperature receptors terminate in the pre-optic area and the brain stem. Skin thermoreceptors can be stimulated by application of menthol to the skin, eliciting a cooling sensation making the body feel cooler. It has been suggested that some aspects of peripheral cooling sensation occur centrally. Nakamura *et al.* (2008) suggested that the qualitative differences in the thermal comfort of various body areas seen in their study could not be solely explained by the distribution of peripheral thermoreceptors, thereby implicating processing mechanisms within the central nervous system. Frank *et al.* (1999) demonstrated that core temperature and skin temperature

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contribute equally to levels of thermal comfort in men, assessed by a ten point visual analogue scale. Therefore, by artificially manipulating the perception of skin temperature to lower than its true temperature, it may be possible to manipulate thermal comfort.

6.6 Conclusion

Applying 1 % menthol solution to the skin during exposure to a warm environment evokes differing sensations between individuals, varying from a very cold sensation, through no sensation to a very warm sensation. However, no matter what the perceived response to the application of menthol to the skin, there is no alteration in the skin temperature or skin blood flow.

Applying menthol to small patches of skin altered overall thermal sensation and thermal comfort. Improving thermal comfort during heat exposure may improve heat tolerance. Furthermore, if this pattern were to hold true, exercise performance or exercise capacity in the heat could be improved. This is the rationale which we aimed to test in the subsequent experiment, detailed in Chapter Seven.

CHAPTER SEVEN

The influence of the application of a 1 % menthol solution to the skin during a cycling time trial in the heat.

7.1 Abstract

In a warm environment, the application of menthol to the skin caused a warming sensation in some individuals and a cooling sensation in other individuals. It is plausible that altered thermal sensation may have important implications for exercise performance but this has never been investigated. Therefore, the aim of this investigation was to determine in alterations in thermal sensation, without alterations in actual core or skin temperatures could alter endurance exercise performance in the heat.

Six participants who perceived a warming sensation due to menthol and six who perceived a cooling sensation due to menthol completed a sixty minute pre-load bout of cycling at 55 % $\dot{V}O_2$ peak in 30.4 ± 0.3 °C and 44 ± 3 % relative humidity. Patches soaked in 1 % menthol (Menthol) or a control (Control) were then applied to the skin immediately prior to a twenty minute time-trial. During the time-trial, core and skin temperatures, heart rate, rating of perceived exertion, work done and pedal cadence were recorded every five minutes. Local and overall thermal sensation ratings were taken every ten minutes, expired air was collected for the last minute and a blood sample was drawn immediately upon completion of the exercise.

There were no differences ($P > 0.05$) between the Menthol and Control trials for any of the parameters except heart rate, which was slightly higher in the Menthol trials than the Control trials ($P = 0.031$). There was no difference in overall exercise performance (work done) between the cooling group and the warming group ($P = 0.200$). Within the warming group, thermal sensation scores were greater on the Menthol than the Control trials ($P = 0.009$), but this did not affect performance ($P = 0.405$). Within the cooling group thermal sensation scores tended to be lower with Menthol ($P = 0.053$), and there was a tendency for an improvement in performance ($P = 0.071$). This suggests that when participants feel cooler than they actually are, exercise performance in a warm environment may be improved.

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7.2 Introduction

During exercise in the heat there is unequivocal evidence that both endurance exercise capacity and exercise performance are reduced compared to when the same endurance exercise is performed in a cool environment (Galloway and Maughan, 1997; Nybo and Nielsen, 2001B; Tatterson *et al.*, 2000). It is likely that the elevation of core body temperature due to exercise, and the reduced ability to lose the excess heat to the surrounding environment during heat exposure, is a significant factor in the reduced ability to perform exercise in a warm environment (Pitsiladis and Maughan, 1999; Nielsen *et al.*, 1997; Gonzalez-Alonso *et al.*, 1999B). However, another influential factor in such situations may be skin temperature. Indeed, lowering of skin temperatures during exercise in the heat has been shown to greatly reduce the elevated perception of effort associated with elevated core temperatures (Armada-da-Silva *et al.*, 2004). Furthermore, it has been shown to modulate the effects of high core temperatures on hormonal responses to exercise in the heat (Bridge *et al.*, 2003B) and possibly even improve endurance exercise capacity (Marvin *et al.*, 1999).

Frank *et al.* (1999) showed that core temperature and skin temperature contribute equally to levels of thermal comfort in men assessed by a ten point visual analogue scale. Therefore, by artificially manipulating the perception of skin temperature to lower than the true physiological temperature, it may be possible to manipulate thermal comfort and consequently improve heat tolerance. If this hypothesis holds true during exercise in a warm environment, endurance exercise performance or capacity may be improved. Equally, by artificially manipulating the perception of skin temperature to higher than its true value, endurance exercise performance or capacity may be reduced.

Several products containing the plant extract menthol are emerging onto the market claiming to make people feel cooler. Menthol is widely used in many medicinal and cosmetic products due to the cooling sensation it causes when applied to the skin or mucous membrane and then making other stimuli, such as air or water, also feel cool (Watson *et al.*, 1978).

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The application of menthol on the skin does not elicit its effects through either evaporation from the skin nor any form of localised vasodilation or vasoconstriction. According to Eccles (1994) it was deemed as far back as the nineteenth century, by Goldsheider (1886) that the warming and cooling sensations were caused by stimulation of sensory nerve endings. Cool sensations can be caused by either the stimulation of cold receptors or the inhibition of warm receptors; conversely, warm sensations can be caused by either the stimulation of warm receptors or the inhibition of cold receptors. Existing research on topically applied menthol is limited and has been carried out at rest (Yosipovitch *et al.*, 1996; Kozyreva and Tkachenko, 2008; Green, 1992).

While the studies by Yosipovitch *et al.* (1996) and Kozyreva and Tkachenko (2008) were carried out at ambient temperatures of 18 - 23 °C, it is likely that the skin temperature is of greater importance to the thermal sensation elicited by the application of menthol than the environmental temperature. Green (1992) conducted a thorough analysis of the responses of participants to the application of menthol to the skin at different skin temperatures. He found that at a mid-range of skin temperatures (27 - 31 °C) menthol resulted in a greater incidence of reporting a cold sensation. At lower skin temperatures (21 - 27 °C) he found that menthol did not affect the incidence of reporting a cold sensation, and at higher temperature (37 - 41 °C) menthol reduced the incidence of reporting a hot sensation. However, these sensation ratings were all taken locally, at the site at which skin temperature was manipulated (forearm only), whilst the participant remained seated. Ambient temperature and overall thermal sensation ratings were either not measured or not reported.

In a recent study by Mundel and Jones (2010) it was shown that cycling exercise capacity in the heat (34 °C) was improved by 9 ± 12 % when swilling a menthol solution around the oral cavity, compared to swilling a placebo solution (63 ± 14 minutes compared to 58 ± 16 minutes with the menthol and placebo solutions respectively). Central ratings of perceived exertion (defined by the authors as the RPE specifically relating to the cardiopulmonary system)

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was significantly lower for the initial forty minutes and pulmonary ventilation was greater after twenty and forty minutes on the menthol trials compared to the placebo trials.

Mundel and Jones (2010) postulated that the mechanism for the improvement in exercise capacity was an alteration in the sensation of oropharyngeal temperature through alterations in psychophysical processes. The reduction in central RPE was likely due to the improved ventilation, as there were no differences in any of the circulatory or metabolic parameters measured. The oropharyngeal cold receptors of the mouth were likely to have been stimulated by the menthol and this may have altered the perception of the effort required to breath. Anecdotal evidence from comments made by participants also suggested that the menthol caused a sensation that was described as 'refreshing' and 'stimulating' (Mundel and Jones, 2010).

The effect of 1 % menthol application to the skin during resting heat exposure was assessed in Chapter Six. It was found that some participants reported a warming sensation while other reported a cooling sensation elicited by the menthol application. Therefore, the aim of this investigation was to determine if the manipulation of thermal sensation through the application of menthol to the skin would alter exercise performance in the heat.

7.3 Methods

7.3.1 Participants

Twelve healthy male volunteers participated in this study. The physical characteristics of the participants (mean ± SD) were: height 1.81 ± 0.07 m, body mass 79.1 ± 10.3 kg, $\dot{V}O_2$peak 59.0 ± 9.9 ml / kg / min and age (median (range)) 23 (20 - 31) y.

7.3.2 Experimental protocol

Participants undertook one preliminary trial involving a patch test and a maximal oxygen uptake test (details can be found in Chapter Two), followed by two familiarisations and two experimental trials. The familiarisations and

experimental trials were performed at least seven days apart and the experimental trials were performed in a blinded, randomised, counter-balanced, crossover design. Briefly, the experimental trials involved participants completing a sixty minute cycle ergometer exercise bout at 55 % of their $\dot{V}O_2$peak in a warm environment. Immediately after that, patches containing either a 1 % menthol solution or a control solution were applied to the skin on the forehead, upper and lower back, and the forearms (see Chapter Two for details). As soon as all the patches were in place the participants started a time-trial where they were instructed to complete as much work as possible in twenty minutes. A schematic of the experimental protocol is presented in *Figure 7.1*.

During both of the familiarisation trials and one experimental trial all of the patches applied contained the 0 % control solution (Control). On the other occasion all of the patches contained the 1 % menthol solution (Menthol), except one patch which was applied to the left forearm. The patch that did not contain the menthol solution instead contained the control solution, to allow the direct comparison of the two solutions on the same day. Participants were blinded to the different solutions used in the forearm patches during the Menthol trial.

The preliminary testing was done at least three days prior to the first familiarisation. The patch test involved participants sitting in a warm environment (30 °C and 45 % relative humidity) for twenty minutes to allow the adjustment of skin temperature. A patch soaked in the 1 % menthol solution was then applied to the skin of their right forearm and a patch soaked in a control solution was applied to the skin of their left forearm. These remained in place for a further twenty minutes before participants were presented with two 10 cm visual analogue scales with verbal anchors at either end ("Cold" and "Hot") and asked to rate their right and left forearm temperatures (see Appendix D).

Figure 7.1. A schematic representing the protocol employed during the main trials in Chapter Seven.

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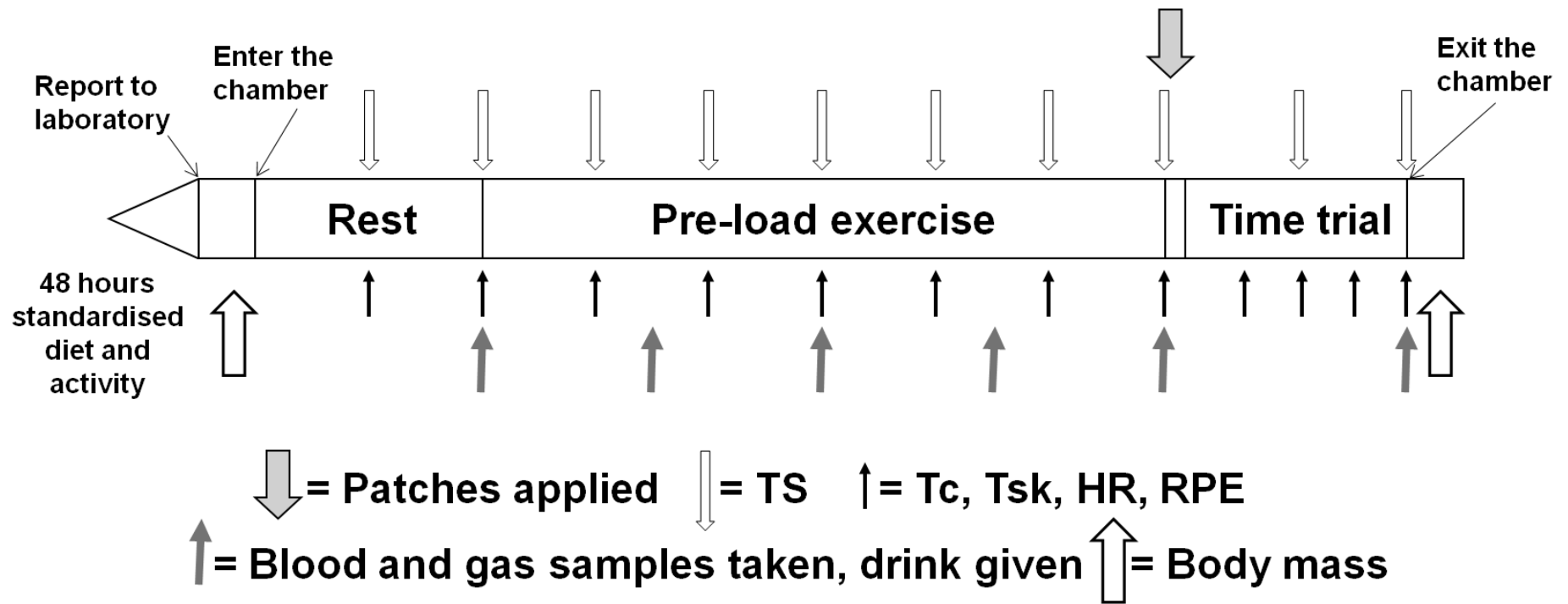
The aim of this patch test was twofold; firstly to ensure that there was no adverse reaction to the patches or the menthol solution used in the main trials and secondly, to allow the investigators to determine if the participant perceived a warming sensation or a cooling sensation due to the menthol. Six participants who perceived a warming effect of the menthol and six who perceived a cooling effect were recruited to the study. No other measures were taken during the patch test. After the patch tests the $\dot{V}O_2peak$ test was completed (for details see Chapter Two).

For the familiarisation and experimental trials, participants reported to the lab at either 0600 or 0900 hours. Upon arrival, participants were asked to empty their bladder and a small sample of the urine passed was retained. Post-void, nude body mass was taken and participants inserted a rectal thermometer to allow the measurement of core body temperature (Tc). A heart rate telemetry band was then positioned and skin temperature was measured using an infrared laser thermometer. Skin temperature was measured at four sites (chest, triceps, thigh and calf) for the calculation of weighted mean skin temperature and estimation of body heat content as described in Chapter Two. Participants wore only shorts and trainers for the duration of the trials.

Upon entering the climatic chamber, maintained at 30.4 ± 0.3 °C and 44 ± 3 % relative humidity, participants were seated for twenty minutes during which measures of core temperature, skin temperature, heart rate and subjective feelings were taken every ten minutes. The subjective feelings questionnaire measured local (forearm) thermal sensation of the right and the left forearms, as in the preliminary testing, and also overall thermal sensation using visual analogue scales (see Appendix D).

During this resting period an indwelling cannula was inserted into a superficial forearm vein (see Chapter Two for details) and a 5 ml baseline blood sample was drawn at twenty minutes. Participants were then given 150 ml of plain water, served at room temperature, to consume. After this, participants started cycling at an equivalent of 55 % $\dot{V}O_2peak$. During this one hour

preload phase, measures of core and skin temperature, heart rate, rating of perceived exertion and thermal sensations were taken every ten minutes while samples of exhaled air and blood were taken every fifteen minutes. Also, after every fifteen minutes participants drank 150 ml of plain water served at room temperature. The total work done at the end of the sixty minutes was noted.

Immediately at the end of the sixty minute pre-load exercise period the skin was dried as thoroughly as possible and the patches were applied (see Chapter Two for details). As soon as the patches were secured, the time-trial commenced. Participants began the exercise at a workload that equated to 75 % of their $\dot{V}O_2$peak and were allowed to alter the workload throughout the time-trial as they liked. The bike was set in hyperbolic mode, meaning that participants could cycle at any pedal cadence and the workload would remain the same. The participants altered the workload by pressing buttons on the console of the ergometer but they received no feedback on their workload or any other performance or physiological measures. The participants could see a timer during the trial but were given no other information.

During the time-trial, measures of core and skin temperature, heart rate and rating of perceived exertion were taken every five minutes. Thermal sensations were taken after ten and twenty minutes, a gas sample was taken during the final minute and a venous blood sample was drawn as soon as exercise was finished. The power output (W), cumulative work done (kJ), and pedal cadence (rpm) data displayed by the bike were also recorded every five minutes during the time-trial.

At the end of the test period, participants left the heat chamber to recover. Once they felt ready, the patches, cannula, heart rate monitor and the rectal probe were removed. Finally, participants were again weighed nude before dressing and being given food and beverages. They were then allowed to leave the laboratory.

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7.3.3 Blood handling and analysis

All blood samples (5 ml) were collected into dry syringes. Samples were analysed for glucose and haemoglobin concentrations, haematocrit values and serum osmolality. Details of the techniques used can be found in Chapter Two.

7.3.4 Statistical analysis

All data sets were investigated for normality of distribution using the Shapiro-Wilk test, and skewness and kurtosis. Where the data were analysed within participants, i.e. between the two conditions, paired t-tests or repeated measures ANOVA were used. Where the data were analysed between the two groups of responses on the Menthol trial, i.e. between those who felt a warming sensation and those who felt a cooling sensation, independent samples t-tests were used or a repeated measures ANOVA mixed design where appropriate. In this part of the analysis the participant who perceived no effect of the treatment was omitted from the investigation. All analysis was performed on SPSS version 17.0 and statistical significance was set at $P < 0.05$.

7.4 Results

7.4.1 Pre-load data

There were no differences between the trials for any of the measured parameters during the sixty minute preload phase of the trials ($P > 0.05$). This result was to be expected since no intervention had been applied at that stage. It also implies that the participants started both time-trials in the same physiological state. Most parameters increased over the duration of the sixty minute pre-load exercise, including: core temperature ($P < 0.001$), weighted mean skin temperature ($P < 0.001$), estimated body heat content ($P < 0.001$), heart rate ($P < 0.001$), right and left arm thermal sensation scores ($P < 0.001$ for both), overall thermal sensation scores ($P < 0.001$), ratings of perceived exertion ($P < 0.001$), glucose concentration ($P = 0.008$), serum osmolality ($P < 0.001$) and fat oxidation rates ($P < 0.001$), while carbohydrate oxidation rates fell during the exercise ($P < 0.001$).

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There was no order effect of the trials (P = 0.695). There was no difference in body mass loss over the duration of the tests between the Menthol and the Control trials (1.65 ± 0.32 kg Menthol and 1.72 ± 0.39 kg Control; P = 0.184). There was no difference in urine osmolality between the Menthol and the Control trials (549 ± 241 mosmol / L Menthol and 552 ± 284 mosmol / L Control; P = 0.948), indicating that participants were in a similar hydration state for both trials.

7.4.2 Time trial protocol

The co-efficient of variation between the first and second familiarisations was 4.7 ± 11.0 % (r = 0.956). The co-efficient of variation between the second familiarisation and the control trial was 3.9 ± 9.6 % (r = 0.976).

7.4.3 Menthol *vs.* Control

There was no difference in the total work done during the time-trial between the Menthol and the Control treatments (249.3 ± 40.7 kJ Menthol and 248.9 ± 42.0 kJ Control; P = 0.925). Also, the distribution of work over the twenty minutes, analysed in five minute blocks, was not different between the trials (P = 0.925) nor over the duration of the trials (P = 0.299).

There was no difference between the treatments for pedal cadence, which was noted every five minutes. There was however, an effect of time, spiking from 93 ± 10 rpm and 95 ± 11 rpm at fifteen minutes to 111 ± 25 rpm and 116 ± 24 rpm at twenty minutes for the Control and Menthol trials respectively (P = 0.014; *Figure 7.2*). Self-selected power output, also noted every five minutes, showed no difference between trials (P = 0.559) nor a significant effect of time (P = 0.067), although there was a tendency for this to increase in the final five minutes (203 ± 40 W and 205 ± 39 W at fifteen minutes increased to 253 ± 91 W and 241 ± 76 W at twenty minutes for the Control and Menthol trials respectively; *Figure 7.3*).

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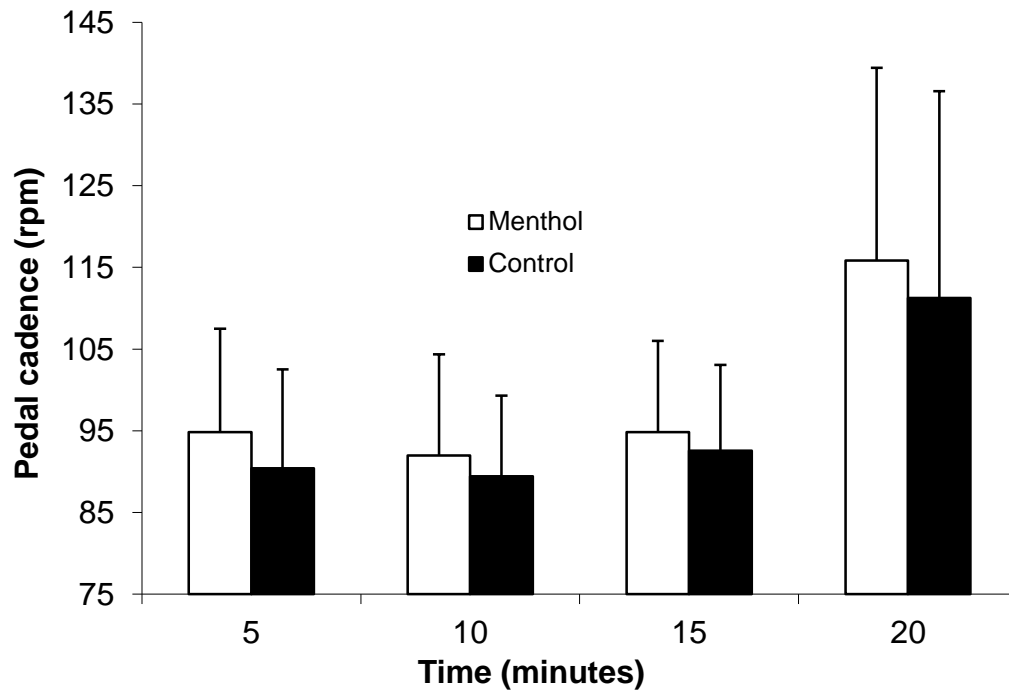
Figure 7.2. Pedal cadence during the Control and the Menthol treatments, noted at five minute intervals. A significant increase in pedal cadence occurred at the end of the time-trial in both conditions (P=0.014).
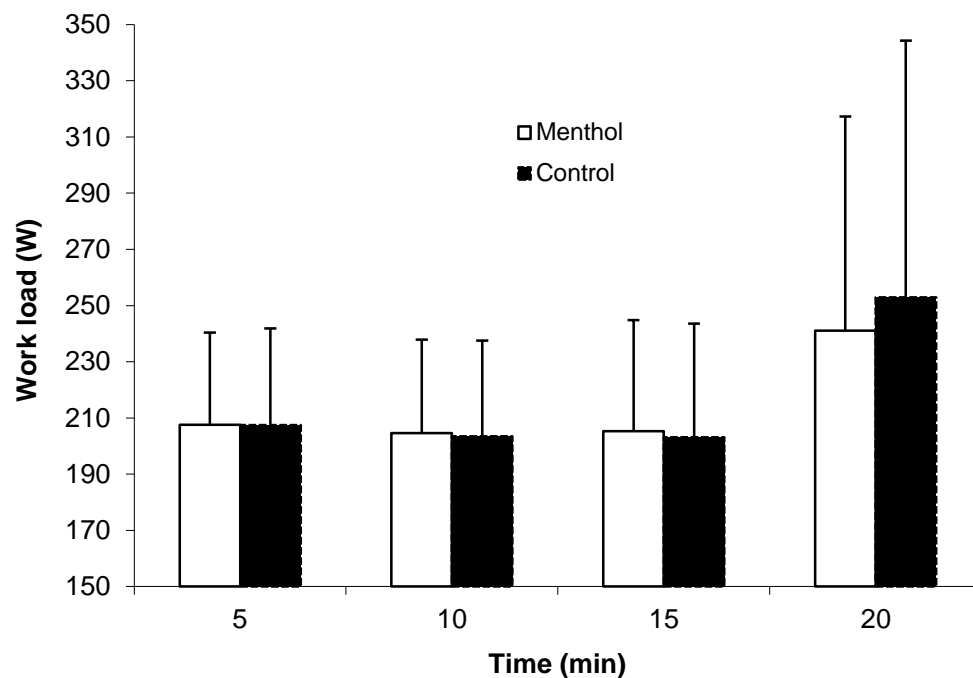
Figure 7.3. Power output during the Control and the Menthol treatments, noted at five minute intervals. There was a tendency for an increase in the power output at the end of the time-trial in both conditions (P=0.067).

As with the pre-load phase of the trials, core temperature continued to increase during the time-trial (P < 0.001) but was not different between the trials (P = 0.619; *Figure 7.4*). Skin temperature showed a similar pattern, increasing over time (P = 0.001), but again showing no influence of menthol application (P = 0.588), and estimated body heat content also reflected this pattern with a significant rise over time (P < 0.001), but no difference between the trials (P = 0.667).
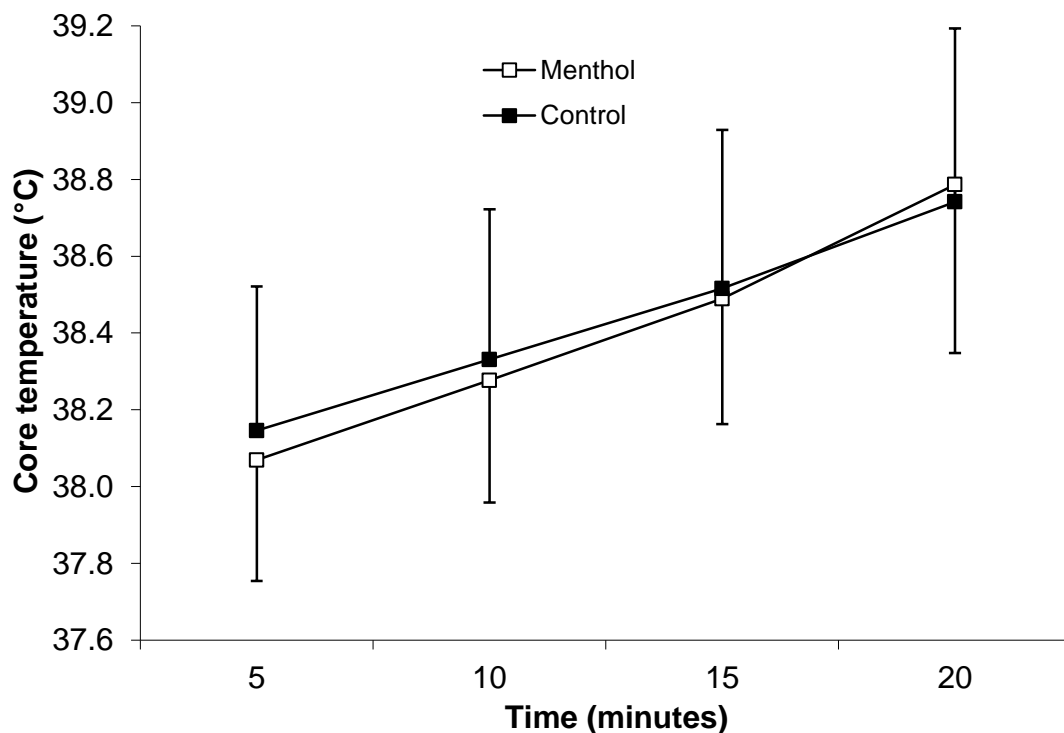
Figure 7.4. Core temperature over the time-trial. There was no difference between the trials (P = 0.619) but a significant increase over time (P < 0.001).

Ratings of perceived exertion increased during the time-trial (15.6 ± 1.3 and 14.9 ± 1.6 at five minutes to 18.8 ± 1.0 and 18.9 (17-20) at twenty minutes for the Control and Menthol trials respectively; P < 0.001), but did not differ between trials (P = 0.339). Right arm (1 % menthol solution) thermal sensation scores increased over time (P = 0.012), but were not affected by the trial (P = 0.470). Left arm (control solution) and overall thermal sensation

scores were not affected by time (P = 0.222 and P = 0.107 respectively), or trial (P = 0.108 and P = 0.372 respectively).

Blood glucose concentrations were not different between trials at the end of the time-trial (Control 4.95 ± 0.89 mmol / L and Menthol 5.28 ± 0.53 mmol / L; P = 0.136). Serum osmolality was also not different between trials (P = 0.244), nor were plasma volume changes from baseline (P = 0.399), carbohydrate oxidation rates (P = 0.632) or fat oxidation rates (P = 0.537).

Heart rate, however, was consistently slightly higher on the Menthol trial than the Control trial (P = 0.031), and increased over time (P < 0.001) from 161 ± 12 and 164 ± 12 beats per minute at five minutes rising to 180 ± 11 and 182 ± 10 beats per minute at twenty minutes for the Control and the Menthol trials respectively (*Figure 7.5*).
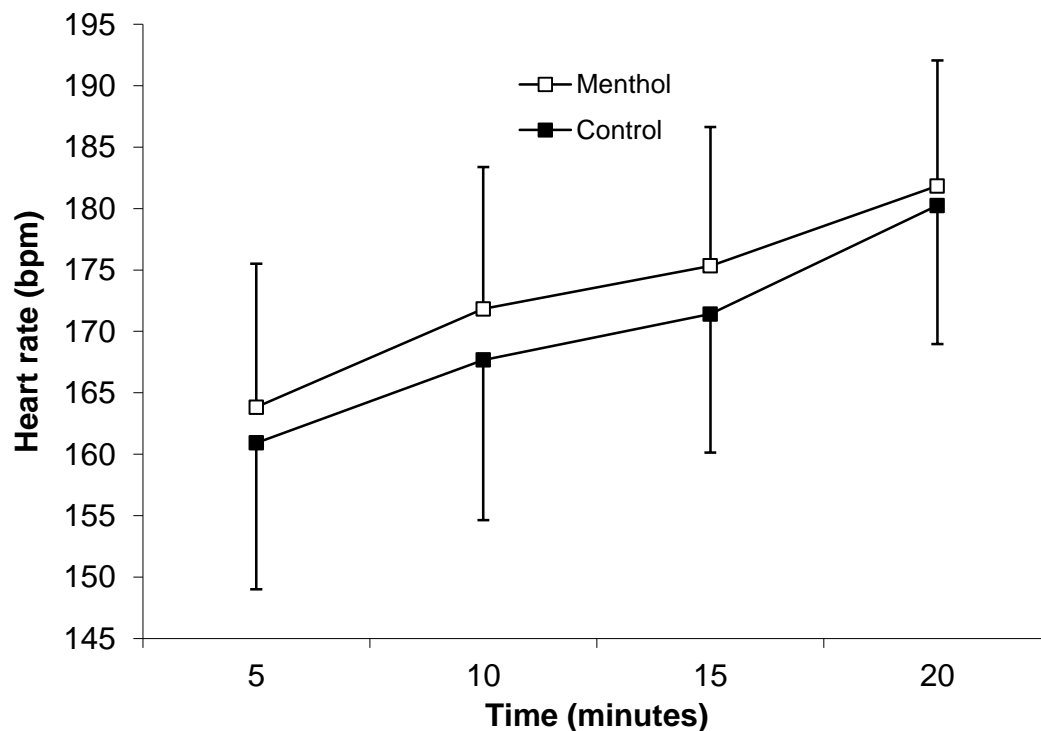
Figure 7.5. Heart rate over the time-trial. There was an increase in heart rate over time (P < 0.001) and the Menthol trial produced consistently slightly higher heart rates than the Control trial (P = 0.031).

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<u>7.4.4 Warming *vs.* cooling</u>

From the effects of the menthol solution on local thermal sensation, assessed during the preliminary trial, six participants who perceived the menthol to have a warming sensation and six who perceived it to have a cooling sensation were recruited to the study. However, analysis of the thermal sensation data generated in the main trials does not reflect the local sensations perceived in the preliminary trial. Therefore, participants have been reclassified based on their overall thermal sensation ratings ten minutes into the time-trial on the Menthol trial, compared to the Control trial. Six participants perceived an overall warming effect (n = 6) and five perceived an overall cooling effect (n = 5) of the menthol solution. The details of these participants, grouped by thermal sensation, are presented in *Table 7.1*. One participant felt no effect of the menthol solution on their overall thermal sensation and when analysing differences between or within the sensation groups his data has been omitted.

Table 7.1. Participant information grouped by overall thermal sensation after ten minutes of the cycling time trial, and the difference between the groups.

	Body mass (kg)	Height (m)	Age (y)	$\dot{V}O_2peak$ (ml / kg / min)
Cooling	84.1 ± 14.2	1.82 ± 0.10	23 ± 3	56.9 ± 9.8
Warming	76.1 ± 5.1	1.79 ± 0.06	23 ± 4	58.2 ± 9.5
P value	0.287	0.582	0.937	0.833

Figure 7.6 shows the difference in perceived overall thermal sensation between the Menthol trial and the Control trial, within the warming and the cooling groups. There was a strong difference (P = 0.004) between the warming and the cooling group (*Figure 7.6*). The overall thermal sensation scores of those in the warming group compared to those in the cooling group on the Menthol trial also tended to be different (P = 0.064; *Figure 7.7*), as did the right arm (1 % menthol solution) local thermal sensation scores (P = 0.086). By analysing the difference between the local thermal sensation scores on the Menthol and Control trials, there was a strong tendency for a difference between the groups (P = 0.060), supporting the re-grouping.

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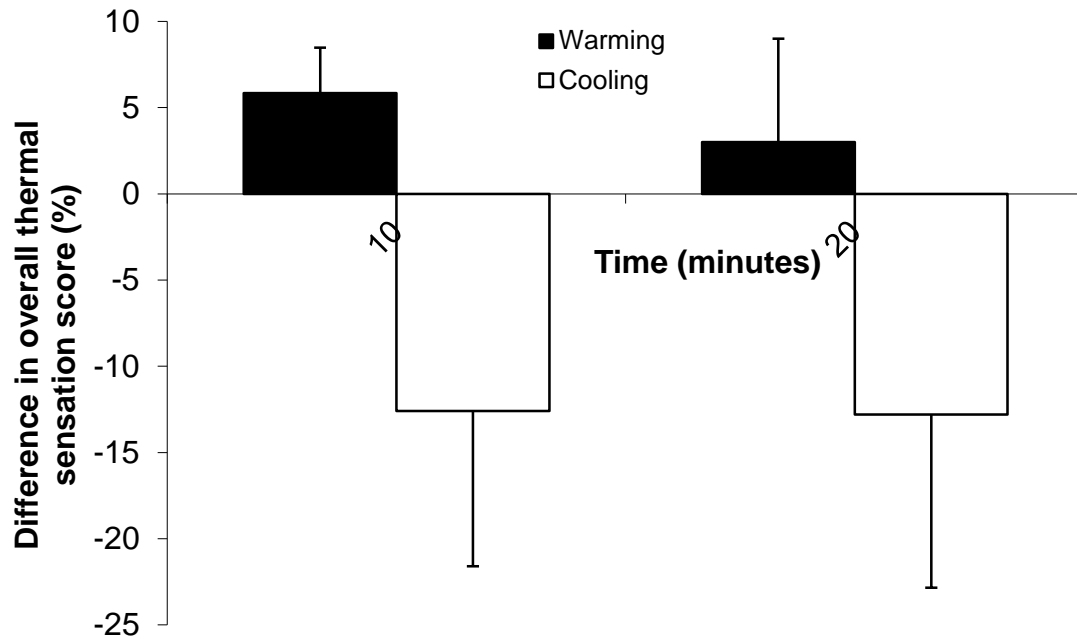
Figure 7.6. Difference between Menthol and Control trials for overall thermal sensation in the cooling (n = 5) and warming (n = 6) groups. There was a significant difference in the overall thermal sensation between the groups (P = 0.004) but no effect of time.
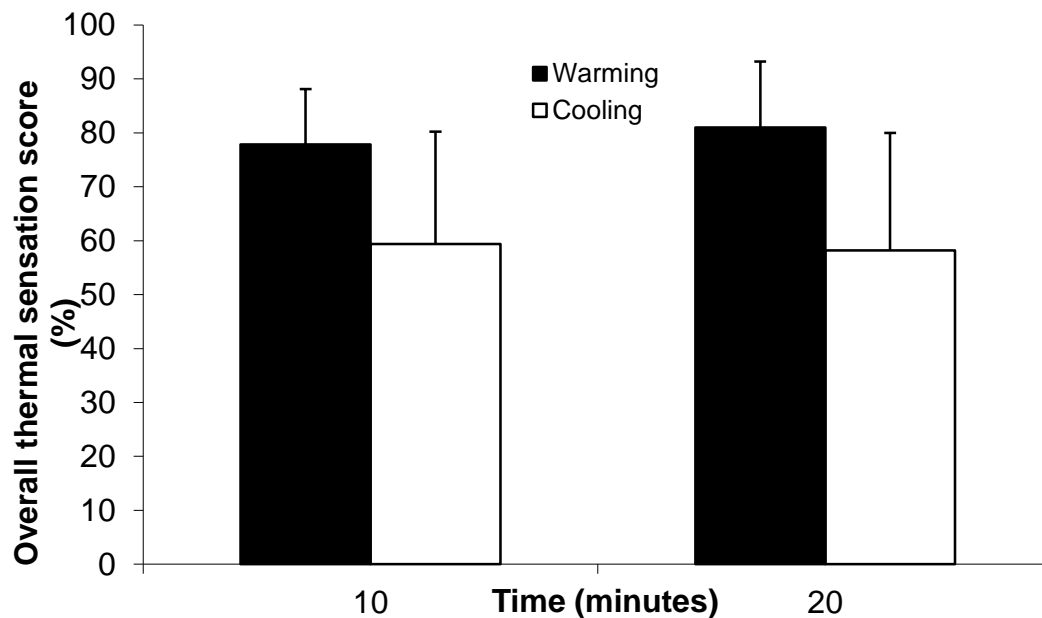
Figure 7.7. Overall thermal sensation scores on the Menthol trial for the warming (n = 6) and the cooling (n = 5) groups. The difference between the groups just failed to reach statistical significance (P = 0.064) and there was no effect of time (P = 0.643).

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Other than the thermal sensation data however, there were no differences between the warming and the cooling groups. There was no difference in body mass loss (P = 0.941) suggesting that the perception of increased warmth did not evoke a thermoregulatory cooling mechanism, such as an increase in sweating. Most importantly there was no difference in the total work done between the groups (P = 0.200), nor in the distribution of the work during the time-trial between groups (P = 0.215) or over time (P = 0.509). Pedal cadence increased over time in both groups but, once again, there was no difference between the groups.

Core temperature increased over the duration of the time-trial in both groups (P < 0.001) but did not differ according to overall thermal sensation (P = 0.559). Again, skin temperature and body heat content followed the same pattern as core temperature, increasing over the time-trial (P = 0.017; P < 0.001) but without differing between the groups (P = 0.443; P = 0.189) for skin temperature and body heat content respectively. Heart rate and RPE also both increased during the time-trial (P < 0.001 for both parameters) but neither differed between groups (P = 0.931 for heart rate; P = 0.712 for RPE). Blood glucose concentration (P = 0.765), serum osmolality (P = 0.162) and plasma volume (P = 0.760) were all similar between groups and there were no differences in fat (P = 0.422) and carbohydrate (P = 0.787) oxidation rates.

7.4.5 Sensation *vs*. treatment

The two trials (Menthol and Control) have been compared to each other. The participants, having been split into two groups depending upon their overall perception of thermal sensation in response to the application of menthol, have also been compared to each other. The final comparisons to be drawn are those within the same participant between the two trials. Once again, the participant who perceived no difference in overall thermal sensation between the Menthol and the Control trials has been omitted from this analysis.

For those who perceived a warming sensation (n = 6), their overall thermal sensation was higher on the Menthol trial compared to the Control trial (P = 0.009) and this increased over the time-trial (P = 0.022; *Figure 7.8*). However,

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the increased thermal sensation did not negatively influence performance as there was no difference in the total work done in the time-trial (P = 0.400; *Figure 7.9*), nor in the cumulative work done, assessed every five minutes during the time-trial (P = 0.405; *Figure 7.10*).
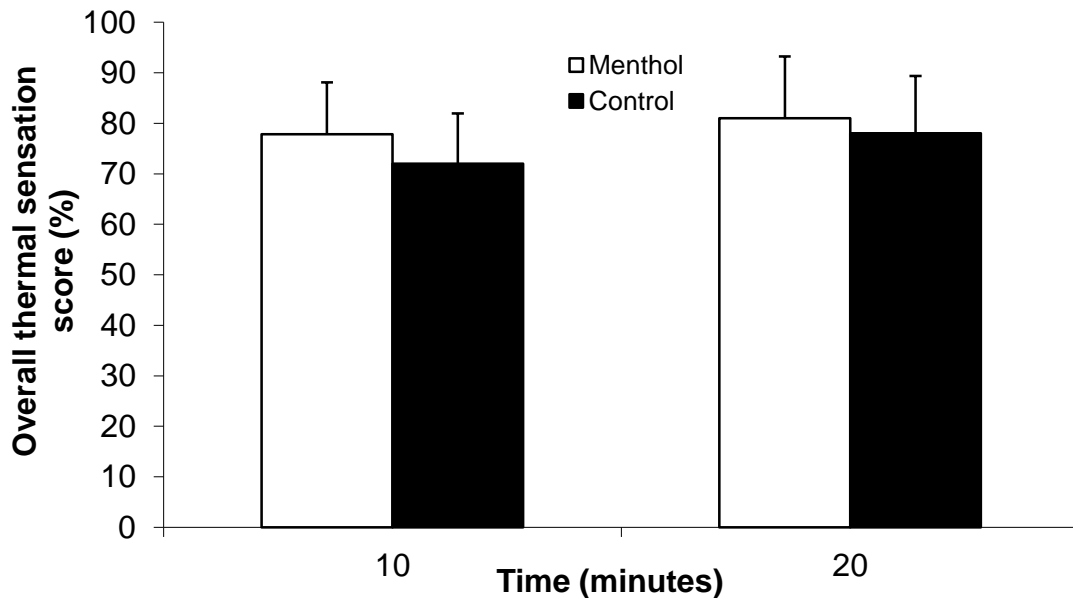
Figure 7.8. The overall thermal sensation scores in the warming group (n = 6) for the Menthol and the Control trials.
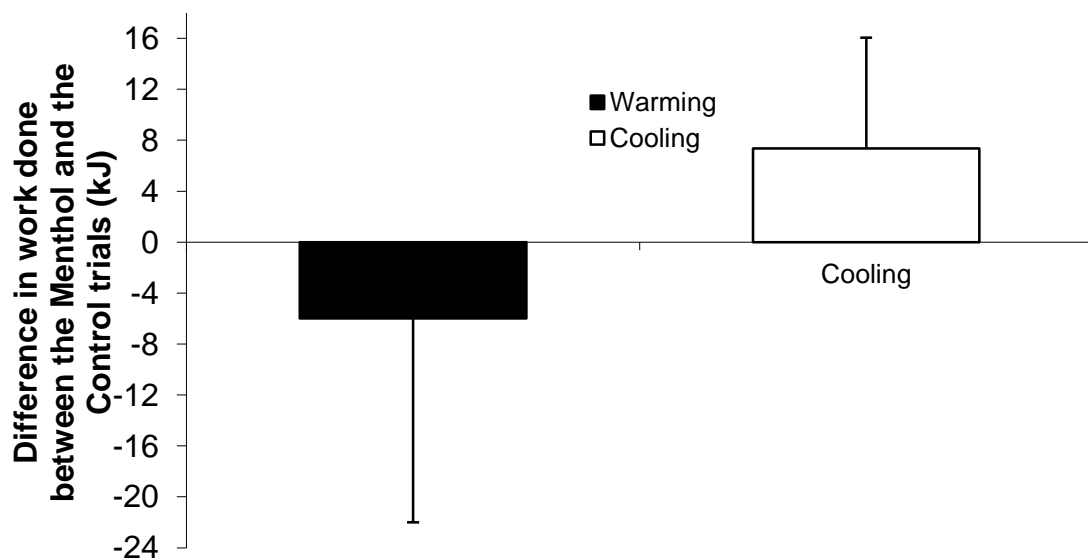
Figure 7.9. The difference in work done between the Menthol and Control trials in the warming group (n = 6) and the cooling group (n = 5). There was no difference between the groups (P=0.400).

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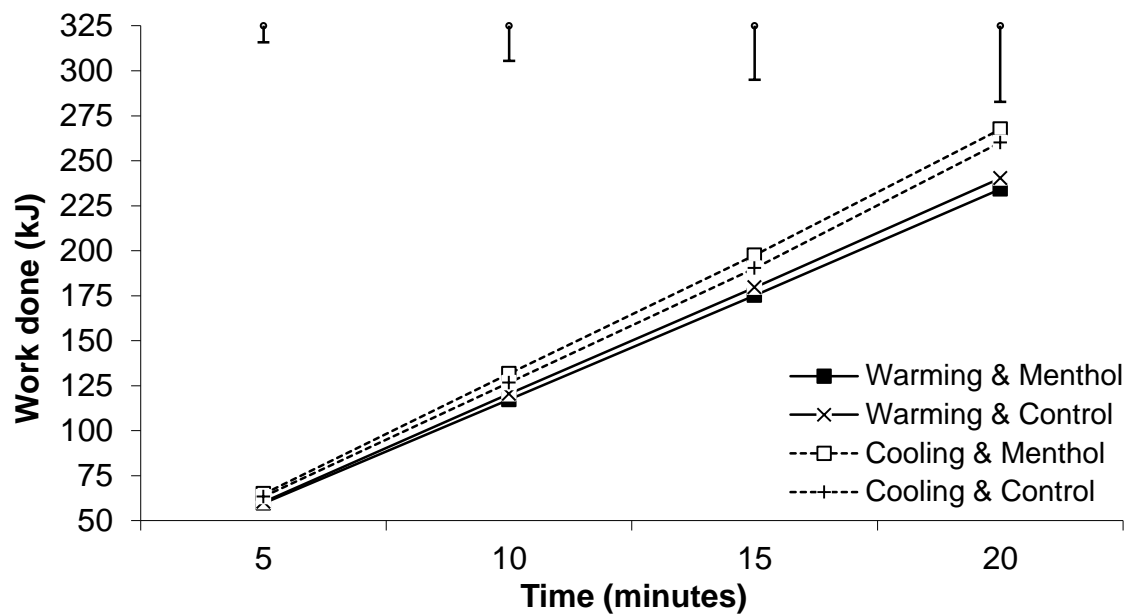
Figure 7.10. Cumulative work done during the time-trial for the warming (n = 6) and cooling (n = 5) groups of both the Menthol and the Control trials. The error bars above the data display the variability of the data.

For those participants who perceived the menthol to have an overall cooling effect there was a strong tendency for them to feel cooler overall, when they had the Menthol patches applied, compared to the Control patches (P = 0.054; *Figure 7.11*). When these participants then performed a time-trial there was a tendency (P = 0.071; *Figure 7.10*) for the cumulative work done to be greater on the Menthol trial than on the Control trial, although the total work done was not different between treatments (P = 0.130; *Figure 7.9*).

There were no other differences in any of the measured parameters between the Menthol and the Control trials in either the warming group or the cooling group, suggesting that the tendency for any difference in performance that may have occurred was not due to thermoregulatory or metabolic differences induced by the treatments.
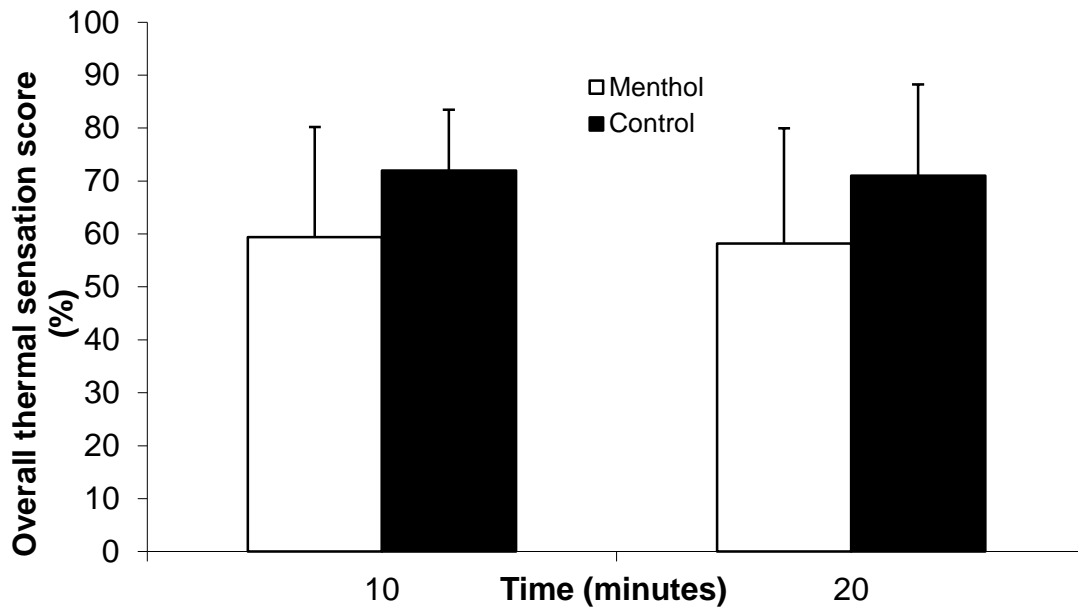
Figure 7.11. The overall thermal sensation scores in the cooling group (n = 5) for the Menthol and the Control trials. The Menthol trial tended to elicit lower thermal sensation scores than the Control trial (P = 0.054).

7.5 Discussion

The preliminary tests employed at the start of this investigation were not a suitable means of assessing participants' perception of the 1 % menthol solution during exercise in the heat. Although the preliminary tests were undertaken in the heat after a twenty minute period to allow for acclimation of skin temperature, clearly this did not reflect the conditions seen after sixty minutes of steady state exercise in the same warm environmental conditions. The effect of applying menthol to the skin when it is in different physiological states has not been investigated. Alterations in the perceived sensation effects of the menthol between the preliminary tests and the main trials in several participants suggests that the perception of the effect of menthol on the skin may be influenced by the condition of the skin, although this was not the focus of this investigation and is therefore purely speculative. Also, since the main aim of this investigation was to analyse the performance effects of altered perception of thermal sensation, conclusions can still be drawn based on the present data.

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The main finding of this study was that altering thermal sensation through application of menthol to the skin during exercise in the heat did not affect performance. This is in contrast to the findings of Mundel and Jones (2010) who found that swilling a 0.01 % menthol solution around the oral cavity increased endurance exercise capacity in the heat (34 °C) by 9 ± 12 % (63 ± 14 and 58 ± 16 minutes for the menthol and the placebo trials respectively), and resulted in improved exercise capacity in eight of the nine participants.

The menthol mouth swill resulted in greater pulmonary ventilation after twenty (72 ± 12 *vs.* 63 ± 7 L / min) and forty (75 ± 12 *vs.* 67 ± 8 L / min) minutes of exercise, compared to a placebo mouth swill trial respectively (Mundel and Jones, 2010). They also reported that while local (i.e. muscular) RPE did not differ between trials, they said global (i.e. overall) RPE tended to be lower (P = 0.09) and central (i.e. cardiovascular) RPE was lower (P = 0.01) on the menthol trials than on the control trials. The authors attribute the increase in exercise capacity to these differences, since there were no other differences between the trials, in any of the measured parameters. During the present investigation, no differences were found in any of the measured parameters. This may have occurred due to the shorter duration of the exercise test, or the different modality and site of menthol application, or, perhaps more likely, a combination of these factors.

Rating of perceived exertion in this experiment did not differ between the sensation groups, or between the trials within the sensation groups. This may suggest that the alteration in thermal sensation due to the menthol solution was not sufficient to alter the perception of the effort required to perform the exercise task. Indeed, facial cooling, using a mist of cold water, has been shown to blunt the elevation in rating of perceived exertion seen without facial cooling during cycling exercise in the heat (Mundel *et al.*, 2007). However, menthol does not actively cool the skin and this could be a reason for the lack of response in the cooling group to the application of menthol to the skin, including the facial skin. Also, with a time-trial protocol participants are asked to give their maximum effort over the twenty minutes. Therefore no matter

what their workload, it is not surprising that the ratings of perceived exertion scores did not differ between groups, or between trials within groups.

Although there was a tendency (P = 0.071) for the menthol solution to increase cumulative work done in the cooling group compared to the warming group, this failed to reach statistical significance. However, as this was the first investigation into the effect of applying menthol to the skin during exercise in the heat, it might be possible, through certain methodological changes, to show an effect of altering thermal sensation by applying menthol to the skin on exercise performance.

If an exercise to exhaustion protocol had been employed, or possibly a longer time-trial with regular re-application of the menthol patches, a difference in exercise capacity or performance may have become apparent. Indeed, since the cooling sensation of menthol remains even once the menthol is removed from the skin or mucous membrane (Eccles, 1994; Burrow *et al.,* 1983), it is possible that by uncovering or removing the patches after an initial exposure, significant changes in performance may have been seen. This, however, is purely speculative.

It should also be noted that the estimated average mean body surface area of the participants, calculated using the equation formulated by Mosteller (1987), was 1.99 ± 0.16 m^2, while the total surface area covered by the patches was only 0.05 m^2 meaning that only approximately 2.5 % of body surface area was exposed to the menthol. Therefore, it is possible that if a greater surface area had been covered by the menthol patches, a greater response to the alteration of thermal sensation may have been elicited, which in turn may have caused significant alterations in exercise performance. Indeed, many of the menthol infused clothing products available vaguely claim to "reduce the physiological factors that affect performance whilst exercising in the heat" (www.skins.net). Such clothing will cover a much greater surface area than was covered here, thereby increasing the chance of seeing effects of applying menthol to the skin. However, a balance needs to be met as increasing the

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surface area covered by the patches (or some types of clothing) decreases the opportunity for evaporative heat loss (Parsons, 2003A).

Furthermore, if a strong cooling sensation was to be elicited through the application of menthol to the skin it is possible that this could actually have a negative effect on prolonged exercise capacity or performance in the heat. The application of a 10 % solution of menthol to the skin of the whole trunk of mice led to an increase in core temperature of 1.3 °C that persisted for ninety minutes, compared to when the control solution was applied in the same way (Tajino *et al.*, 2008). The authors suggest that menthol, through activating the skin thermoreceptors, evoked heat gain responses including peripheral vasoconstriction and shivering-like responses. These responses have been seen before as a result of menthol application to the skin of mice (Tajino *et al.*, 2007). If a similar physiological response was to be evoked in humans performing prolonged exercise in the heat, it is likely that performance would be impaired. Therefore, it is important to note that, while sufficient menthol needs to be applied to elicit a cooling sensation and therefore possibly an improvement in exercise performance in the heat, an excessive activation of the peripheral thermoreceptors may result in heat gain responses, to the detriment of exercise performance in the heat.

Heart rate was consistently slightly, but significantly ($P = 0.031$), higher on the Menthol trials than on the Control trials. One possible explanation for this could be that the menthol caused an increase in blood flow to the skin. This would have required an increased cardiac output which may have been satisfied by a slight elevation in heart rate. However, previous published literature (Yosipovitch *et al.*, 1996), and the data shown in Chapter Six, show that the application of menthol to the skin has no effect on skin blood flow (see Chapter Seven for more details). Furthermore, there was no difference in the skin temperature between the Menthol and Control trials. Therefore, such a theory is unlikely to be the true explanation for the elevated heart rate seen on the Menthol trial.

In fact, the elevated heart rate on the Menthol trials is more surprising, as the cooling of facial skin through cold water (4 - 6 °C) immersion stimulates trigeminal receptors, altering vagal tone and producing a bradycardia (Finley *et al.*, 1979). Therefore, by activating the facial cooling thermoreceptors through the application of a menthol patch to the forehead, it may have been expected that heart rate would have been slightly lower on the menthol trials. However, with an average difference of only three beats per minute after five minutes of the time-trial and two beats per minute after twenty minutes of the time-trial, it is unlikely that this slight elevation would have had any physiological influence on participants or their performance.

There was a significant effect of time on pedal cadence during the time-trial, regardless of treatment. At fifteen minutes pedal cadence was 94 ± 11 revolutions per minute, while at twenty minutes pedal cadence was 114 ± 24 revolutions per minute. Of the twenty four time-trials completed throughout this study, pedal cadence increased during the last five minutes in twenty of the trials, despite participants being told very clearly at the start of each time-trial that the amount of work completed was independent of pedal cadence, as the ergometer was left in hyperbolic mode for the entire investigation. Whether this increase in pedal cadence would still have been seen if the ergometer was in linear mode, where power output is directly related to pedal cadence, is unclear. Sewell and McGregor (2008), upon whose protocol the present study is based, do not report pedal cadence at any point during their trials.

The hypothalamus co-ordinates the thermoregulation of the body. Skin temperature receptors terminate in the pre-optic area of the hypothalamus, and core temperature receptors terminate in the pre-optic area and the brain stem. The thermoregulatory responses to these inputs are the outputs of the serotonergic and dopaminergic pathways of the hypothalamus, which are also responsible for the elevation in prolactin concentrations, in response to high core temperatures.

Mills and Robertshaw (1981) found that, in resting males there is an association between mean body temperature and plasma prolactin concentration where when body temperature increases so does plasma prolactin concentration. Bridge and colleagues (2003B) showed that prolactin release during exhaustive cycling at 73 % $\dot{V}O_2$max was significantly lower from ten minutes onwards when the exercise was performed in 20 °C compared to 35 °C. They postulated therefore, that skin temperature, which was ~4 °C lower in the cooler climatic conditions, may modulate prolactin release, and not core temperature which increased similarly regardless of the climatic conditions. Armada-da-Silva *et al.* (2004) showed that facial cooling by 2 °C during exercise in the heat attenuated the rise in plasma prolactin concentration seen with no facial cooling, although this was not statistical significance. In a similar study, facial cooling did attenuate the increase in prolactin release seen in response to exercise in a warm environment reducing the elevation from a six-fold increase in the control condition to a two-fold increase in the facial cooling condition (Ansley *et al.*, 2008).

Prolactin release during exercise, and its blunted release with facial cooling, suggests that the hypothalamus or associated areas of the brain are involved in exercise fatigue (Ansley *et al.*, 2008). Although menthol does not actually cool skin, it does stimulate the thermoreceptors of the skin which terminate in the hypothalamus. It is therefore possible that the application of menthol to the skin may slow the rate of prolactin release during exercise in the heat. Plasma prolactin concentrations were not measured in the present study. However, it might be plausible to speculate that those who perceived a warming effect of the menthol would have had greater plasma prolactin concentrations in the Menthol trial than the Control trial. Conversely, those who perceived a cooling effect of the menthol may have had lower prolactin concentrations in the Menthol trial than the Control trial. This is an avenue for further research.

7.6 Conclusion

The application of a 1 % menthol solution to the skin during exercise in the heat did not alter exercise performance compared to a control solution when assessed by a pre-loaded twenty minute time-trial. This was found to be true whether analysed between treatments, between sensation groups, or within sensation groups between treatments. It was also found that there was a slight tendency for those who perceived the menthol to have a cooling effect to do more work during the time-trial.

CHAPTER EIGHT

General discussion and conclusions.

8.1 Discussion

There is a vast volume of literature on the peripheral physiological effects of prolonged exercise and the peripheral factors that cause fatigue. The central mechanisms of fatigue, and the role of the serotonergic system in the reduction in exercise performance, particularly in the heat, are yet to be fully clarified. The studies in this thesis aimed to investigate potential central limiting factors to exercise performance and possible manipulations of exercise related to this problem.

Based on their findings, Watson *et al*. (2004) purported that the exercise capacity of some individuals may be improved with branched-chain amino acid supplementation, while other individuals will not benefit from the same supplementation regimen. Therefore, the first experimental study in this thesis aimed to investigate this theory by using a protocol in which participants underwent the supplementation regimen twice, and the control regimen twice, and the difference in the response to branched-chain amino acid supplementation during an exercise capacity test in the heat was assessed.

None of the participants recruited can categorically be stated as having benefitted from an ergogenic effect of branched-chain amino acid supplementation. One participant did exercise for longer on the BCAA trials (168.5 minutes) than on the Control trials (157.9 minutes), and the duration of this individuals' exercise test was longer than that of all other participants (group mean of 105 minutes). Mittleman and colleagues (1998) also showed that during prolonged exercise in the heat, exercise capacity is improved with branched-chain amino acid supplementation (153.1 ± 13.3 minutes) compared to a placebo supplement (137.0 ± 12.2 minutes). However, none of the other participants showed an improvement in performance as a result of the supplementation protocol, which is in line with research from several authors (Blomstrand *et al*., 1995; Blomstrand *et al*., 1997; Galiano *et al*., 1991; Struder *et al*., 1998; Van Hall *et al*., 1995; Watson *et al*., 2004).

Indeed, an increase in brain tryptophan uptake during prolonged exercise is suggested to contribute to an elevation in central serotonergic activity (Blomstrand *et al.*, 1989). This has been suggested to reduce arousal levels and increase in the perception of tiredness and lethargy during exercise (Blomstrand *et al.*, 1997). By reducing the free tryptophan to branched-chain amino acid ratio in the blood, there should be a reduction in the uptake of the serotonin precursor into the brain. This may lead to a reduced production of serotonin, and consequently, theoretically, a delay in the onset of feelings of fatigue. Based on this theory, the ratings of perceived exertion might have been expected to be lower on the branched-chain amino acid trials where the ratio of free tryptophan to branched-chain amino acids was reduced. However, the ratings of perceived exertion in Chapter Three were not different between trials, a finding that corresponds well with other research (Watson *et al.*, 2004; Mittleman *et al.*, 1998; Struder *et al.*, 1996).

Improving exercise capacity by reducing the transport of tryptophan across the blood brain barrier using branched-chain amino acids has proven largely unsuccessful. Despite being used in several psychology studies, the effect of acute tryptophan depletion on exercise capacity has never been investigated. Based on the same principles as branched-chain amino acid supplementation, acute tryptophan depletion aims to reduce the entry of tryptophan into the brain, thereby reducing serotonin production, and theoretically delaying fatigue. However, when acute tryptophan depletion was undertaken by the participants in Chapter Four, there was no improvement in exercise capacity in the heat. In fact, the exercise times to exhaustion tended to be shorter with acute tryptophan depletion, compared to when the control amino acid load was administered (P=0.088). Possible explanations for this unexpected finding can be proposed, based on the supporting physiological data collected. Serotonin is known to play a critical role in the control of heart rate (Jordan, 2005; Ramage and Fozard, 1987). Van der Veen and colleagues (2008) found that heart rate did not fall on acute tryptophan depletion trials when negative feedback was given as it did on the control trials. It is possible therefore that acute tryptophan depletion may lead to a relative increase in heart rate. Put another way, the increases seen in heart

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rate during exercise, such as in the control trial of Chapter Four, may naturally be slightly blunted due to the presence of serotonin in greater concentrations than during trials designed to reduce serotonin concentration, such the tryptophan depletion trial in Chapter Four. This hypothesis is backed up by the trend throughout the tryptophan depletion trials for a higher heart rate than on the control trials ($P=0.053$).

It is possible that if acute tryptophan depletion did cause a reduction in serotonin activation, it could have led to the tendency ($P=0.069$) for rectal temperatures to be higher in the tryptophan depletion trials of Chapter Four compared to the control trials. There are several sub-groups of serotonin receptors, and the 5-HT_{2C} receptor is thought to be related to thermoregulation (Schwartz et $al.,$ 1995). When a 5-HT_{2C} receptor antagonist (Pizotifen) was administered to healthy human participants it resulted in an elevated resting core temperature (Strachan et $al.$, 2005). The aim of both acute tryptophan depletion and administration of serotonin receptor antagonists is theoretically the same, i.e. a reduction in serotonin release, and both methods have now been shown to result in elevations in resting core temperatures.

In combination, the tendencies for a higher heart rate and core temperature on the tryptophan depletion trials in Chapter Four may have led to the trend for exercise capacity to be impaired compared to the control trials. It is likely that any benefit which may have been seen due to the influences of the acute tryptophan depletion on serotonin synthesis or release were overridden by the trends for a higher heart rate and core temperature that acute tryptophan depletion evoked.

Serotonin has an influential role in factors which may ultimately affect exercise performance or capacity, but does not appear to influence it directly. It is possible that manipulation of influential factors, such as core temperature and heart rate, along with the ratio of serotonin to other neurotransmitters, may still hold interesting opportunities for further exploration, as suggested in section 8.3. Based on the possible involvement of serotonin in fatigue, the

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focus of the research conducted in this thesis moved from the nutritional manipulation of serotonin production to the transport of serotonin at the synapse.

After serotonin is synthesised and transported to the nerve terminal in vesicles, it is released into the synapse where it then participates in a plethora of responses. The concentration of serotonin in the synapse is altered by serotonin transporters. A reduction in the concentration of serotonin in the synapse could be achieved by either an increased number of serotonin transporters, an increase in the activity levels of such transporters, or possibly a combination of these two factors.

However, due to obvious restrictions, the direct examination of the transporters on the neurones is near impossible in humans. Fortunately, serotonin transporters are found not only in the serotonergic neurones but also on the membranes of blood platelets. The blood platelets offer an imperfect yet simplistic, accessible and consistent model for the serotonergic neuron. Previous research has shown that there are elevated levels of serotonin transporters on the membranes of platelets of endurance trained athletes compared to sedentary controls (Strachan and Maughan, 1998). What remained unclear was whether these differences were a state marker (an aspect which can adapt under the influence of one or more factors; Rieder, 1978) or a trait marker (an aspect which is fixed and cannot be influenced by other factors).

Chapter Five shows that those who follow an elite level training program, particularly for endurance events, have a greater number of serotonin transporters on the membranes of their blood platelets (CE: 1090 ± 76 fmol / mg protein; CS: 953 ± 68 fmol / mg protein) compared to those who had previously trained to a similar standard in a similar discipline (RE: 892 ± 88 fmol / mg protein; RS: 866 ± 58 fmol / mg protein), or those who have never done any training (I: 885 ± 58 fmol / mg protein).

The data suggests that platelet serotonin transporter density is a state marker as the maximum number of binding sites increases as a result of training but the adaptation is lost once training has stopped. Therefore, if the platelet model is transferable to the situation in the serotonergic neuron, it is possible that the perception of fatigue is altered in those currently undertaking a training regimen, compared to those who are not currently undertaking a training regimen.

Strachan (1996) offered this as a explanation for why some individuals choose to exercise regularly, while other choose not to. The data from Chapter Five refutes this hypothesis, and perception of fatigue can only be one reason in a plethora of reasons as to why an individual will not undertake regular exercise. However, it must also be noted that research into depression has found that psychological state does not influence platelet serotonin transporter density, implying that it is a trait marker and not a state marker of the illness (Lawrence *et al.,* 1993; Lawrence *et al.*, 1994).

Chapter Six looked at the effects of applying a 1 % menthol solution to the skin during heat exposure. Thirteen participants perceived a cooling sensation of menthol application, while eight perceived a warming sensation and three perceived no change in thermal sensation when compared to a control solution. There were no differences in skin temperature, core temperature or skin blood flow between any of the trials. This agrees with previous research in which menthol has been shown to elicit both warming and cooling sensations when applied to human skin, without a concomitant change in skin temperature or skin blood flow (Yosipovitch *et al.,* 1996; Kozyreva and Tkachenko, 2008).

However, the findings in human studies conflict with findings in animal studies where a 10 % menthol solution, applied to the skin on the whole trunk of mice, caused an increase in core temperature of 1.3 °C in fifteen minutes that then persisted for over ninety minutes (Tajino *et al.,* 2008). The authors suggest that the menthol, through activating the skin thermoreceptors, evoked heat gain responses such as shivering-like muscle contractions, tail

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vasoconstriction and heat seeking behaviours (Tajino *et al.,* 2007), resulting in the increase in core temperature seen in the mice.

These results, although opposing in some aspects, both imply that alterations in perceived thermal sensations occur centrally as a result of the stimulation of skin thermoreceptors. These thermoreceptors terminate in the pre-optic area of the hypothalamus, the region of the brain responsible for co-ordinating thermoregulation. Indeed, based on their qualitative data, Nakamura *et al.* (2008) suggested that the central nervous system must be involved in the processing of peripheral thermal sensation information.

Frank *et al.*, (1999) demonstrated that core temperature and skin temperature contribute equally to levels of thermal comfort in men, assessed by a ten point visual analogue scale. By artificially manipulating the perception of skin temperature to lower than its true temperature during heat exposure, Chapter Six showed that ratings of thermal comfort and thermal sensation also tended towards being colder. Conversely, by artificially manipulating the perception of skin temperature to be higher than its true temperature, ratings of thermal comfort and thermal sensation tended towards being warmer. Chapter Seven aimed to further investigate these findings and discover if the responses persisted during exercise in the heat, and if exercise performance would be influenced.

In Chapter Six the cooling sensation of the menthol seemed to be stronger than the warming sensation. Significant differences in overall thermal sensation and overall thermal comfort seen in the cooling group between the menthol and control trials were not present in the warming group. However, in Chapter Seven the opposite effect was seen; within the warming group the thermal sensation scores were greater with the menthol than the control treatment (P=0.009), while in the cooling group thermal sensation scores only tended to be lower with the menthol treatment (P=0.053).

Performance was not altered due to the menthol in those who perceived a warming sensation (P=0.405); those who felt a cooling sensation tended to

perform better on the menthol trial compared to the control trial (P=0.071). This suggests that when participants feel cooler than they actually are, exercise performance in a warm environment may be improved. Mundel and Jones (2010) found that swilling a menthol solution around the oral cavity increased endurance exercise capacity in the heat by 9 ± 12 %. They reported that overall ratings of perceived exertion tended to be lower (P=0.09) and cardiopulmonary ratings of perceived exertion were lower (P=0.01) on the menthol trials than on the control trials.

Ratings of perceived exertion in this experiment did not differ between the sensation groups, or between the treatments within the sensation groups. This may suggest that the alteration in thermal sensation due to the menthol solution was not sufficient to alter the perception of the effort required to perform the required exercise. However, with a time-trial protocol participants are asked to give their maximum effort over the twenty minutes and therefore no matter what their workload was, it is not surprising that the rating of perceived exertion scores did not differ between groups, or between treatments within groups.

It should also be noted that only approximately 2.5 % of body surface area was exposed to the menthol solution in Chapter Seven. Therefore, it is possible that if a greater surface area had been covered by the menthol patches, a greater response to the alteration of thermal sensation may have been elicited, which in turn may have caused significant alterations in exercise performance. However, future research in this area should also consider the results of Tajino *et al.,* (2008) who found that applying menthol to a large surface area and thus stimulating many peripheral cold thermoreceptors, albeit of mice, elicited heat gain responses which would be likely to have a detrimental effect on exercise performance.

The thermoregulatory responses to the inputs from skin thermoreceptors are outputs from the serotonergic and dopaminergic pathways of the hypothalamus. Once again it appears that serotonin many have a role to play in exercise capacity and performance through its effects on related

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physiological systems. Indeed, the serotonergic and dopaminergic pathways are responsible for the elevation in prolactin concentrations at high core temperatures. Bridge and colleagues (2003B) postulated that skin temperature, and not core temperature, may modulate prolactin release. Although menthol does not actually alter skin temperature, it does stimulate the thermoreceptors of the skin and mucous membranes which terminate in the hypothalamus. It is therefore possible that those who perceived a warming sensation due to menthol would have had higher plasma prolactin concentrations in the menthol trial than the control trial; while conversely, those who perceived a cooling sensation may have had lower prolactin concentrations in the menthol trial than the control trial.

Despite being used for many years, it was not until 1989 that a study was conducted with the sole purpose of assessing the reliability of the time to exhaustion exercise capacity test. Krebs and Powers (1989) showed that the day to day variability of high intensity cycle to exhaustion tests had a within participant variability ranging from 5 % to 56 %. However, these findings were widely dismissed due to the lack of a familiarisation trial, minimal standardisation of pre-test conditions and because the exercise intensity was not accurately measured meaning that different workloads could have been performed on each trial. The findings were also dismissed as much lower levels of variability were also reported (7.0 ± 5.1 %; Maughan *et al.,* 1989). The issue of the reliability of time to exhaustion tests was again brought to the fore when Jeukendrup and colleagues (1996) showed that, while time-trial protocols had a coefficient of variation of around 3.5 %, time to exhaustion tests had a coefficient of variation of around 26.6 %.

Indeed, the data presented in this thesis does back-up their findings to some extent, with time to exhaustion trials showing a greater variation (11.0 ± 11.2 % in Chapter Three and 11.5 ± 12.4 % in Chapter Four) than a pre-loaded time-trial protocol (3.9 ± 9.6 % in Chapter Seven). However, the variation in the cycle to exhaustion data in the Jeukendrup study (1996) seems questionably large. For example, one participant exercised for 43.8 minutes on one occasion, and then 111.8 minutes on another occasion. It is possible

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that there were underlying reasons for such dramatic alterations in ability, but it is not possible to explore this avenue due to the lack of supporting physiological data presented in their study.

Although Coyle *et al.* (1991) have shown that performance in a one hour laboratory-based exercise test is closely related to the performance of a forty kilometre time-trial, Palmer and colleagues (1996) found that forty kilometre time-trial tests using competitive cyclists were approximately 8 % faster when done in a laboratory environment than in an outdoor competitive setting. Reasons for this will include environmental conditions, air resistance, road surface, terrain and route. These factors will always exist outside of a laboratory, causing athletes to slow down slightly. These findings also suggest that the competitiveness of real race environments is not sufficient to override such external influences.

A purported problem of time-trial tests is that they are influenced by pacing strategies (Baron *et al.,* 2009) and due to the infinite number of strategies available (St Clair Gibson *et al.,* 2006), sometimes these pacing strategies do not produce optimal performance (Hinckson and Hopkins, 2005) and merely add noise to any data. However, Atkinson and Nevill (2005) argue that pacing strategies are an inherent component of real exercise and sporting performance and therefore cannot be dismissed as a disadvantage of the time-trial method. Unfortunately, in exercise physiology the re-creation of a sporting situation is not usually the aim of the research, it is often conducted to explore or elucidate physiological mechanisms and try to generate explanations of responses seen due to interventions.

It has been suggested that one of the reasons that time to exhaustion tests appear to have greater variability is simply because a small change in a participants' ability to produce power will result in a large change in time to exhaustion (Hopkins *et al.*, 2001). Through sophisticated mathematical models Hinckson and Hopkins (2005) have successfully shown that time to exhaustion can actually be a highly reliable measurement (coefficients of variation of only 1 – 2 %), allowing the detection of small changes in capacity

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caused by an intervention. However, the complexity of the methodology required to generate data that is suitable for such mathematical modelling is greatly increased.

Some studies appear to find no learning effect of the time-trial tests (Currell *et al.*, 2006; Jeukendrup *et al.,* 1996) while others find that coefficient of variation decreases as the number of trials increases (Marino *et al.,* 2002; Laursen *et al.,* 2003), as was seen in all of the exercise chapters in this thesis. In the data presented here, all coefficient of variation values were reduced by the inclusion of a familiarisation trial (Chapter Three 21.7 ± 20.4 % to 11.0 ± 11.2 % , Chapter Four 14.4 ± 14.8 % to 11.5 ± 12.4 %, Chapter Seven 4.7 ± 11.0 % to 3.9 ± 9.6 %) indicating that there is a learning effect with both protocols.

8.2 Conclusions

1) The nutritional manipulation of the ratio of free tryptophan to branched-chain amino acids or the total tryptophan concentration in the plasma had no effect on prolonged exercise capacity in the heat.

2) Endurance training appears to increase the number of serotonin transporter sites on the blood platelet, which can be used as a suitable peripheral model for the central serotonergic neuron.

3) Menthol can elicit warming or cooling sensations when applied to the skin during heat exposure. If a warming sensation is perceived, exercise performance in the heat does not seem to be impaired. However, if a cooling sensation is perceived, there is a tendency for exercise performance in the heat to improve.

4) Exercise performance measures in the heat seem more reliable than exercise capacity measures, although the aims of an investigation should be considered when choosing an exercise test protocol.

8.3 Future areas of research

There are still a great number of unanswered questions regarding the origin of fatigue during exercise. This thesis has tried to investigate just a small selection of the potential avenues for research in this field. Firstly, the purported alterations to exercise capacity that amino acid supplementation could theoretically cause were investigated. The thesis went on to look at the role that training could play in bringing about adaptations to improve resistance to central fatigue. Finally, the role that altering temperature perception, through the use of menthol, could have both physiologically and in an exercise performance setting was investigated. Based on the conclusions outlined above in section 8.2, this section will raise research questions which would be interesting to attempt to answer and may provide further insight in to the role of the brain during exercise fatigue.

From the findings presented in the first two experimental chapters (Chapters Three and Four), the Newsholme theory of central fatigue appears to be too simplistic. The concept of altering serotonin production solely through the manipulation of tryptophan delivery across the blood brain barrier lacks supporting evidence in human participants. It seems fruitless to continue research down this avenue with the aim of improving exercise capacity or performance. However, research employing pharmacological agents offers a much more direct avenue of exploration into serotonin-mediated fatigue. Furthermore, pharmacological agents exist which can influence more than just the serotonergic system, and the role of the dopaminergic system, and the ratio in the activity of the dopaminergic and serotonergic systems, in fatigue during prolonged exercise warrants further systematic investigation.

Chapter Five suggests that endurance training may lead to adaptations within the central nervous system; potentially altering an individual's the perception of fatigue. Several interesting avenues of research could grow from these findings. Is there a dose-response relationship in terms of training history or training load / intensity / duration / frequency and the increases seen in serotonin transporter density on the blood platelet? Is this apparent adaptation to training possibly linked to ethnicity, thereby providing an

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explanation for the dominance of those of North and East African descent in distance running? What is the role of Vmax, the maximum rate of binding, within the blood platelet and does this differ between sporting disciplines or alter with training? What is the time course for this apparent adaptation? The answers to all of these questions will undoubtedly be difficult to come by and involve time-consuming, expensive and extensive studies, but this does not mean that such questions should be ignored.

Chapter Six and Chapter Seven investigated the effects of manipulating the perception of skin temperature without a concomitant alteration in actual skin temperature. The altered afferent sensory input, received by the pre-optic area of the anterior hypothalamus from the peripheral thermoreceptors, after an application of menthol may influence the thermoregulatory responses initiated based on the information received. Future research based on Chapter Seven could consider covering a larger surface area in the menthol solution. If assessment of thermoregulatory responses is of interest then a longer exercise period after the application of the menthol to the skin could also be employed. A compound which targets warm receptors, such a capsaicin, could also be employed to look at the physiological responses to the manipulation of thermal sensation. The precise relationship between prolactin, heat exposure, and actual and perceived skin temperature is also an avenue for further investigation. Furthermore, the relationship between thermal comfort and exercise performance, independent of core and/or skin temperature, would also be an interesting avenue of exploration, if appropriate methodologies could be developed.

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APPENDICES

A: Gas equations

$SWVP = (1.1001 * \text{gas temperature}) - 4.19\text{mmHg}$

V_{ESTPD} (L/min) $= V_{EATPS} * ((BP - SWVP) / 760) * (273 / (273 + \text{room temp}))$

$F_I N_2$ (%) $= 100 - 20.93 - 0.03$

$F_E N_2$ (%) $= 100 - F_E O_2 - F_E CO_2$

V_I (L/min) $= (F_E N_2 / F_I N_2) * VE$

VO_{2I} (L/min) $= V_I * F_I O_2 / 100$

VO_{2E} (L/min) $= V_E * F_E O_2 / 100$

VCO_{2I} (L/min) $= V_I * F_I CO_2 / 100$

VCO_{2E} (L/min) $= V_E * F_E CO_2 / 100$

VO_2 (L/min) $= VO_{2I} - VO_{2E}$

VCO_2 (L/min) $= VCO_{2E} - VCO_{2I}$

$RER = VCO_2 / VO_2$

Rate of CHO oxidation (g/min) $= (4.585 * VCO_2) - (3.226 * VO_2)$

Rate of fat oxidation (g/min) $= (1.695 * VO_2) - (1.701 * VCO_2)$

Energy expenditure (kJ/min) $=$ (rate of fat oxidation $* 39$) $+$ (rate of CHO oxidation $* 16$)

B: Rating of perceived exertion

6

7   Very very light

8

9   Very light

10

11   Fairly Light

12

13   Fairly hard

14

15   Hard

16

17   Very hard

18

19   Extremely hard

20   Maximum

C: Thermal comfort scale

-10   Cold impossible to bear

-9

-8   Very cold, shivering hard

-7

-6   Cold, light shivering

-5

-4   Most areas of the body feel cold

-3

-2   Some areas of the body feel cold

-1

0   Neutral

1

2   Some areas of the body feel warm

3

4   Most areas of the body feel hot

5

6   Very hot, uncomfortable

7

8   Extremely hot, close to limit

9

10   Heat impossible to bear

Subjective feelings Questionnaire

1) Thinking about your CURRENT thermal sensation, please indicate on the scales below how you feel now.

Right forearm?

a)

Cold   Hot

Left forearm?

b)

Cold   Hot

2) With reference to your whole body, please consider your OVERALL thermal <u>sensation</u>.

Cold   Neutral   Hot

3) With reference to your whole body please consider your OVERALL thermal <u>comfort</u>.

Too much cool   Comfortable   Too much warm

E: Blood volume calculations

In the equations below the subscript B denotes the first sample (Before) and the subscript A denotes the second sample (After).

$$BV_A = BV_B * (Hb_B/Hb_A)$$
$$CV_A = BV_A * Hct_A$$
$$PV_A = BV_A - CV_A$$
$$PV_B = 100 * (1 - Hct_A)$$

The values are reported as a percentage change relative to the first sample collected and are calculated using the equations below:

$$\Delta BV = 100 * (BV_A - BV_B) / BV_B$$
$$\Delta CV = 100 * (CV_A - CVB) / CV_B$$
$$\Delta PV = 100 * (PV_A - PV_B) / PV_B$$