Possible neurological mechanisms of fatigue during prolonged exercise in a warm environment

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POSSIBLE NEUROBIOLOGICAL MECHANISMS OF FATIGUE DURING PROLONGED EXERCISE IN A WARM ENVIRONMENT

By

Phillip Watson

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University

Abstract

Capacity to perform prolonged exercise is reduced in high ambient temperatures, but this premature fatigue is not adequately explained by peripheral mechanisms. The aim of this thesis was to examine some possible underlying mechanisms of central fatigue operating during prolonged exercise in a warm environment.

The first series of experiments investigated the effect of nutritional manipulation of central serotonergic activity through alterations to the plasma concentration ratio of free-tryptophan to branched-chain amino acids (f-TRP:BCAA). In contrast to previous reports, acute BCAA supplementation failed to alter perceived exertion and delay the onset of fatigue (Chapter 3). This response was similar when exercise was preceded by an exercise and diet regimen designed to reduce glycogen availability (Chapter 4). The ingestion of meals containing added carbohydrate and fat did not alter f-TRP:BCAA at rest (Chapter 5).

Acute dopaminergic / noradrenergic reuptake inhibition with bupropion increased exercise performance by 9% in warm conditions (30 °C), but this effect was not apparent at 18 °C (Chapter 6). This response was accompanied by attainment of a higher core temperature and heart rate towards the end of the bupropion trial in the heat despite no detectable difference in perceived exertion and thermal stress. These data suggested that maintenance of catecholaminergic activity may dampen inhibitory signals from the CNS due to the attainment of a high core temperature, allowing power output to be maintained.

The blood-brain barrier (BBB) regulates the exchange of substances between the cerebral interstitial fluid and the blood to maintain a stable environment for the CNS. If the BBB is compromised this may adversely influence normal brain function. Serum S100b, a proposed peripheral marker of BBB permeability, was increased following exercise in a warm environment (Chapter 7). These data indicate that exposure to combined exercise and heat stress may result in a loss of BBB integrity.

Keywords: central nervous system, serotonin, catecholamines, blood-brain barrier, heat stress, core temperature
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Publications

The findings of the studies reported in this thesis have been published as follows:

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List of Abbreviations

List of abbreviations contained in this thesis. Abbreviations are defined in the text in the first instance.

5-HIAA 5-hydroxyindoleacetic acid
5-HT 5-hydroxytryptamine (serotonin)
5-HTP 5-hydroxytryptophan
ACTH adrenocorticotropic hormone
ANOVA analysis of variance
BBB blood-brain barrier
BCSFB blood-cerebrospinal fluid barrier
BCAA branched-chain amino acids (sum of leucine, isoleucine and valine)
CHO carbohydrate
Cl chloride
CNS central nervous system
CO₂ carbon dioxide
CSF cerebrospinal fluid
CV coefficient of variation
DA dopamine
DOPA dihydroxyphenylalanine
DOPAC dihydroxyphenylacetic acid
K₂EDTA potassium ethylenediaminetetraacetic acid
ELISA enzyme linked immunosorbant assay
f-TRP free tryptophan
f-TRP:BCAA plasma concentration ratio of f-TRP to BCAA
g gram
h hour
HPA-axis hypothalamic-pituitary-adrenocortical-axis
HR heart rate
HVA homovanillic acid
K potassium
K₂EDTA potassium ethylenediaminetetraacetic acid
kcal  kilocalorie
kg    kilogram
kilodalton  kDa
L     litre
LNAA  large neutral amino acids (sum of leucine, isoleucine, valine, tyrosine, phenylalanine, methionine and histidine)
m    metre
MAO   monoamine oxidase
mg    milligram
mL    millilitre
mmol  millimole
min   minute
Na    sodium
NA    noradrenaline
NH₃   ammonia
O₂    oxygen
PCA   perchloric acid
PO/AH preoptic anterior hypothalamus
PRL   prolactin
RER   respiratory exchange ratio
RPE   rating of perceived exertion
s     second
SD    standard deviation
STPD  standard temperature, pressure, dry
SSRI  selective serotonin reuptake inhibitor
Tcore Core temperature
TRP   tryptophan
Tskin weighted mean skin temperature
TYR   tyrosine
VE    expired gas volume
VMA   3-methoxy-4-hydroxymandelic acid
VO₂ max maximum oxygen uptake
VO₂ peak peak oxygen uptake
\( W \)  
watt

\( W_{\text{max}} \)  
maximal workload

\( ^\circ \text{C} \)  
degrees celsius
Chapter 1

General Introduction
1.1 – Fatigue during prolonged exercise

The development of fatigue during prolonged submaximal exercise has featured as a predominant area of exercise-related research, yet a number of unanswered questions remain. Fatigue has been defined as the inability to maintain the required or expected power output that leads to a loss of performance in a given task (Edwards, 1981). This definition can be sub-categorised into central and peripheral components, relating to the location of the inhibition. Central fatigue refers to a loss of neural drive, resulting in a reduction in motor unit recruitment. While peripheral fatigue encompasses events that occur independently of central fatigue, including disturbances to neuromuscular transmission, sarcolemma excitability and excitation-contraction coupling (Davis, 2000).

Factors thought to be important in the development of peripheral fatigue during prolonged exercise include the depletion of muscle glycogen (Bergstrom et al., 1967) limiting the rate of ADP rephosphorylation (Maughan et al., 1997) and the loss of body fluids resulting in increased cardiovascular, metabolic and thermoregulatory strain. The latter is thought to be particularly important when exercise is performed in warm ambient conditions, as it elevates body heat storage, accelerating the development of hyperthermia (Sawka et al., 1992). The attainment of a high core temperature is associated with a reduction in motivation and drive to continue exercise and is thought to be a key factor in the development of fatigue during exercise in the heat, thus serving as a form of safety mechanism limiting further heat production (Bruck and Olschewski, 1987; Nielsen, 1992).

The notion that the CNS is involved in the development of fatigue is not new. Early work conducted by Alessandro Mosso (1904) crudely demonstrated a reduced capacity to perform repeated muscle contractions following a mental effort and resulted in the development of the term ‘mental fatigue’ (Mosso, 1904). Since this original work many advances have been made to clarify the role of the CNS in the development of fatigue (for a review see Gandevia, 2001). While there have been a number of neurobiological mechanisms proposed to explain the apparent loss of neural drive referred to as central fatigue, the neurotransmitter hypothesis put forward by Newsholme and colleagues (1987) has received the greatest academic recognition.
1.2 - Central Neurotransmission

Neurotransmitters are a group of chemicals released from nerve endings to transmit impulses across synapses to other neurons or across gaps that exist between the nerves and the muscles or glands that they supply (Cooper et al., 2003). Four criteria have been proposed for a compound to qualify as a neurotransmitter (Kandel et al., 2000): 1) it has to be synthesised by a neuron 2) it is present in the presynaptic terminal and when released exerts an action on a postsynaptic neuron / effector organ 3) when exogenously administered it mimics the action of the endogenous transmitter 4) a mechanism exists to remove it from the site of action. At present around 60 biological substances have been classified as neurotransmitters, including acetylcholine (ACh), biogenic amines (serotonin, dopamine, noradrenaline), neuropeptides (endorphins, melatonin etc), some amino acids (glutamate, GABA etc) and gases (nitric oxide). Neurotransmitters act by either binding to receptors located on the post-synaptic membrane producing a rapid change through ion channels within the cell, whereas others exert an effect through the activation of secondary messenger systems subsequently influencing enzymatic reactions inside the cell (Cooper et al., 2003). Although all neurotransmitters play important roles within the CNS, for the purpose of this review only serotonin (5-hydroxytryptamine; 5-HT), dopamine (DA) and noradrenaline (NA) will be discussed in detail, as these have been implicated in the genesis of central fatigue during prolonged physical exertion.

1.2.1 - Serotonin

Within the mammalian brain, serotonergic neurons comprise one of the smallest but most widely distributed neuronal systems. 5-HT cell bodies originate primarily in clusters found in the brainstem, with dense collections of cells present in, or near, the raphe nuclei (Jacobs and Azmitia, 1992). These have been divided into two functionally significant groups: the superior group supplying the forebrain and the inferior group largely projecting into the spinal cord (Jacobs and Fomal, 1997). Serotonergic fibres project outwards throughout the brain and spinal cord, enabling the innervation of almost all areas of the brain. Despite its apparent central importance, 5-HT has a relatively small presence in the CNS. Around 1 in every 1
million CNS neurons is classified as serotonergic, with only 1 - 2% of the body's 5-HT content present within the CNS (Cooper et al., 2003). Many non-nervous cells, including the chromaffin cells of the gastrointestinal tract, mast cells and blood platelets are capable of synthesising and / or transporting this biogenic amine (Struder and Weicker, 2001a).

The activity of central serotonergic neurons changes throughout the sleep-wake-arousal cycle, exhibiting a strong positive relationship with level of behavioural arousal (Trulson and Jacobs, 1979). The investigation of 5-HT neuronal firing rate in the dorsal raphe nuclei (DRN) of the domestic cat has demonstrated that serotonergic neurons exhibit a slow regular discharge rate during quiet waking state that increases significantly during periods of arousal. An elevation in the firing rate of serotonergic neurons during periods of arousal and stress has been associated with increased brain 5-HT content (Chaouloff et al., 1985; Chaouloff et al., 1986a). Whilst sleeping a progressive fall in neuronal discharge rate is apparent, with almost no activity measurable during rapid eye movement (REM) sleep. It is this characteristic pattern of neuronal activity that has led to the suggestion that the serotonergic system acts as a neuromodulator, rather than directly mediating behavioural and physiological responses (Gandevia, 2001; Jacobs and Azmitia, 1992; Trulson and Jacobs, 1979). This premise may be important to the ideas presented in this thesis, and will be discussed further.

As 5-HT is charged at physiological pH (pK = 9.8), it is unable to cross the blood-brain barrier (BBB) or readily diffuse across cell membranes from the extracellular space. Therefore, neurons are required to synthesize it for themselves, and it is the presence of enzymes required in this process that specifically characterises the serotonergic system (Cooper et al., 2003). The initial step in this process is the cerebral uptake of the amino acid tryptophan (TRP), which acts as the substrate in the formation of 5-HT. TRP delivered to serotonergic neurons first undergoes hydroxylation by the rate-limiting enzyme tryptophan hydroxylase to form 5-hydroxytryptophan (5-HTP). 5-HTP is then decarboxylated into 5-HT by aromatic L-amino acid decarboxylase. This step in 5-HT synthesis occurs rapidly, so little 5-HTP is present within the brain at any one time. Under resting conditions in healthy individuals tryptophan hydroxylase is approximately 50% saturated. Therefore an
increase or decrease in TRP transport across the BBB produces a corresponding change in the rate of 5-HT synthesis within the CNS (Hamon et al., 1981). The fate of increased 5-HT synthesis is discussed below.

After release into the synapse from presynaptic vesicles, the action of 5-HT is terminated by reuptake into the presynaptic nerve terminal (Cooper et al., 2003). Pharmacological agents that selectively inhibit this uptake process (selective serotonin reuptake inhibitors; SSRI) increase the concentration of 5-HT present at the postsynaptic receptors and have been widely administered in the treatment of various psychiatric disorders, in particular depression. Following reuptake, serotonin may return to the synaptic vesicles for reuse or be metabolised by monoamine oxidase A (MAO A) to 5-hydroxyindole acetaldehyde and then oxidised by aldehyde dehydrogenase to 5-hydroxyindoleacetic acid (5-HIAA). The concentration of 5-HIAA present in the brain and CSF has been used as marker of serotonergic activity in psychiatric disorders. The metabolic pathways involved in the synthesis and metabolism of serotonin are shown in Figure 1.1.
Figure 1.1: The metabolic pathway for synthesis and metabolism of 5-HT (Cooper et al., 2003)

The rate of 5-HT synthesis is largely dependent upon the peripheral availability of the essential amino acid, TRP, which is supplied from dietary proteins (e.g. meats, nuts, milk, eggs etc). It was initially believed that the concentration of plasma TRP was the only determinant of 5-HT synthesis (Fernstrom and Wurtman, 1971), but subsequent work has gradually revealed that the situation is complicated by additional factors relating to the transport of TRP across the BBB.

A series of studies undertaken by Fernstrom and colleagues has helped clarify the role of competing amino acids in limiting TRP uptake across the BBB, and demonstrated that brain TRP content is not wholly dependent upon levels of circulating plasma TRP (Fernstrom and Faller, 1978; Fernstrom et al., 1976; Fernstrom and Wurtman, 1971;
Fernstrom and Wurtman, 1972; Leathwood and Fernstrom, 1990; Pan et al., 1982). A marked increase in brain 5-HT levels was reported following the ingestion of a CHO-containing meal, resulting from a transient fall in plasma large neutral amino acids (LNAA) concentrations caused by the release of insulin (Fernstrom and Wurtman, 1971). This response was attenuated when protein was added to the ingested meal (Fernstrom and Wurtman, 1972). It is now accepted that TRP uptake into the CNS occurs via a specific BBB transporter shared in competition with other LNAA (Hargreaves and Pardridge, 1988). This work led to the suggestion that the plasma concentration ratio of TRP to competing amino acids is important in determining the rate of cerebral TRP uptake, with an increase in this ratio leading to increased brain TRP and 5-HT content (Fernstrom, 1983).

However, this situation is further complicated by a degree of controversy regarding the availability of plasma TRP for uptake into the CNS by the LNAA-carrier (Bloxam et al., 1980; Fernstrom et al., 1976; Knott and Curzon, 1972; Madras et al., 1974a; Pardridge, 1979). Following intestinal absorption, around 80–90% of TRP circulates in the blood loosely bound to albumin (McMenamy and Oncley, 1958), with the remaining fraction circulating in a free form (free-tryptophan; f-TRP). Originally it was believed that albumin-bound TRP was not available for CNS uptake (Knott and Curzon, 1972), but subsequent findings have failed to identify a significant association between changes in the size of the f-TRP pool and brain TRP content (Fernstrom et al., 1976; Madras et al., 1974a; Madras et al., 1974b; Pardridge, 1979). While Bloxam and colleagues (1980) subsequently reported that TRP binding to albumin was important in determining brain TRP uptake, the balance of evidence suggests that under resting conditions total-TRP concentration closely reflects the availability of TRP for uptake into the CNS (Fernstrom, 1983; Pardridge, 1983). The apparent poor correlation between plasma f-TRP and brain TRP content may be explained by the ability of the LNAA-transporter to directly disassociate TRP from albumin during passage through the brain capillaries (Pardridge, 1983). Changes in cerebral blood flow and BBB transport may alter the kinetics of amino acid transport during exercise. This is considered in Section 1.3.

While the studies described above contributed to knowledge regarding the influence of diet on central serotonergic neurotransmission, it is important to recognise the
limitations of this early work. These findings are based on the association between concentrations of circulating amino acids and changes in the brain TRP and 5-HT content, but an increase in 5-HT synthesis may not produce a comparable change in synaptic 5-HT release. As early studies analysed the tissue content of brain homogenates, what is unclear from these data is how the kinetics of transmitter storage and release are influenced by an upregulation of 5-HT synthesis (Meeusen et al., 2001a). The development of in vivo brain microdialysis has enabled the direct analyses of extracellular neurotransmitters and metabolites from the brain of resting and active animals with limited tissue trauma. Meeusen and co-workers (1996) demonstrated that increased TRP availability resulted in an elevation in extracellular 5-HT and 5-HIAA concentrations in 24-hour fasted, but not fed, rats. The apparent discrepancy between these metabolic states has been ascribed to an increased TRP delivery and an upregulation in serotonergic neuron firing rate in food-deprived animals. It is important to note that microdialysis studies only consider changes in a single brain structure (e.g. hippocampus, hypothalamus etc) and these findings may not reflect changes in other areas of the brain. Additionally, the effect of changes in substrate supply on the balance between neurotransmitter storage and release may vary across species.

It is naïve to believe that the only regulator of 5-HT release and synthesis is the delivery of TRP to a serotonergic neuron. A number of subtle control factors have been proposed to influence 5-HT synthesis, including the availability of oxygen and pteridine - cofactors that are required in the hydroxylation of TRP (Cooper et al., 2003). Serotonin release is thought to be influenced by the activity of serotonergic and other neurotransmitter systems, including DA and GABA as well as cerebral glucose availability (Bequet et al., 2002). Additionally, increases in extracellular 5-HT concentrations have been demonstrated to activate 5-HT_{1A} (and potentially 5-HT_{1B/1D}) autoreceptors producing a negative feedback, normalising 5-HT levels due to a reduction in the firing rate of the neuron (Artigas et al., 1996). This response is thought to be largely responsible for the delayed therapeutic response observed following the administration of SSRI in the treatment of symptoms of depression.
1.2.2 – Dopamine and Noradrenaline

The primary catecholaminergic neurotransmitters important to the study of brain neurochemistry are dopamine (DA) and noradrenaline (NA). While the existence of dopamine has long been known, its function as a neurotransmitter and distribution within the brain has only been explored relatively recently. Dopaminergic neurons within the brain originate from the ventral tegmental area (VTA) and substantia nigra in the midbrain and project to areas of the striatum, frontal cortex and the limbic system (Cooper et al., 2003). The cells of the NA system are found in clusters around the brain stem, originating from the locus ceruleus and lateral brain stem tegmentum. These nerve fibers give rise to the five major NA tracts that innervate almost every part of the brain, including the cerebral cortex, thalamus, hypothalamus, cerebellum, midbrain and the spinal cord. Noradrenaline is also released by sympathetic neurons of the autonomic nervous system, playing an important role in regulation vascular tone (Astrand et al., 2003).

The catecholaminergic neurotransmitters are synthesised through a shared metabolic pathway, with the amino acid tyrosine (TYR) acting as the precursor (Figure 1.2). TYR is found in protein-rich dietary sources, including chicken and milk, but unlike TRP it is a non-essential LNAA that can also be synthesised from phenylalanine in the liver. Cerebral uptake of TYR is subject to competitive transport across the BBB by the LNAA-carrier system, which is shared with TRP and the other LNAA as discussed above (Fernstrom, 1983). The first step in the synthesis of DA and NA is the conversion of TYR to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. This is the rate-limiting step, with the other enzymes in the biosynthetic pathway possessing an activity 100 – 1000 times greater than tyrosine hydroxylase (Cooper et al., 2003). Tyrosine hydroxylase is thought to be fully saturated with substrate under resting conditions (unlike tryptophan hydroxylase), so an increase in TYR delivery into the CNS would produce little effect on rates of dopamine synthesis (Davis and Bailey, 1997; Nestler et al., 2001). Although there is some evidence from studies employing in vivo microdialysis that extracellular DA concentrations are responsive to increased precursor supply at rest (During et al., 1989). DOPA is then converted to DA by DOPA decarboxylase. Noradrenergic neurones are characterised, and differ
from dopaminergic neurons, by the presence of dopamine β-hydroxylase, which is responsible for the hydroxylation of DA to NA.

![Chemical diagram of the metabolic pathway for synthesis and metabolism of DA and NA](image)

**Figure 1.2:** The metabolic pathway for synthesis and metabolism of DA and NA (Cooper et al., 2003)

The action of both DA and NA are terminated by reuptake into the presynaptic nerve terminal (Cooper et al., 2003). Once inside the axon terminal the catecholamines may return to the synaptic vesicles for reuse or be metabolised. DA is metabolised to 3,4-dihydroxyphenylacetic acid (DOPAC) by MAO and aldehyde dehydrogenase, which can be further reduced to homovanillic acid (HVA) by catechol-O-methyltransferase.
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(not shown in Figure 1.2). In a similar manner to 5-HIAA, the accumulation of HVA in the brain and CSF has been employed as an indirect index of the activity of the dopaminergic neurons. The enzymes MAO, aldehyde dehydrogenase and catechol-O-methyltransferase are responsible for the catabolism of NA to its metabolite 3-methoxy-4-hydroxymandelic acid (VMA).

The synthesis and turnover of both DA and NA are highly dependent on neuronal discharge activity (Cooper et al., 2003). Catecholamine synthesis is significantly upregulated during arousal and stress where a marked increase in catecholaminergic activity is apparent (Milner and Wurtman, 1986). Prolonged exposure to stress is thought to result in a partial depletion of catecholamines in some brain regions, including the hippocampus, striatum and midbrain (Bailey et al., 1992; Owasoyo et al., 1992; Palkovits et al., 1975). This reduction in extracellular catecholamine concentrations may be important in the loss of motor control often observed under these conditions (Davis, 2000). The regulation of catecholamine synthesis appears to be largely mediated by a change in the activity of tyrosine hydroxylase through end product inhibition, negative feedback from presynaptic dopamine receptors and the activity of the catecholaminergic neurons (Cooper et al., 2003).

1.3 – Behavioural Responses to Changes in Central Neurotransmission

Physiological and behavioural responses modulated or mediated through changes in central serotonergic and / or catecholaminergic neurotransmission are triggered by ligand binding to postsynaptic receptors (Dinan, 1996). To date more than ten 5-HT receptors (5-HT₁⁻⁷) have been classified, distributed in different areas of the brain. There are fewer subtypes of catecholamine receptors (Dopamine - D₁, D₂; Noradrenaline - α₁, α₂, β).

Serotonergic neurones play a key role in various behavioural and autonomic functions such as the sleep-wake-arousal cycle, feeding, temperature regulation, nociception, sexual activity, activation of the hypothalamic-pituitary-adrenal axis and locomotion (Jacobs and Fornal, 1997). Neurotransmitters, in particular 5-HT, have been
implicated in the aetiology of a wide variety of psychiatric disorders, and the pharmacological manipulation of central monoamine activity forms the basis of many modern treatments. A large body of evidence supports a role of nutrition in mediating changes in arousal, mood, mental function and perception of fatigue (Spring et al., 1986; Young, 1991). While these responses were thought to be mediated through manipulation of serotonergic neurotransmission, it is likely that changes in brain glucose availability may also be important (Bequet et al., 2002). While, 5-HT is often implicated in almost all behavioural or physiological responses, evidence usually suggests that it is not directly responsible (Jacobs and Azmitia, 1992). This has led to the suggestion that 5-HT primarily acts as a neuromodulator, possessing little intrinsic activity, but exerting a response by influencing other neurotransmitter systems (Cooper et al., 2003).

The DA system plays an important role in motivation, memory, reward and attention (Davis and Bailey, 1997). Evidence suggests that animals are motivated to perform behaviours that stimulate DA release in the VTA (Burgess et al., 1991) and addiction is common feature of a number of dopaminergic drugs. The DA activity in the caudate and accumbens nuclei appears to be involved in the control of voluntary movement and locomotion (Freed and Yamamoto, 1985). NA neurons seem to be important to the regulation of attention, arousal and sleep-awake cycles as well as learning and memory, anxiety, pain, mood and brain metabolism (Cooper et al., 1994). In a similar manner to the dopaminergic system, noradrenergic mechanisms are involved in feelings of reward. NA has also been implicated in aetiology of depression (Ascher et al., 1995).

1.4 - Exercise, Serotonergic Neurotransmission and Newsholme's Central Fatigue Hypothesis

As mentioned in Section 1.1, the notion that the CNS is involved in the development of fatigue during various activities including mental and visual tasks as well as physical activity is not a new proposition. Work conducted over the past 25 years has established a clear link between exercise and improved mental health, leading to the prescription of exercise as an alternative treatment for depression, anxiety and stress.
This led to the suggestion that exercise may directly influence the synthesis and metabolism of central monoamines, in particular DA, NA and 5-HT.

The effects of exercise on the central serotonin levels of rats was extensively investigated by Chaouloff and colleagues during the 1980's (Chaouloff et al., 1985; Chaouloff et al., 1986a; Chaouloff et al., 1986b; Chaouloff et al., 1987). This work demonstrated that treadmill exercise resulted in a progressive elevation in brain TRP and 5-HIAA content, with 5-HT also tending to increase (Chaouloff et al., 1985; Chaouloff et al., 1986b). As 5-HIAA is employed as an index of brain 5-HT activity, the authors concluded that exercise resulted in an increased 5-HT turnover. The vast majority of the available evidence suggests that whole brain 5-HT and 5-HIAA content is increased following exercise (Bailey et al., 1992; Chaouloff et al., 1985; Romanowski and Grabiec, 1974), but the magnitude of change varies significantly across cerebral structures. 5-HT and 5-HIAA levels are elevated to varying degrees in the brain stem, frontal cortex, hippocampus, hypothalamus, mid-brain and striatum during exercise (Blomstrand et al., 1989; Brown et al., 1979; Dey et al., 1992; Gomez-Merino et al., 2001a). The apparent discrepancy in the magnitude of response reported may be due to differences in the exercise protocol used and the analytical techniques employed to measure changes in neurotransmission (Meeusen and De Meirleir, 1995).

A number of recent studies have confirmed and developed this early work, demonstrating that exercise results not only in an increase in TRP uptake and brain 5-HT content, but also influences extracellular 5-HT and 5-HIAA concentrations (Gomez-Merino et al., 2001a; Meeusen et al., 1996; Wilson and Marsden, 1996). Progressive increases in extracellular 5-HT and 5-HIAA have been reported during exercise, but marked differences in the time-course of changes were apparent between studies. Whereas Meeusen and colleagues (1996) demonstrated a rise in 5-HT in the hippocampus from the onset of treadmill running, other reports suggest a delay in this response with a marked increase in extracellular 5-HT only evident after 60 minutes exercise (Gomez-Merino et al., 2001a; Gomez-Merino et al., 2001b). This apparent discrepancy in the time-course of change in serotonergic neurotransmission has been attributed to differences in the pre-exercise dietary conditions, with the rapid response
observed in 24-hour fasted animals (Meeusen et al., 1996) compared to a delayed response in fed animals (Gomez-Merino et al., 2001a; Gomez-Merino et al., 2001b).

As previously described, there is limited evidence for a relationship between plasma f-TRP and brain TRP content under resting conditions (Fernstrom et al., 1976; Pardridge, 1979), but this situation appears to be altered during exercise. The concentration of plasma f-TRP, rather than total-TRP, was identified as a key factor in cerebral TRP uptake during exercise, with a strong positive relationship reported between changes in plasma f-TRP and brain TRP content (Chaouloff et al., 1985). This led to the suggestion that exercise-induced lipolysis is directly responsible for the increase in brain TRP and 5-HT synthesis (Chaouloff, 1989). The apparent difference in the size of the TRP pool available for transport into the CNS may be result from changes in cerebral blood flow. Cerebral blood flow is markedly increased during exercise (Ide and Secher, 2000) and this increase in blood velocity may limit the effectiveness of the LNAA-carrier at removing TRP from albumin.

The mobilisation of FFA from the adipose tissue by adrenaline-stimulated lipolysis has been proposed to be important to the development of serotonin-mediated fatigue during prolonged exercise (Newsholme et al., 1987). Plasma FFA concentrations typically increase progressively throughout prolonged low- to moderate-intensity exercise, particularly following a period of food deprivation (e.g. an overnight fast). Although there appears to be a near linear relationship between plasma FFA and the rate of FFA utilisation (Issekutz et al., 1967), this increase occurs as the mobilisation of FFA from the adipose tissue often slightly exceeds uptake by the working muscles (Spriet, 2002). When muscle and liver glycogen stores are nearing depletion, FFA mobilisation can increase disproportionately over the rate of transport into the muscle, resulting in a marked elevation in plasma FFA concentrations (Newsholme and Castell, 2000). As FFA molecules bind to albumin, conformational changes occur that result in the liberation of TRP from its binding site (Curzon et al., 1974), consequently increasing the proportion of TRP circulating in a free form.

Based on the literature presented above, the underlying mechanism behind the central fatigue hypothesis as proposed by Newsholme and co-workers (1987) can be divided into two interrelated sections:
1. Under resting conditions, the majority of TRP circulates in the blood loosely bound to albumin, a transporter shared with free fatty acids (FFA). The shift in substrate mobilisation occurring as exercise progresses causes an increase in plasma FFA concentration. This displaces TRP from binding sites on albumin, leading to a marked increase in f-TRP. Free tryptophan, unlike albumin bound tryptophan, is then readily available for transport across the BBB.

2. Plasma BCAA concentrations either fall (Blomstrand et al., 1988; van Hall et al., 1995a) or are unchanged (MacLean et al., 1994) during prolonged exercise. Since f-TRP and BCAA share a common transporter across the BBB, a reduction in competing LNAA would increase the uptake of TRP into the CNS. Plasma concentrations of FFA and f-TRP are typically low at the onset of exercise, so the rate of cerebral TRP uptake and central 5-HT synthesis are also relatively low (Figure 1.3A). The metabolic events described above result in an increased plasma concentration ratio of f-TRP to BCAA during exercise, favouring the entry of TRP into the CNS. As the rate of 5-HT synthesis is largely dependent upon the delivery of TRP into the brain (see Section 1.2.1), serotonergic activity increases as exercise duration progresses (Figure 1.3B). According to Newsholme's hypothesis, this change in brain neurochemistry results in subjective sensations of lethargy and tiredness, causing a loss of drive and motivation to continue exercise. This could provide a feedback link between substrate availability and the feelings of fatigue that occur during prolonged exercise (Newsholme and Castell, 2000).

While the underlying tenets of this hypothesis appear biochemically sound, recent human work fail to support the suggestion that cerebral TRP uptake is elevated during prolonged exercise (Nybo et al., 2003). These data indicate that despite a 50% increase in plasma f-TRP, a net uptake of TRP was apparent in only half the subjects at the end of exercise. Despite this observation the authors suggest that serotonin levels in the brain may increase when exercise elevates the plasma f-TRP concentrations, based on the observation of a positive correlation between the arterial f-TRP concentration and the a-v difference of TRP across the brain. However, as there is a physiological link between arterial f-TRP and the a-v difference of f-TRP, this conclusion may be flawed (Atkinson et al., 2004).
Figure 1.3: An overview of the proposed key events involved in the development of central fatigue at rest (A) and during exercise (B).
1.5 - Experimental support for serotonin-mediated fatigue

Support for 5-HT-mediated fatigue arises largely from animal and human studies employing pharmacological agents to manipulate central serotonergic neurotransmission. Many modern antidepressant treatments exert their therapeutic effect through a modification of extracellular 5-HT concentrations in the brain, with SSRI widely prescribed. SSRI drugs act through the inhibition of the presynaptic 5-HT transporter, effectively limiting the reuptake of 5-HT from the synaptic cleft and prolonging the action of serotonin present within the synapse. In healthy individuals the reuptake process prevents over stimulation of receptors in the synapse, but some psychiatric disorders are thought to be characterised by an altered sensitivity of the postsynaptic receptors (Owens and Nemeroff, 1994). In particular, clinical depression is thought to be characterised by an upregulation of receptor sites, which consequently results in a deficiency of neurotransmitter present in the synapse. The therapeutic benefit of antidepressant treatments are thought to arise through a chronic increase in extracellular 5-HT, causing a normalisation of receptor sensitivity.

A series of studies by Bailey and co-workers examined the effects of pharmacological manipulation of brain 5-HT levels in rats through the administration of specific 5-HT agonists and antagonists (Bailey et al., 1992; Bailey et al., 1993a; Bailey et al., 1993b). This early work provided good evidence for a role of 5-HT in the development of fatigue, with a dose-dependent reduction in exercise capacity reported when central 5-HT activity was augmented by the acute administration of a general 5-HT agonist (Bailey et al., 1992). Brain 5-HT and DA content progressively increased during exercise, but at the point of exhaustion a marked fall in tissue DA content was apparent. Furthermore, exercise capacity was enhanced by a 5-HT antagonist (LY-53857), although this was apparent only when the highest dose was administered (Bailey et al., 1993a; Bailey et al., 1993b). These alterations to exercise capacity occurred without any change in core temperature, circulating metabolites or stress hormones (Bailey et al., 1993a). Additionally, muscle glycogen concentrations at fatigue were higher when a 5-HT agonist (quipazine dimaleate) was administered, suggesting that the accelerated fatigue did not occur as a result of limited glycogen availability.
While these data look promising, the significance of these findings has been questioned. It is important to note that the higher doses of drugs given in these studies were many times greater than would normally be administered. It is probable that some of the animals displayed symptoms of serotonin-syndrome, including loss of coordination, dizziness, confusion and disorientation that would almost certainly negatively affect exercise capacity (Struder and Weicker, 2001b).

Based on these observations, Davis and Bailey (1997) developed Newsholme's original hypothesis. Rather than the proposition that central fatigue was exclusively mediated through changes in 5-HT, the findings of this series of studies led to the suggestion that the brain content ratio of 5-HT to DA was important to the fatigue process. The revised central fatigue hypothesis suggests that an increase in 5-HT:DA is associated with feelings of tiredness and lethargy, accelerating the onset of fatigue, whereas a low ratio favours improved performance through the maintenance of motivation and arousal (Davis and Bailey, 1997). The importance of catecholaminergic neurotransmission to the development of fatigue will be discussed in Section 1.7.

Wilson and Maughan (1992) were first to investigate the effects of an acute dose of a 5-HT agonist on the capacity to perform prolonged exercise in humans. Paroxetine administration resulted in a 19% reduction in time to exhaustion when compared to a placebo. In the absence of a clear drug effect on the metabolic, cardiovascular and thermoregulatory responses to exercise, the reduction in exercise capacity was attributed to an upregulation of serotonergic activity caused by the treatment. Subsequent work has provided support of these initial findings, with reduced exercise capacity reported following an acute dose of fluoxetine (Prozac; Davis et al., 1993), buspirone (Marvin et al., 1997) and paroxetine (Struder et al., 1998). Newsholme's hypothesis suggested that a change in serotonergic neurotransmission might alter an individual's perception of effort. The work of Davis et al. (1993) and Marvin et al. (1997) reported a marked increase in perceived exertion, with drug ingestion in these studies associated with a feeling of heaviness in the legs during exercise. No effect on RPE was apparent following paroxetine administration, despite a reduction in exercise capacity (Wilson, 1994).
Although much of the initial pharmacological manipulation work provided good evidence in support of the mechanisms proposed by Newsholme and colleagues (1987), a number of studies have failed to replicate these early findings. This includes studies that have sought to investigate serotonin-mediated fatigue through the administration of drugs to increase 5-HT neurotransmission (Meeusen et al., 2001b; Piacentini et al., 2002a; Strachan, 2002) as well as agents to reduce central 5-HT release (Meeusen et al., 1997a; Pannier et al., 1995).

The equivocal findings of these pharmacological studies may be explained by the complexity of the actions of the drugs employed. Although there is good evidence that acute doses of drugs influencing central neurotransmission can transiently influence neurotransmission in rodents (Bailey et al., 1993a; Piacentini et al., 2003a; Piacentini et al., 2003b), the effect of these drugs on the human CNS is not clear at present. Evidence from clinical studies suggests that an acute administration of a monoamine reuptake inhibitor may produce little change in extracellular neurotransmitter concentrations, due to a reduction in cell firing rate caused by presynaptic 5-HT$_{1A}$ autoreceptor-mediated feedback inhibition (Artigas et al., 1996). This is supported by the observation that there is a delayed increase in plasma prolactin concentration following chronic administration of paroxetine in healthy volunteers (Cowen and Sargent, 1997). While there is some evidence that acute SSRI ingestion can reduce an individual’s capacity to perform prolonged exercise (Davis et al., 1993; Struder et al., 1998; Wilson and Maughan, 1992), it has been suggested that this occurred due to a disturbance in regulatory homeostasis of the 5-HT system perhaps via a disruption in pre- or postsynaptic receptor function (Struder and Weicker, 2001b), rather than an increase in the activity of the serotonergic neurons (Davis et al., 1993; Wilson and Maughan, 1992).

1.6 - Nutritional Manipulation of 5-HT Neurotransmission during Exercise

Much of the attraction of the hypothesis described by Newsholme and co-workers (1987) was the potential for nutritional manipulation of neurotransmitter precursors to delay the onset of central fatigue, potentially enhancing performance. In recent years a
number of studies have attempted to attenuate the increase in central 5-HT levels through dietary supplementation with specific nutrients, including BCAA and CHO.

BCAA are unique amongst the amino acids in that they largely avoid hepatic uptake and are metabolised primarily in the skeletal muscle, where they serve as an energy substrate and also a precursor for the synthesis of new proteins (Wagenmakers and van Hall, 1996). The overall contribution of protein to energy production is thought to be around 3 to 6 % during prolonged exercise (Gibala, 2001), and is typically overlooked when estimating substrate utilisation from gas exchange (Astrand et al., 2003). Despite the observation that exercise and BCAA ingestion independently and additively increase the activation of the branched-chain 2-oxoacid dehydrogenase complex (van Hall et al., 1996; Wagenmakers et al., 1991), evidence suggests that BCAA oxidation contributes a relatively insignificant portion of energy expenditure (~1 %; Wagenmakers et al., 1991). On the basis of these findings it is unlikely that BCAA supplementation would benefit exercise performance through glycogen sparing (Wagenmakers and van Hall, 1996).

As described in Section 1.2, a number of amino acids, collectively termed the large-neutral amino acids (LNAA), share a common transporter across the BBB (Fernstrom, 1983). As both TRP and BCAA compete for cerebral uptake via the same active transporter, the plasma concentration ratio of f-TRP to BCAA is thought to dictate the rate of TRP entry into the brain and the rate of 5-HT synthesis during exercise. Ingestion of exogenous BCAA would therefore be expected to reduce this ratio, through a marked increase in plasma BCAA levels, effectively blocking the cerebral uptake of TRP (Figure 1.4). The resulting change in brain TRP delivery has been proposed to limit the exercise-induced increase in 5-HT synthesis, attenuate the development of central fatigue and enhance exercise performance (Blomstrand et al., 1991a).
Figure 1.4: The effect of BCAA supplementation on the development of central fatigue as proposed by Newsholme and colleagues (1987). BCAA supplementation results in a marked reduction in plasma concentration ratio of f-TRP to BCAA.

The first investigation undertaken to test the efficacy of BCAA supplementation at attenuating serotonin-mediated fatigue was a field study of the physical and mental performance of male volunteers competing in either a marathon or a 30 km cross-country race (Blomstrand et al., 1991a). The findings suggested that both physical (race time) and mental (colour and word tests) performance were enhanced in those receiving BCAA prior to exercise. Enhanced exercise performance was only witnessed in subjects completing the marathon in times slower than 3 hours 5 minutes. The authors suggested that a lack of effect in the faster runners might have been due to their increased resistance to the feelings associated with central and peripheral fatigue. The reliability of these results has been subsequently questioned, largely due to a number of methodological problems (Davis and Bailey, 1997). In particular, fluid and CHO ingestion was not controlled during exercise, subjects were not matched to controls in terms of previous performance and the retrospective
division of subjects into groups relating to their performance in the trial has also been criticised as statistically incorrect.

Work conducted by Mittleman and colleagues (1998) has provided further evidence in support of these early findings and the apparent role of 5-HT in the fatigue process. A 14 % increase in capacity to perform low intensity (40 % \( VO_2 \text{max} \)) exercise was reported following BCAA supplementation when compared to a polydextrose placebo. No difference in peripheral markers of fatigue was reported between the two exercise bouts. The authors concluded that the supplementation regimen was successful in limiting the entry of TRP into the CNS, attenuating serotonin-mediated fatigue. The unique aspect of this study was that the trials were undertaken in a warm environment (34.4 °C), with subjects seated at rest for 2 hours before exercise in these conditions. BCAA supplementation began 60 minutes prior the start of exercise, resulting in a 2 to 3-fold reduction in the plasma concentration ratio of f-TRP to BCAA. The potential involvement of the CNS when exercising in warm environmental conditions will be discussed in detail in Section 1.11. The effect of chronic administration of BCAA on exercise performance has also been examined (Hefler et al., 1995). Following 14-days of BCAA supplementation (16 g.day\(^{-1}\)) performance of a 40 km cycle time trial in temperate conditions was improved by 12 % compared to the same period of placebo ingestion. Despite these apparently positive findings, these data do not appear to have been published as a full manuscript and the abstract does not provide any supporting metabolic data. These factors make it difficult to accept the findings. The influence of chronic BCAA supplementation on exercise performance warrants further investigation.

While there is some additional evidence of BCAA ingestion influencing perceived exertion (Blomstrand et al., 1997) and mental performance (Blomstrand et al., 1991b; Hassmen et al., 1994), the results of several apparently well-controlled laboratory studies have not demonstrated a positive effect on exercise capacity or performance. No ergogenic benefit has been reported during prolonged fixed intensity exercise to exhaustion (Blomstrand et al., 1995; Blomstrand et al., 1997; Galiano et al., 1991; Struder et al., 1998; van Hall et al., 1995a), prolonged time trial performance (Hassmen et al., 1994; Madsen et al., 1996), incremental exercise (Varnier et al., 1991).
1994) or intermittent shuttle-running (Davis et al., 1999). These findings question whether the attenuation of TRP uptake through the provision of exogenous BCAA can influence central neurotransmission, but methodological differences make comparisons between studies difficult. With the exception of the studies conducted by Heffler et al. (1995) and Mittleman et al. (1998), BCAA supplements have been provided immediately prior to, and during exercise, in quantities ranging from 6 to 18 g. There is some limited evidence that a delay may exist between changes in the plasma concentration ratio of TRP to BCAA, the uptake of TRP into the CNS and the resulting modification to serotonergic neurotransmission (Curzon and Knott, 1974). Administering BCAA supplements immediately prior to the start of exercise may have limited their efficacy at preventing cerebral TRP uptake from the onset of exercise.

A possible explanation for a failure to observe an ergogenic effect in many BCAA studies, despite a good rationale for their use, is an increase in ammonia (NH₃) production (Davis and Bailey, 1997). During prolonged exercise, increased NH₃ production is primarily thought to result from the deamination of BCAA, rather than the deamination of AMP to IMP (Greenhaff et al., 1991; van Hall et al., 1995b; Wagenmakers et al., 1991). This response appears to be amplified by reduced glycogen availability (Czarnowski et al., 1995; Wagenmakers et al., 1991), hyperthermia (Febbraio et al., 1994; Marino et al., 2001) and the ingestion of BCAA (MacLean et al., 1994; van Hall et al., 1995a). The BCAA aminotransferase reaction draws carbon groups from the TCA cycle by using 2-oxoglutarate as an amino group acceptor (Wagenmakers and van Hall, 1996). This may attenuate anaplerosis, potentially limiting ATP production and negatively affecting exercise performance, particularly when muscle glycogen availability is low (Wagenmakers et al., 1991; Wagenmakers et al., 1990). Prolonged exhaustive exercise has also been suggested to produce a state of 'acute NH₃ toxicity' that may disrupt brain metabolism and alter the pH of the nervous tissue, potentially impairing motor control and coordination (Banister and Cameron, 1990; Davis and Bailey, 1997). The brain buffers NH₃ by the amination of glutamate to glutamine, which is then released into the peripheral circulation (Struder and Weicker, 2001a). Elevated plasma NH₃ concentrations have also been demonstrated to alter the transport kinetics of amino acids across the BBB (Mans et al., 1983). Whether this response contributes to the development of central
fatigue by enhancing the uptake of neurotransmitter precursors (Banister and Cameron, 1990) or acts as a compensatory response to normalise cerebral amino acid uptake despite a marked increase in blood flow through the brain microvessels is not clear at present.

Following work with patients with myophosphorylase deficiency (McArdle's disease) (Wagenmakers et al., 1990) suggested that BCAA supplementation under conditions of reduced muscle glycogen availability could result in a reduction in endurance performance in healthy individuals (Wagenmakers and van Hall, 1996). Recent work suggests, however, that BCAA ingestion does not appear to impair the increase in TCA intermediates during exercise in a glycogen depleted state (Gibala et al., 1999). It appears that the highest concentrations of plasma NH₃ observed in healthy individuals during prolonged exercise (~150 µmol.L⁻¹) are not sufficient to limit exercise capacity through peripheral or central mechanisms (Gibala, 2001).

While support for a benefit of BCAA ingestion in humans is limited, particularly when exercise is performed in temperate conditions, the response appears to be different in animals, where a clear increase in exercise capacity (Calders et al., 1999; Calders et al., 1997) and free running activity (Smriga et al., 2002) has been shown. A study conducted by Verger and co-workers (1994) observed no difference in time to exhaustion following BCAA ingestion in rats when compared to a placebo condition, but it is not clear why these results fail to agree with those of Calders and colleagues. Good evidence for the role of BCAA in limiting TRP entry into the CNS and attenuating the increase in 5-HT has been reported in rodents using in-vivo brain microdialysis (Gomez-Merino et al., 2001b). During the placebo trial (saline infusion) a progressive increase in extracellular 5-HT was apparent in the hippocampus as exercise continued, but this elevation was abolished when exercise was preceded by an infusion of valine. Perhaps a difference between species in sensitivity to manipulation of the 5-HT system may account for these different findings.

The opposite of supplementing with BCAA to limit 5-HT synthesis would be a reduction in exercise capacity following TRP ingestion. The administration of TRP in food-deprived rats has been reported to elevate extracellular 5-HT and 5-HIAA
concentrations in the hippocampus by 55% (Meeusen et al., 1996). TRP infusion before exercise resulted in an additive response, supporting the claims of Newsholme and co-workers (1987) by significantly amplifying the exercise-induced increase in 5-HT. While there are reports of TRP supplementation producing a marked reduction in the exercise capacity of horses (Farris et al., 1998) and rodents (Soares et al., 2003), ingestion of between 1.2 and 3.0 g of TRP immediately prior to the start of exercise produced no effect on exercise capacity in human subjects (Alves et al., 1995; Stensrud et al., 1992; van Hall et al., 1995a). Paradoxically, a 49% increase in exercise capacity at 80% \( \dot{V}O_2 \) max has been observed following TRP supplementation when compared to a placebo (Segura and Ventura, 1988). A non-significant reduction in RPE was apparent that led the authors to propose that TRP supplementation may attenuate nociception, increasing the tolerance of pain associated with the performance of strenuous exercise. The findings of Segura and colleagues (1988) are difficult to accept in the light of subsequent work in this area and such a large improvement in exercise capacity.

Carbohydrate feeding suppresses lipolysis, consequently lowering the circulating concentration of plasma FFA. Recognising this, Davis and colleagues (1992) suggested CHO ingestion as a means of reducing cerebral TRP uptake. A five- to sevenfold increase in the plasma concentration ratio of f-TRP to BCAA was reported under placebo conditions. Supplementation with a 6 or 12% CHO solution attenuated the increase in plasma FFA and f-TRP, reducing the plasma concentration ratio of f-TRP to BCAA in a dose-dependent manner. Exercise capacity during CHO trials was increased over the placebo, suggesting CHO ingestion as an effective means of delaying the onset of central fatigue, but it is difficult to interpret the contribution of central factors from the widely reported benefits of CHO at attenuating peripheral fatigue.

1.7 – Exercise and Catecholaminergic Neurotransmission

While the majority of work conducted in the area of exercise and brain neurotransmission has focused on changes in 5-HT, many early studies were interested the effects of exercise on the catecholaminergic neurotransmission. The
primary catecholamines implicated in the development of fatigue during prolonged exercise are DA and NA. As mentioned in Section 1.2, DA and NA are synthesised from the amino acid precursor tyrosine, through a similar metabolic pathway (Fernstrom, 1983). Whereas increased 5-HT activity is associated with feelings of tiredness and lethargy, an elevation in catecholaminergic neurotransmission is linked to arousal, motivation and reward (Davis, 2000). A change in regional dopamine metabolism has also been implicated in the control of locomotion and posture in moving animals (Freed and Yamamoto, 1985).

Early studies conducted by Gordon and colleagues (1966) using radiolabelled tyrosine reported a marked increase in NA synthesis and turnover during exercise, but no change in central DA synthesis. In contrast to these initial findings, subsequent work provides good evidence that prolonged exercise results in a progressive increase in tissue DA, DOPAC and HVA content in a number of cerebral regions including midbrain, hippocampus and striatum (Bailey et al., 1992; Bailey et al., 1993a; Chaouloff et al., 1987; Heyes et al., 1988). Recent data have demonstrated an elevation in extracellular DA (+28 - 50 %) and NA (+60 - 66 %) concentrations during exercise (Gerin and Privat, 1998; Meeusen et al., 1997b). During recovery, extracellular DA in the stratum remained elevated above baseline levels for over 100 minutes, whereas NA concentrations returned to resting values soon after the cessation of exercise (Meeusen et al., 1997b).

Studies investigating neurotransmission during exhaustive exercise (Bailey et al., 1992; Bailey et al., 1993a) and prolonged exposure to stress (Palkovits et al., 1975) have reported a fall in brain DA content. Bailey and co-workers (1992, 1993) observed a parallel increase in both 5-HT and DA after one hour of exercise. While brain 5-HT and 5-HIAA content continued to increase as exercise progressed, DA and DOPAC concentrations appeared to return to resting levels at exhaustion. These findings have led to the suggestion that the partial depletion of catecholamines in regions of the brain may be linked to a loss of physical and mental performance during prolonged exercise (Davis and Bailey, 1997) and exposure to stress (Owasoyo et al., 1992; Palkovits et al., 1975).
1.8 - Experimental Support for Catecholamine-Mediated Fatigue

Early pharmacological manipulation of central neurotransmission to improve exercise performance focused largely on the effects of amphetamines, with studies of this nature undertaken by German scientists during the 2nd World War (Chaouloff, 1991). Amphetamine is a close analogue of DA and NA, thought to act directly on catecholaminergic neurones to produce a marked elevation in extracellular dopamine concentrations. This response is believed to be mediated through the stimulation of DA release from storage vesicles, inhibition of dopamine reuptake and the inhibition of DA metabolism by MAO (Cooper et al., 2003). Amphetamines may also limit the synthesis of 5-HT through a reduction in tryptophan hydroxylase activity and a direct interaction between DA release and serotonergic neurotransmission (Breese et al., 1974; Chaouloff et al., 1987).

Studies have demonstrated a clear performance benefit following the administration of amphetamine to both rodents (Gerald, 1978) and humans (Chandler and Blair, 1980). Gerald (1978) reported a dose-dependent increase in time to exhaustion, up to a dose of 2.5 mg.kg\(^{-1}\), with further doses progressively reducing exercise capacity. The ergogenic action of amphetamine is thought to be mediated through the maintenance of DA release late in exercise, through the mechanisms described above. These positive findings have led to the widespread use of amphetamines in many endurance events, with a long history of abuse in cycling events in particular. Further evidence for a role of DA in the development of central fatigue is provided by work conducted by Heyes and colleagues (1985). Infusion of apomorphine (a DA agonist) has been shown to prolong exercise capacity, and partially restore exercise capacity following destruction of dopaminergic neurons with 6-hydroxydopamine (Heyes et al., 1985).

Intracranial self-stimulation (ICSS) has been employed as a model to induce exercise in rodents, removing the need to administer aversive electric shocks (Burgess et al., 1991). ICSS involves the implantation of an electrode into the ventral tegmental area (VTA), the origin of the dopaminergic projection, which triggers electrical stimulation to this area of the brain when the animal maintained a pre-determined running speed. The dopaminergic reward associated with ICSS has been reported to enable rats to run...
around 50% longer when compared to the use of electric shock (Burgess et al., 1991),
while producing no effect on peripheral variables relating to cardiovascular, metabolic
or thermoregulatory function (Burgess et al., 1993). Studies investigating changes in
central DA using electric shock grids to stimulate running should be viewed with
cautions as the stress associated with this form of motivation has been demonstrated to
significantly increase dopamine release (Chrapusta et al., 1997).

Despite the apparent link between exercise and catecholaminergic neurotransmission
demonstrated in animals, there has been relatively little work conducted to assess the
effects of DA and NA manipulation on exercise capacity in humans. This may relate
to the relatively limited number of safe pharmacological agents available to influence
central catecholamine concentrations, and the belief that the provision of tyrosine
does not influence DA and NA synthesis due to a saturation of tyrosine hydroxylase
(Davis and Bailey, 1997). Recently the development of a new wave of psychiatric
treatments, including reuptake inhibitors that act on NA (reboxetine), 5-HT/NA
(venlafaxine) and DA/NA (bupropion) have enabled the role of these
neurotransmitters in the fatigue process to be further examined.

A series of studies conducted by Piacentini and colleagues reported no change in the
performance of a 90 minute cycle time trial following the acute administration a NA
reuptake inhibitor (Piacentini et al., 2002b), a dual 5-HT and NA reuptake inhibitor
(Piacentini et al., 2002a) or a dual DA and NA reuptake inhibitor (Piacentini et al.,
2004). The pituitary-hormone response to exercise was significantly altered by drug
ingestion, leading the authors to suggest that acute administration of these agents
resulted in the desired central effect (Piacentini, 2003). The influence of central
neurotransmission on the regulation of pituitary-hormonal release is discussed in
Section 1.10. It is worth mentioning at this stage that a reduction in exercise capacity
was observed following the administration of buspirone (Marvin et al., 1997). While
this drug is thought to primarily act as a 5-HT1A receptor agonist, it also produces a
limited dopaminergic D2 antagonism (Bridge et al., 2003), which may have played a
significant role in mediating this response.

As stated in Section 1.2, increasing the availability of TYR for uptake into the CNS
may not influence the rate of DA and NA synthesis at rest (Davis, 2000), but limited
evidence suggests that the provision of exogenous TYR can increase central DA synthesis under basal conditions (During et al., 1989). Certainly exposure to stress results in a marked increase in catecholaminergic activity resulting in an enhanced activation of tyrosine hydroxylase, making these neurons more responsive to changes in precursor availability (Milner and Wurtman, 1986).

The administration of L-DOPA, an intermediate in the catecholamine pathway, has been employed to effectively bypass the rate-limiting step in DA and NA synthesis. L-DOPA has been used in the management of Parkinson’s disease, a disorder characterised by a loss of motor control and coordination, to maintain central dopamine neurotransmission. Meeusen and co-workers (1997) examined the effect of L-DOPA on exercise capacity in endurance-trained males. Ingestion of L-DOPA 24 hours and immediately before commencing exercise had no effect on submaximal time to exhaustion or peripheral cardiovascular or metabolic measures during exercise.

TYR supplementation during sustained military operations exceeding 12-hours, often involving severe sleep deprivation and fatigue, reduced stress-related decrements in mood and task performance (Owasoyo et al., 1992). Evidence of an ergogenic benefit of TYR supplementation during prolonged exercise, however, is limited. Work conducted by Struder and colleagues (1998) failed to observe any change in the capacity to perform prolonged exercise following the ingestion of TYR immediately before (10 g) and during exercise (10 g). It has been suggested that the dose of TYR administered in this study may have resulted in an inhibition of dopamine synthesis, but a recent report administering half the dose employed by Struder and colleagues (1998) also produced no effect on time trial performance (Chinevere et al., 2002). Interestingly a marked elevation in plasma prolactin concentration was observed by Struder et al. (1998) throughout exercise following TYR ingestion. This is unexpected as DA is thought to play an inhibitory role in the regulation of prolactin release from the anterior pituitary gland (Checkley, 1980).
1.9 – The hypothalamic-pituitary-adrenal (HPA) axis and Hormone Release

It is difficult to directly determine changes in central neurotransmission in human subjects, but the recent development of imaging techniques (e.g. positron emission tomography) has opened new avenues to monitor the effects of pharmacological, nutritional and physiological challenges on the brain. While these methods are increasingly being employed in a clinical setting, their use is restricted in exercise physiology due to expense, access to experienced operators and logistical problems associated with performing exercise in or close to the equipment. For a number of years the measurement of changes in circulating concentrations of peripheral hormones has been employed as an index of central neurotransmission (Checkley, 1980).

The premise that circulating hormones may be used to determine changes in central neurotransmission is based on the role of central monoamines in the control of hormone release from the anterior and posterior pituitary gland. The paraventricular nucleus of hypothalamus is the major integrating link between the nervous and endocrine systems, with inputs from several areas of the brain governing the release of pituitary hormones through the infundibulum stalk. Changes in the activity of 5-HT, DA and NA neurons innervating the hypothalamus stimulates the synthesis of releasing and inhibiting hormones that act directly on areas of the pituitary to trigger the release of hormones into the peripheral circulation. Adrenocorticotropic hormone (ACTH) released from the pituitary also exhibits control over the adrenal gland, influencing cortisol release. This has led to the term the hypothalamic-pituitary-adrenal (HPA) axis.

While the hypothalamus receives input from a number of cerebral structures, it is clear that the stimulation of different receptors within the neuroendocrine control sites produces a differing action on hormone release (Checkley, 1980; Van de Kar, 1997). The application of neuroendocrine tests to disturb central neurotransmission has provided insight into the relative contributions of 5-HT, DA and NA in the regulation of pituitary hormone release, but there remains a degree of uncertainty regarding the coordination of the endocrine response particularly under conditions of stress (Dinan, 1996).
1.9.1 – Serotonergic control of pituitary hormone release

There is a considerable body of evidence to indicate a role for central serotonergic
neurons in the regulation of prolactin (PRL), ACTH, cortisol, and growth hormone
(GH) secretion (Checkley, 1980; Dinan, 1996; Van de Kar, 1997).

While basal secretion of PRL is regulated by tonic inhibition by dopamine,
serotonergic regulation originates from cells in the dorsal raphe nucleus with
activation of 5-HT$_{1A}$, 5-HT$_{2A/C}$, 5-HT$_3$ receptors thought to stimulate the secretion of
PRL (Van de Kar, 1997). Administration of fenfluramine, a potent 5-HT releaser and
uptake inhibitor, has been demonstrated to produce a marked increase in prolactin
release. While BCAA ingestion does not appear to influence PRL concentrations at
rest, except when large quantities (60 g) are ingested (Carli et al., 1992; Gijsman et
al., 2002), around 10 g BCAA attenuated the PRL response to exercise (Carli et al.,
1992). Early evidence demonstrated a clear relationship between serotonergic activity
and plasma concentrations of PRL during exercise (De Meirleir et al., 1985; Fischer et
al., 1991). These findings have resulted in the widespread use of PRL as a peripheral
index of changes in central serotonergic activity during exercise (Pitsiladis et al.,
2002; Strachan, 2002; Struder et al., 1998). Recently it has become clear that PRL
release during activity is not governed solely by 5-HT, but through a complex
interaction between a number of neurotransmitter systems (Meeusen et al., 2001b;
Piacentini, 2003). Evidence also suggests that elevated brain temperature may provide
a stimulus for PRL release during exercise (Radomski et al., 1998).

There is good evidence to link increased 5-HT activity with the stimulation of ACTH
and consequently cortisol release (Dinan, 1996). The administration of TRP,
5-hydroxytryptophan and fenfluramine have been demonstrated to elevate plasma
concentrations of both ACTH and cortisol in healthy individuals (Siever et al., 1984).
The role of 5-HT in the regulation of GH release is less certain, although it is thought
to exert an excitatory influence (Checkley, 1980).
1.9.2 - Catecholaminergic control of pituitary hormone release

Prolactin release is clearly inhibited by dopamine (Checkley, 1980), with drugs that increase extracellular DA producing a progressive reduction in circulating PRL concentrations in the rat (Piacentini et al., 2003a). Ingestion of a large dose of BCAA (60 g) has been reported to significantly elevate plasma prolactin: this was thought to be mediated through the competitive inhibition of TYR entry into the CNS, consequently limiting DA synthesis (Gijsman et al., 2002). Evidence for a role of DA in the control of ACTH and cortisol release is limited in humans, but DA is responsible for the stimulation of GH release (Checkley, 1980).

Regulation of hormone release by NA is complicated by the type of adrenoceptor subtype activated, with conflicting responses produced by alpha and beta receptors. While ACTH release is stimulated by activation of α1 adrenoceptors, α2 and β adrenoceptors have been demonstrated to inhibit ACTH secretion. Prolactin release does not appear to be greatly influenced by NA activity, whereas NA-stimulated α adrenoceptor activation enhances GH release (Checkley, 1980).

Table 1.1: Influence of neurotransmitters on the release of hormones from the anterior pituitary. ↑ represents a stimulatory influence, ↓ represents an inhibitory influence, ? represents a questionable / unknown influence (adapted from Checkley, 1980 and Van de Kar, 1997).

<table>
<thead>
<tr>
<th></th>
<th>5-HT</th>
<th>DA</th>
<th>NA α</th>
<th>NA β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin</td>
<td>↑</td>
<td>↓</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>ACTH</td>
<td>↑</td>
<td>?</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Cortisol</td>
<td>↑</td>
<td>?</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Growth Hormone</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓?</td>
</tr>
</tbody>
</table>

The regulation of hormone release by central neurotransmission is summarized in Table 1.1. Monitoring the hormonal response of individuals to a nutritional,
pharmacological or exercise / heat stress intervention has become an important tool in both a clinical setting and in exercise physiology to provide information regarding alterations in central neurotransmission. As all the neurotransmitter systems are functionally interconnected it is difficult to attribute changes in the peripheral concentration of a particular hormone to a single monoamine. While the understanding of the central control of hormone release is growing, caution should be exercised when interpreting hormone responses as it is unclear how the release of hormones is governed by neurotransmitters, particularly under conditions of stress including physical activity.

1.10 – The Blood-Brain Barrier (BBB)

1.10.1 – Structure and function of the BBB: relevance to exercise

Under normal physiological conditions the CNS functions within a relatively stable environment, largely distinct from the compositional fluctuations occurring in the peripheral circulation. This regulation is essential to ensure normal brain function and the operation of the organism as a whole, as well as to protect the brain from pathogens and other harmful substances. As a function of this, the capillaries supplying the brain differ structurally from those found in the periphery (Figure 1.5), forming a barrier to the entry of blood-borne substances, known as the blood-brain barrier (BBB). The BBB is a dynamic structure formed primarily by microvascular endothelial cells. These are characterised by the presence of overlapping tight junctions and restricted vesicular transport (Kandel et al., 2000). The foot processes of astrocytes surround around 99 % of cerebral vessels and along with the presence of pericytes these cell serve to reinforce the tight junctions (Pardridge, 2003). The BBB functions through these specialised structures to maintain a stable environment for the CNS by tightly regulating the exchange of substances between the cerebral interstitial fluid and the peripheral circulation.
There is also a blood-to-CSF barrier (BCSFB) that regulates the exchange of substances between the blood and CSF (Marchi et al., 2003a). While both barriers possess tight junctions, the BCSFB consists of epithelial rather than endothelial cells. Despite this structural difference, both barriers tightly limit the passage of substances into and out of the CNS. Additionally, some areas of the brain lack the protection of the BBB, and are therefore subject to changes occurring in the periphery. These are referred to as the circumventricular organs, which include the hypothalamus, the pineal and pituitary glands and some other nearby structures (Kandel et al., 2000). The lack of a BBB in these leaky regions largely relates to their function in the regulation of peripheral actions through hormone release. The circumventricular organs are isolated from the rest of the brain by specialised ependymal cells that limit the exchange between these structures and the cerebrospinal fluid (CSF).

The relative impermeability of the BBB helps to maintain a stable environment for the CNS by regulating exchange between the brain / CSF and the extra-cerebral environment. The transport of substances across the BBB is the result of a complex interaction between active efflux systems, active transporters and enzymes distributed on the structures that comprise the BBB (Pardridge, 2003). Entry into the CNS across the BBB is primarily mediated through three mechanisms (Kandel et al., 2000): 1) Passive diffusion of lipid-soluble molecules, including lipid soluble gases such as O₂ and CO₂. 2) Selective, carrier-mediated transport of specific water-soluble substances, including glucose (GLUT 1) and amino acids (L-transporter) by Na⁺-K⁺-ATPase dependent exchange 3) Electrolyte transport through active ion channels.
While the BBB is largely resistant to changes in permeability there are situations where the function of the BBB may be compromised. Changes in the permeability of this barrier may allow the entry or exit of species that can affect the metabolism of the brain and thus influence a wide range of homeostatic mechanisms (Shivers and Wijsman, 1998). Situations where the BBB may be breached include infections and fever (Chaudhuri, 2000), neuronal damage (Greenwood, 1991), physiological and emotional stress (Hanin, 1996) and prolonged exposure to hyperthermia (Sharma and Dey, 1987; Wijsman and Shivers, 1993). Paradoxically, there are situations where transient BBB disruption is desirable, to enable the delivery of drugs that would not otherwise cross the BBB (Rapoport, 2000).

Prolonged exercise may lead to increased BBB permeability. Animal studies have established that the BBB can be widely disrupted following 30 minutes forced swimming, with an albumin-tracer complex detected in a number of cerebral regions at the end of exercise (e.g. cerebral cortex, cerebellum, hippocampus and hypothalamus) (Sharma et al., 1991; Sharma et al., 1996). These changes were found to be relatively short lasting, with normal BBB function restored 2 hours after the end of exercise. When a pharmacological agent (p-chlorophenylalanine) was administered prior to exercise to deplete central 5-HT, this response was abolished. As 5-HT is a potent vasoactive agent, it is possible that changes in BBB permeability may have been serotonin-mediated. Interestingly, this response was only apparent in young animals (8 - 9 weeks), with little change in permeability observed in rats 30 to 32 weeks old (Sharma et al., 1996).

It is not clear how an increase in BBB permeability may influence exercise. A degree of change in the exchange of substances across the barrier is probably desirable during exercise, to facilitate the transport of metabolites and other substances into the CNS when cerebral blood flow is elevated. While there is no direct evidence for this suggestion at present, it is clear that adrenaline-stimulated vasodilation of brain capillaries (Abdul-Rahman et al., 1979) and NH$_3$ facilitated amino acid transport (Mans et al., 1983) may be important in this response during prolonged exercise. However, a marked disturbance in BBB function during exercise may adversely affect normal brain function and contribute to the development of central fatigue by allowing increased brain-blood interfacing. This may modify the transport kinetics of
neurotransmitter precursors and other metabolites or allow the accumulation of unwanted substances in the CNS.

While it is accepted that the BBB integrity can be altered by a wide range of physiological disturbances, the molecular and cellular mechanisms behind these changes in vivo are not clear at present (Gloor et al., 2001). Factors that may be important in changes to BBB permeability during prolonged exercise include a persistent elevation in core temperature (Sharma and Hoopes, 2003; Shivers and Wijsman, 1998). Wijsman and Shivers (1993) demonstrated that BBB permeability of anesthetized mice was increased when subjected to 45 minutes passive heat exposure. Colonic temperature throughout the protocol was maintained at 40.5 °C, with brain temperature possibly maintained at a higher level (Nybo et al., 2002). This is clearly a stressful situation that could not be ethically reproduced in humans, but the level of disruption resulted in considerable damage to the endothelial cells of the BBB. The use of an anaesthetic would have impaired thermoregulation in these animals creating an artificial situation, but whole-body hyperthermia can also influence BBB function in free living rats although a longer exposure was required to increase core temperature and consequently observe a response (Sharma and Dey, 1987).

As mentioned previously, manipulation of central serotonergic neurotransmission has been reported to alter the BBB response to exercise, suggesting a role for 5-HT in the regulation of BBB integrity during exercise (Sharma et al., 1991; Sharma et al., 1996). Increases in circulating interleukin-6 (IL-6), IL-8, IL-10 and tumour necrosis factor-α (TNF-α) concentrations have been reported following bouts of strenuous exercise (Nieman et al., 2001). An upregulation in pro-inflammatory cytokine production has also been implicated in changes in BBB permeability occurring during exercise (de Boer and Breimer, 1998). Cytokines are cellular hormones that play important roles in the growth, differentiation and function of many cells, as well as mediating inflammation. Some cytokines have been proposed to influence BBB permeability through an inflammatory component (IL-1β, IL-6, TNF-α), producing functional changes in the barrier. An energy sensing role has been proposed for IL-6 (Steensberg et al., 2000). Increased muscle IL-6 production during exercise, modulated by the muscle glycogen content, has been shown to stimulate lipolysis and increase fat.
oxidation (Pedersen et al., 2003). In this way, IL-6 may act as a negative feedback mechanism to the CNS contributing to the development of central fatigue (Gleeson, 2000). Additionally, there is a growing body of evidence to support the idea that feelings of tiredness and fatigue are triggered by the production of pro-inflammatory cytokines (Dantzer, 2004). This communication between the periphery and the brain has been termed sickness behaviour and is thought to be a natural homeostatic reaction to promote recovery from infection by limiting non-essential activities.

Strenuous exercise (Pals et al., 1997) and hyperthermia (Lambert et al., 2002) have also been reported to produce a loss of tight junction control in the gastrointestinal (GI) tract, resulting in an increase to gut permeability. Impaired GI tract permeability result in inflammatory events that alter gut structure and function that may manifest in intestinal bleeding and feelings of bloating and nausea (Gisolfi, 2000). It is important to note, however, that the histology of the intestinal mucosa differs markedly from that of the cells comprising the BBB, and changes in permeability control are likely to be mediated through different molecular and cellular mechanisms.

1.10.2 - S100b: A proposed peripheral marker of BBB permeability

As the protein content of the CSF is relatively low compared to that of the plasma, diagnostic methods of assessing BBB integrity often rely on the detection of plasma proteins in the CSF following disruption. This typically requires the use of sophisticated equipment (e.g. gadolinium-enhanced MRI), specialist operators and often invasive procedures (e.g. lumbar puncture). Recently it has been proposed that the appearance of CSF-specific proteins, including S100b, in the circulation may be used as a peripheral marker of BBB function (Kanner et al., 2003; Kapural et al., 2002; Marchi et al., 2003b). S100b (also referred to as S100β, S100beta) is a low molecular weight protein (21 kDa) expressed predominantly within the CNS by astrocytes and Schwann cells. It is typically found in low concentrations in the circulating serum, with a CSF (≈1.5 μg.L⁻¹) to serum (≈0.08 μg.L⁻¹) ratio of approximately 18:1 (Reiber, 2001). While the role of S100b is not clear at present, it is thought is to regulate protein phosphorylation, cell proliferation and it may be important in the regulation of cerebral energy metabolism (Zimmer et al., 1995).
Serum S100b concentrations markedly increase following brain tumours, strokes, head injury and multiple sclerosis, leading to the widespread use of this protein as a peripheral marker of brain damage. These events result in a marked release of S100b from the cytosol of damaged astrocytes, causing an up to 3- to 5-fold elevation in CSF concentrations (Petzold et al., 2003). As most neurological disorders are accompanied by BBB disruption, markers used to indicate neuronal damage may actually indicate changes in BBB function (Marchi et al., 2003b). In fact, when brain damage is induced in pig while the BBB was intact, no change in serum S100b was apparent, despite a marked elevation in CSF S100b concentrations.

Recently, Kapural et al. (2002) demonstrated that a significant elevation in serum S100b occurred in the absence of structural neuronal / brain tissue damage following osmotic opening of the BBB by intra-arterial mannitol infusion (1.4 M). The authors proposed that S100b present in the perivascular space moves into the systemic circulation in areas of BBB disruption. Thus, an elevation in serum S100b may be an important marker of changes in BBB function. Mathematical modelling conducted by Marchi et al. (2003) established a serum S100b concentration of $\sim 0.34 \mu g.L^{-1}$ as a cut off following an opening of the BBB in the absence of neuronal damage. This appears to fit well as serum S100b concentrations of greater than $0.7 \mu g.L^{-1}$ are typically associated with poor patient recovery in clinical settings (Ali et al., 2000).

Figure 1.6: A graphical representation of the use of S100b as a peripheral marker of BBB disruption.
To confirm these initial findings, Kanner and co-workers (2003) examined the relationship between changes in serum S100b and the assessment of BBB function using gadolinium-enhanced MRI following osmotic opening of the BBB. Changes in serum S100b were directly associated with the extent and time-course of BBB disruption determined using neuroimaging, suggesting that S100b is a suitable marker of changes in BBB permeability under these conditions.

S100 proteins circulating in the periphery are cleared by the kidneys and appear in the urine, with a serum half-life of around two hours (Ali et al., 2000). The beta subunit of this protein is highly specific to the CNS, it is also expressed in small quantities in peripheral tissues including bone, heart and adipose tissue (Zimmer et al., 1995). While peripheral sources have been identified, evidence suggests that the expression of S100b in these sites is around 5% of the astrocytic production (Marchi et al., 2003b). Additionally, recent work suggests that S100b is liberated into the circulation only when significant trauma occurs to these tissues, for example the tissue damage encountered during surgery (Anderson et al., 2001; Pelinka et al., 2003). At present a peripheral role of S100b is not apparent.

As there are clearly logistical and ethical problems associated with the use of imagining and lumbar-puncture techniques to monitor changes in BBB function during exercise. The recent identification of serum S100b as a peripheral marker of BBB permeability may provide useful information regarding potential changes in barrier function during exercise.

1.11 – Exercise in a warm environment

The capacity to perform prolonged exercise is reduced in a warm environment (Galloway and Maughan, 1997; Parkin et al., 1999). Galloway and Maughan (1997) reported that the exercise capacity of non-heat acclimated males was greatest at 11 °C, with a progressive fall in time to fatigue as ambient temperature was increased. Similar findings have been reported by subsequent work, with an inverse relationship reported between ambient temperature and exercise capacity (Parkin et al., 1999). Additionally, a 6.5% reduction in mean power output during a cycle time trial was
reported when the ambient temperature was increased from 23 °C to 32 °C (Tatterson et al., 2000). This response occurred despite little difference in rectal temperature between trials. Despite an understanding of the influence of ambient conditions on prolonged exercise capacity, the underlying mechanisms behind the deleterious effects of heat stress are not clear at present.

During exercise, muscle contraction required to produce locomotion results in heat production. In cool-moderate ambient conditions, body temperature is typically maintained at a relatively constant level (within 1 °C), by the dissipation of excess heat through a number of routes of heat loss (e.g. radiation, conduction, convection and evaporation). As ambient temperature rises, the effectiveness of these routes becomes compromised, and heat loss becomes increasingly dependent upon the evaporation of sweat from the skin's surface. The water that forms the majority of sweat is derived primarily from the blood plasma, with fluid mobilised from intracellular fluid compartments to maintain the circulating blood volume (Nose et al., 1988). High rates of sweat loss, typically encountered when exercising in hot and humid conditions, can result in a progressive reduction in blood volume and stroke volume, consequently limiting muscle blood flow. This situation is confounded by the need to supply an increased skin blood flow, required to transport heat away from the deep tissues to the periphery (Rowell, 1974).

The loss of fluids occurring through increased sweat losses, results in a steady-state condition of decreased body water content, termed hypohydration (Greenleaf, 1992). Body water losses have been demonstrated to result in elevated exercise heart rate and the impairment of thermoregulation during exercise (Sawka et al., 1985; Sawka et al., 1992), leading to an elevation in core temperature at the same absolute intensity. Hypohydration during exercise in the heat is also associated with a reduction in stroke volume, cardiac output and blood pressure (Gonzalez-Alonso et al., 1997) as well as a marked decline in blood flow to the working muscles (Gonzalez-Alonso et al., 1998).

Undertaking prolonged exercise in the heat appears to increase the reliance upon carbohydrate oxidation, accelerating muscle glycogen utilisation and resulting in a greater accumulation of intramuscular lactate (Febbraio et al., 1994). These metabolic alterations appear to result from changes in muscle temperature influencing the
activity of key enzymes responsible for energy production and elevated plasma catecholamine concentrations (Febbraio, 2001). While fatigue during prolonged exercise (65 – 85% \( \dot{V}O_2\text{max} \)) in temperate conditions is largely associated with the depletion of muscle glycogen (Bergstrom et al., 1967), this relationship is not apparent at high ambient temperatures. There appears to be an inverse relationship between muscle glycogen concentration at fatigue and ambient temperature (Parkin et al., 1999), suggesting that CHO availability is not limiting during prolonged exercise in the heat.

While it appears that fat oxidation remains relatively unchanged in response to exercise in a warm environment (Febbraio, 2001), there is some evidence that protein metabolism may be increased. Elevated intramuscular (Febbraio et al., 1994) and plasma (Marino et al., 2001) ammonia accumulation has been reported during exercise in the heat. During prolonged exercise ammonia production is thought to arise from the oxidation of BCAA rather than purine catabolism (Greenhaff et al., 1991; van Hall et al., 1995b). Although the contribution of protein oxidation to energy production is typically small, around 3 – 6 % of total energy expenditure (Gibala, 2001), this may be elevated when exercising in warm ambient conditions (Febbraio, 2001). An increased skeletal muscle uptake and utilisation of BCAA during exercise in the heat may have implications in the development of central fatigue through the mechanisms discussed previously (see Section 1.4).

These peripheral changes do not adequately explain the reduction in performance, leading to the suggestion that the CNS may be involved (Nielsen, 1992; Nielsen and Nybo, 2003; Strachan and Maughan, 2002). Fatigue during prolonged exercise in a warm environment may coincide with the attainment of a critical core temperature (Gonzalez-Alonso et al., 1999; Nielsen et al., 1993; Walters et al., 2000), suggesting that there may be a thermal limit to exercise performance. Nielsen and co-workers (1993) first proposed this concept following a comprehensive investigation of the effects of the physiological adaptations associated with repeated exposure to the heat. Following a period of heat acclimation exercise capacity at 60% \( \dot{V}O_2\text{max} \) was increased by 40 %, with fatigue occurring at a similar oesophageal temperature of 39.7 ± 0.15 °C. The period of acclimation attenuated the rate at which core
temperature increased and appeared to lower resting levels, allowing performance to be extended prior to the attainment of this critical value. This concept has been supported by recent findings in humans (Gonzalez-Alonso et al., 1999) and rodents (Walters et al., 2000), although Sawka and colleagues (1992) observed that around 75% of individuals appear to fatigue at a rectal temperature of 39.1 °C. While it is currently unclear how an elevated body temperature contributes to the development of fatigue, it seems possible that a critical core temperature may serve as a protective mechanism preventing potential damage to the body by limiting further heat production.

Recent work suggests that hyperthermia may have a direct affect on the CNS (Nielsen et al., 2001; Nybo and Nielsen, 2001a; Nybo and Nielsen, 2001b; Nybo and Nielsen, 2001c; Nybo et al., 2002). Neilsen and co-workers (2001) demonstrated that prolonged exercise in the heat was characterised by a progressive reduction in electroencephalogram (EEG) activity from the prefrontal cortex (Nielsen et al., 2001), with an increase in the ratio of α to β frequency bands. This shift towards lower-frequency α-bands is associated with feelings of tiredness and fatigue and is observed during the transition from waking to sleep. Whether exercise-induced hyperthermia influences neuromuscular function is not clear at present, with studies reporting a marked reduction (Nybo and Nielsen, 2001a) or little change (Ftaiti et al., 2001; Nielsen et al., 1993) in force generation capacity. This discrepancy appears to relate to differences in the duration of contraction, with the fall in voluntary activation occurring faster when body temperature is elevated (Nybo and Nielsen, 2001a). In addition, a decline in cerebral blood flow has been reported during exercise with hyperthermia (Nybo and Nielsen, 2001b). Perceived exertion is also significantly elevated by high core temperature compared to exercise at the same intensity under normothermic conditions (Armada-Da-Silva et al., 2004; Nybo and Nielsen, 2001c). This has been demonstrated to occur despite little change to peripheral indices of fatigue, suggesting that a high body temperature may reduce motivation and drive to continue exercise (Bruck and Olschewski, 1987; Nielsen, 1992). The underlying neurobiological mechanisms for these responses are not clear at present.
Thermoreceptors in the preoptic anterior hypothalamus are responsible for regulation of body temperature, with changes in body temperature detected through inputs from peripheral osmoreceptors and pressure receptors as well as the temperature of blood flowing to the brain (Gleeson, 1998). As serotonergic and catecholaminergic projections innervate areas of the hypothalamus, a change in the activity of these neurons may be expected to contribute to fatigue when core temperature is elevated (Gandevia, 2001). The suggestion that serotonin-mediated fatigue is important during exercise in the heat is partially supported by the work of Mittleman and colleagues (1998). The findings of this study are outlined in Section 1.6, but it is important to note that core temperature at fatigue (< 38 °C) was significantly below values suggested as limiting. There is also limited evidence that combined exercise and heat stress results in an increase in serotonergic neurotransmission (Pitsiladis et al., 2002). Additionally, high levels of dopaminergic activity have been correlated with an increased tolerance to exercise in the heat (Bridge et al., 2003). On the basis of these findings it is possible to speculate that the central ratio of 5-HT to DA may be important to the development of fatigue during prolonged exercise in a warm environment.

Serotonin and dopamine have been implicated in the control of thermoregulation at rest (Lin et al., 1998; Lipton and Clark, 1986) and during exercise (Hasegawa et al., 2000; Strachan, 2002). Pharmacologically-induced increases in serotonergic activity have been demonstrated to transiently elevate core temperature in free living rats, with the pattern of change in hypothalamic 5-HT and 5-HIAA concentrations mirroring almost exactly the change in body temperature (Lin et al., 1998). Strachan (2002) reported a marked elevation in core temperature at rest and during exercise following pizotifen (a 5-HT2C receptor antagonist) administration, suggesting a role for the 5-HT2C receptor in the regulation of core temperature. Dopamine has also been implicated in thermoregulation, with a clear relationship reported between dopaminergic activity in the preoptic anterior hypothalamus (PO/AH) and changes in core temperature during exercise (Hasegawa et al., 2000).

While many investigations have contributed to our understanding of the increased thermoregulatory and metabolic demands experienced during prolonged exercise in the heat, it is clear that the causes of fatigue are yet to be fully understood. While
early work focused primarily on the increase in circulatory and thermoregulatory strain, there is now a considerable body of evidence to suggest that central fatigue may be accelerated when exercise is performed in a warm environment. An overview of the factors currently thought to be important in the development of fatigue during prolonged exercise in a warm environment is illustrated in Figure 1.7.

![Diagram](image)

**Figure 1.7:** Potential interaction between hyperthermia, physiological function and performance during exercise in the heat (Hargreaves and Febbraio, 1998).

### 1.12 - Summary and Aims

Support for a central component of fatigue mediated through changes to central neurotransmission is relatively poor under temperate environmental conditions. It appears likely that the development of fatigue during prolonged exercise can be largely explained by mechanisms peripheral to the CNS, including the depletion of muscle glycogen (Bergstrom et al., 1967), and the development of hypohydration (Maughan and Shirreffs, 1997). This is not to suggest that the brain has no role in fatigue whilst exercising in temperate conditions, as psychological aspects significantly influence exercise performance and ultimately an individual has to make a conscious decision to cease exercise (Kayser, 2003).
Evidence is accumulating for a role of the CNS in the development of fatigue during prolonged exercise in a warm environment (Nielsen and Nybo, 2003). Perhaps this acts as a protective mechanism to limit tissue and cellular damage resulting from hyperthermia, but the neurobiological mechanism behind this response is not clear at present. The aim of this series of studies was to examine the potential role of central neurotransmitter systems and changes in blood-brain barrier function in the development of fatigue during prolonged exercise in a warm environment.

The studies described in chapters 3 and 4 examined the effects of acute BCAA supplementation on the capacity to perform prolonged exercise in a warm environment. These studies were undertaken to further investigate the role of serotonin-mediated fatigue during prolonged exercise in the heat. The work in chapter 5 was undertaken to determine the magnitude and time-course of changes in the plasma concentration ratio of \( f \)-TRP to BCAA at rest, following meals containing added CHO and fat. The work described in Chapter 6 investigated the effect of acute dopamine / noradrenaline reuptake inhibition on exercise performance in temperate and warm environments. Finally, the effect of water immersion and prolonged exercise under temperate and warm conditions on BBB function was considered in Chapter 7.
Chapter 2

General Methods
Chapter 2 General Methods

2.1 – Ethical approval

All work described in Chapters 3, 4 and 5 received ethical approval from the Grampian Research Ethics Committee. The Loughborough University Ethical Advisory Committee approved the experimental protocol employed in Chapter 7. The bupropion study (Chapter 6) was approved by the Research Council of the Vrije Universiteit Brussel, Belgium. Prior to the start of each investigation all potential volunteers were first approached either in person, or contacted via email or poster. Those expressing an interest in taking part received written details outlining the background to the study, information regarding the protocol and any possible discomfort that may arise during the investigation. Following an opportunity to ask any questions, those interested in volunteering signed a written statement of consent. All subjects were fully aware from the outset that they were free to withdraw from the study at any time without providing any reason for doing so.

2.2 – Subjects

Subjects were typically recruited from the University staff and student populations as well as local sports clubs. Due to the physically demanding nature of the investigations described in Chapters 3, 4, 6 and 7, subjects were familiar with the sensation of strenuous exercise, and had regularly taken part in prolonged exercise for a number of years. Subjects recruited to take part in the studies investigating responses to exercise in a warm environment were unaccustomed to exercise in the heat at the time of the investigation. Due to the nature of this series of studies, those with a history of psychiatric illness and / or metabolic disease were excluded. Those individuals that did not fit the inclusion criteria were thanked for their interest and politely told that their help would not be required.
2.3 – Experimental design and standardisation of experimental conditions

The studies described in Chapters 3, 4 and 6 were placebo-controlled trials. Trials were randomised using a Latin-square crossover design to minimise any order effect and administered in a double-blind manner. The studies investigating the effect of meals with added CHO and fat (Chapter 5), and potential changes in blood-brain barrier permeability during exercise (Chapter 7) were randomised crossover trials. Trials were performed at the same time of day to minimise the influence of circadian variation and were also performed on the same day of the week wherever possible to further standardise the pre-exercise conditions. Prior to the start of the experimental trials, at least one familiarisation trial was undertaken to ensure the subjects were accustomed to the procedures employed during the investigations and to minimise any potential learning or anxiety effects. This followed the exact protocol used in the experimental trials. In Chapters 3, 4 and 6, the placebo treatment was administered during the familiarisation. During the BCAA supplementation trials (Chapters 3 and 4) a single expired gas collection was made following 15 minutes of exercise to verify the workload was correct to elicit the desired percent $\text{VO}_2\text{peak}$.

To help ensure metabolic conditions were similar before each experimental trial, subjects were instructed to record all food and fluid intake (household measures technique), as well as any exercise performed, in a diary over the 2 days prior to the first trial. This dietary intake and physical activity was replicated as closely as possible during the 2 days before subsequent trials. Subjects were also asked not to perform any strenuous exercise or consume alcoholic beverages in the 24 hours prior to all trials.

All trials outlined in this thesis were performed following an overnight fast, with the exception of the study described in chapter 6. In the study investigating the influence of bupropion administration on exercise performance, subjects were instructed to ingest a standardised breakfast 90 minutes before entering the lab. Subjects in all investigations were asked to consume 500 mL of plain water around 90 minutes before entering the lab.
The environmental conditions for exercise trials were controlled using a climatic chamber. Thermostat-controlled floor standing heaters were used to elevate the ambient temperature to the desired level prior to each trial. The room temperature was monitored throughout the protocol using a dry-bulb ambient temperature probe (YSI UK Ltd, Hampshire, UK or Gram Corp. LT-8A, Saitama, Japan), with any deviation in temperature corrected to maintain the temperature within at least ± 0.5 °C of the desired temperature. Relative humidity was also determined at regular intervals using a hygrometer. When subjects were required to remain seated at rest, the ambient temperature was maintained within a comfortable range (24 - 25 °C). Trials investigating prolonged exercise in a warm environment (Chapters 3, 4, 6 and 7) were separated by at least 7 days to limit the development of heat acclimation (Barnett and Maughan, 1993).

2.4 – Measurement of peak oxygen uptake (\(\dot{V}O_2\) peak)

To determine the workloads subjects were required to exercise at during the experimental protocols, the determination of peak oxygen uptake (\(\dot{V}O_2\) peak) was first necessary. This was undertaken using either a discontinuous (Chapters 3, 4 and 7) or continuous (Chapter 6), incremental graded exercise test on an electrically braked cycle ergometer. The discontinuous protocol required subjects to complete between 4 to 6 discrete 3 minute increments, beginning at an initial workload of 100 watts (W). Depending upon the subject's performance in the previous stage (e.g. was fatigue evident, heart rate close to age-predicted maximum?), the workload was increased by either 50 or 25 W. Between each bout, a supervised rest period of 3 to 5 minutes was observed, during which the subject was able to walk around and drink plain water, if desired. The test continued until the subject retired through volitional exhaustion. The continuous protocol required subjects to began exercise at an initial workload of 80 W, with the intensity increased by 40 W every 3 minutes until volitional exhaustion. Maximum workload (\(W_{\text{max}}\)) was determined using the following equation: 

\[ W_{\text{max}} = W_{\text{out}} + (t/180) \times 40 \] 

where ‘\(W_{\text{out}}\)’ is the workload of the last completed stage and ‘t’ is the time in seconds of the final stage (Jeukendrup et al.,
1996). The experimenters provided verbal encouragement during both protocols to help ensure a maximal effort.

Expired gas was collected during the last 120 seconds of the first, and the final 60 seconds of the remaining stages of the discontinuous protocol, using the Douglas bag method (described by Astrand et al., 2003). Throughout the continuous test, expired gas was analysed using an automated spirometry system (Metamax, Cortex, Biophysik GmbH, Germany). At the end of each increment in both protocols, heart rate was recorded using telemetry (Polar Beat, Kempele, Finland).

The expired gas collected using Douglas bags was analysed for oxygen (O₂) and carbon dioxide (CO₂) composition by drawing gas through a Servomex paramagnetic O₂ transducer (Sussex, UK) and medical CO₂ analyser (Beckman LB-2, Illinois, USA). Both analysers were calibrated prior to each test using appropriate gases of known concentrations (BOC Gases, Surrey, UK). The remaining expired gas in the Douglas bag was then evacuated using a Harvard dry gas meter (Harvard Apparatus Ltd, Kent, UK), to determine gas volume and temperature (Comark, Sussex, UK). All expired gas volumes were corrected to standard temperature and pressure dry (STPD) prior to integration into further analysis.

The expired gas data were used to calculate oxygen uptake (\(\dot{V}O_2\)), carbon dioxide production (\(\dot{V}CO_2\)), ventilation rate (\(\dot{V}_E\)), and respiratory exchange ratio (RER) for each stage of the protocol. These data were then used to calculate the workloads corresponding to an appropriate percentage of a subject's \(\dot{V}O_2\) peak. Measurement of expired gas composition was also used during experimental trials to estimate rates of substrate oxidation (Peronnet and Massicotte, 1991) and energy expenditure (Chapters 4 and 7).

2.5 - Exercise trials

All the exercise-based investigations outlined in this thesis used cycle exercise on a stationary, electrically braked cycle ergometer as an experimental model (Gould
Corival 300 or Lode Excalibur Sport, Groningen, Holland). This type of ergometer was used as a preset workload can be maintained by the subject independent of pedal frequency (hyperbolic ergometry). During the preliminary incremental test to exhaustion, subjects were instructed to set up the cycle ergometer, as desired (e.g. saddle height etc). These details were recorded and the ergometer was set to their specifications prior to the start of each trial. Wherever possible subjects were supervised by the same experimenters for all experimental trials to minimise the influence of social facilitation.

Trials investigating the effect of BCAA supplementation on exercise capacity (Chapters 3 and 4), used exercise time to volitional exhaustion, defined as an inability to maintain a pedal cadence of ≥ 60 revolutions.min⁻¹, as the outcome measure. To help ensure the achievement of a maximal effort, the experimenters provided verbal encouragement, particularly during the later stages of each trial. To assess the influence of pharmacological manipulation of central dopamine and noradrenergic systems on exercise performance (Chapter 6), a time trial (TT) protocol was employed. The TT required the subjects to complete an amount of work equal to 30 minutes at 75% \( W_{\text{max}} \) as quickly as possible (Jeukendrup et al, 1996). Subjects began the TT at a workload corresponding to 75% \( W_{\text{max}} \) and were free to increase or decrease their power output as desired. During the TT a computer program displayed a bar indicating the percentage of total work completed to give the subject an indication of their progress. No feedback was provided regarding time lapsed, power output, pedal cadence or heart rate.

Throughout the thesis, before and after exercise post-void nude body mass was measured to the nearest 10 g, using a beam balance (Marsdens, London, UK). When body mass was measured following exercise, the subject first towelled them self dry to remove any sweat lying on the skin surface. The difference in body mass before and after exercise, corrected for any urine output and fluid ingestion, was used as an index of sweat losses that had occurred. These data were not corrected for immiscible fluid losses (e.g. respiratory water and losses due to substrate oxidation).
To monitor core body temperature whilst at rest and during exercise, subjects inserted a flexible rectal thermistor (YSI UK Ltd, Hampshire, UK or Gram Corp. LT-8A, Saitama, Japan) 10cm beyond the anal sphincter. Surface skin thermistors (YSI UK Ltd, Hampshire, UK or Gram Corp. LT-8A, Saitama, Japan) were positioned at four sites (chest, upper arm, thigh and calf). Thermistors were held securely in contact with the skin using transpore medical tape (3M, Loughborough, UK). Weighted mean skin temperature was calculated using the methods described by Ramanathan (1964). Throughout the work described in this thesis perceived exertion (RPE) was assessed at regular intervals during exercise using the standard 15-point Borg scale (Borg, 1982). To provide an indication of perceived thermal stress, a 21-point thermal sensation scale was also used (see Appendix III). Heart rate was measured whilst at rest and during exercise using short-range telemetry (Polar Beat, Kempele, Finland).

Where studies involved the ingestion of set meals (Chapters 4 and 5), the nutritional composition of the meals was determined using a computer-based dietary analysis program based on the food composition tables of McCance and Widdowson (Windiets Professional, Robert Gordon University, Aberdeen, UK). The amino acid composition of the meals ingested in Chapter 5 were determined using USDA National Nutrient Database (http://www.nal.usda.gov/fnic/foodcomp). When fluids were provided at regular intervals during exercise (Chapters 3, 4 and 6), these were maintained at a temperature of approximately 21 °C by immersing the drinks bottles in a water bath prior to ingestion.

2.6 – Blood collection, handling and analysis

To enable serial blood samples to be drawn whilst at rest and during exercise, a 21 g butterfly cannula (Terumo, Leuven, Belgium) was introduced into a superficial vein found in either the forearm or back of the hand. Blood was drawn through a three-way tap into dry syringes, except during the bupropion study were vacutainer tubes were used (see Chapter 6). To ensure the cannula remained patent between blood samples, it was flushed with a small volume of heparinised saline after sampling. Where arterialised venous blood was required whilst seated at rest, the subject’s forearm was immersed in warm water (40 – 42 °C) for approximately 10 minutes prior to sampling.
(Nauck et al., 1992). Collected blood was immediately dispensed into plain tubes or tubes containing an anticoagulant (e.g. K$_2$EDTA or lithium heparin) depending on its intended analytical use. Assays used widely throughout this thesis are described below, with assays common to a single study described in the methods section of the appropriate chapter. All biochemical analyses performed throughout this thesis were performed in duplicate, unless otherwise stated.

In all investigations, with the exception of the bupropion study, 2.5 mL of whole blood was dispensed into tubes containing K$_2$EDTA. Duplicate 100 µL aliquots of blood were rapidly deproteinised in 1000 µL of ice-cold 0.3 N perchloric acid. These were centrifuged, and the resulting supernatant was used for spectrophotometric determination of blood glucose on the day of the trial using a commercially available assay kit (God-Perid, Sigma Diagnostics, Poole, UK or God-PAP, Randox, Co. Antrim, UK). Blood lactate was determined fluorimetrically within 48 hours of each trial using the methods outlined by Maughan (1982).

The remaining EDTA-treated whole blood was used for the spectrophotometric determination of haemoglobin (Hb) by the cyanmethaemoglobin method. Packed cell volume (PCV) was measured in triplicate using microcentrifugation (Hawksley, Sussex, UK). Both Hb and PCV were determined within 2 hours after each experimental trial. Using the Hb and PCV values obtained, percentage changes in blood, plasma and red cell volumes relative to the first sample were estimated using the methods proposed by Dill and Costill (1974).

K$_2$EDTA or lithium heparin treated whole blood was centrifuged at 1500 g for 15 minutes at 4 °C, with the visible plasma transferred into plain eppendorf tubes ready for storage at -20 °C. Plasma free-fatty acids (FFA) were analysed using a commercially available test kit (Roche Diagnostics, Mannheim, Germany). Changes in circulating plasma amino acids were determined using enzymatic fluorimetric methods. To isolate plasma free-tryptophan (f-TRP) from albumin bound tryptophan, a 0.5 mL aliquot of plasma was centrifuged at 4 °C for 120 minutes at 400 g through a 12 kDa cellulose tri-acetate membrane (Whatman VectaSpin Micro, Kent, UK). Plasma and ultrafiltrate samples were stored frozen at -20 °C ready for the analysis of
total-TRP and f-TRP concentrations (see Appendix II). Plasma branched-chain amino acid concentrations were determined through the oxidative deamination of L-leucine, L-isoleucine and L-valine by leucine dehydrogenase. NADH is produced stoichiometrically from the oxidation of BCAA in an alkaline reaction medium (Gleeson and Maughan, 1987).

2.7 – Statistical analysis

The statistical analyses of the physiological data collected throughout this series of investigations were performed using Minitab v13.3 for Windows (Minitab Inc. PA, USA). Normality was first determined using the Ryan-Joiner correlation test. All normally and non-normally distributed results are presented as means ± standard deviation and median ± range respectively, unless otherwise stated. Data presented in figures throughout the thesis are also contained in Appendix I as numerical values. To identify differences in normally distributed results, one and two-way (time-by-trial) repeated measures analysis of variance (ANOVA) were employed. Where a significant interaction was apparent, pair-wise differences were evaluated using Tukey’s post hoc procedure. Non-normal data were examined using Kruskal-Wallis tests where appropriate. For the purpose of hypothesis testing, the 95 % level of confidence was predetermined as the minimum criterion to denote a statistical difference (P < 0.05).
Chapter 2

2.8 – Coefficients of variation (CV) of assays

Intra-assay coefficient of variation (SD/mean*100) was determined for the biochemical assays used throughout this thesis (Table 2.1) using at least 20 samples.

Table 2.1: Intra-assay coefficient of variation of biochemical assays used throughout this thesis

<table>
<thead>
<tr>
<th>Assay</th>
<th>Method</th>
<th>Mean</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>Cyanmethaemoglobin</td>
<td>146 g.L⁻¹</td>
<td>0.6</td>
</tr>
<tr>
<td>Packed Cell Volume</td>
<td>Microcentrifugation</td>
<td>45.0 %</td>
<td>0.6</td>
</tr>
<tr>
<td>Blood Glucose</td>
<td>GOD-Perid (Sigma)</td>
<td>5.1 mmol.L⁻¹</td>
<td>1.1</td>
</tr>
<tr>
<td>Blood Glucose</td>
<td>GOD-PAP (Randox)</td>
<td>5.1 mmol.L⁻¹</td>
<td>1.3</td>
</tr>
<tr>
<td>Blood Lactate</td>
<td>Maughan (1982)</td>
<td>1.47 mmol.L⁻¹</td>
<td>0.8</td>
</tr>
<tr>
<td>Plasma BCAA</td>
<td>Gleeson and Maughan (1987)</td>
<td>372 μmol.L⁻¹</td>
<td>1.3</td>
</tr>
<tr>
<td>Plasma free-tryptophan</td>
<td>See Appendix I</td>
<td>4.6 μmol.L⁻¹</td>
<td>1.6</td>
</tr>
<tr>
<td>Plasma total-tryptophan</td>
<td>See Appendix I</td>
<td>43 μmol.L⁻¹</td>
<td>1.9</td>
</tr>
<tr>
<td>Plasma free fatty acids</td>
<td>Half-micro test kit</td>
<td>0.36 mmol.L⁻¹</td>
<td>2.3</td>
</tr>
<tr>
<td>Plasma ammonia</td>
<td>Glutamate dehydrogenase</td>
<td>90.4 μmol.L⁻¹</td>
<td>6.6</td>
</tr>
<tr>
<td>Plasma ACTH</td>
<td>Immulite 2000 (DPC)</td>
<td>23.7 ng.L⁻¹</td>
<td>13.1</td>
</tr>
<tr>
<td>Serum sodium</td>
<td>Flame photometry</td>
<td>143 mmol.L⁻¹</td>
<td>0.7</td>
</tr>
<tr>
<td>Serum potassium</td>
<td>Flame photometry</td>
<td>5.4 mmol.L⁻¹</td>
<td>1.7</td>
</tr>
<tr>
<td>Serum chloride</td>
<td>Coulometric Titration</td>
<td>104 mmol.L⁻¹</td>
<td>1.1</td>
</tr>
<tr>
<td>Serum osmolality</td>
<td>Freezing point depression</td>
<td>295 mOsm.kg⁻¹</td>
<td>0.9</td>
</tr>
<tr>
<td>Serum prolactin</td>
<td>Architect (Abbott)</td>
<td>6.3 ng.mL⁻¹</td>
<td>5.8</td>
</tr>
<tr>
<td>Serum cortisol</td>
<td>Immulite 2000 (DPC)</td>
<td>32.5 ng.mL⁻¹</td>
<td>8.3</td>
</tr>
<tr>
<td>Serum growth hormone</td>
<td>Immulite 2000 (DPC)</td>
<td>3.3 mIU.L⁻¹</td>
<td>8.0</td>
</tr>
<tr>
<td>Serum S100b</td>
<td>Microplate ELISA</td>
<td>0.06 μg.L⁻¹</td>
<td>6.7</td>
</tr>
</tbody>
</table>
Chapter 3

Branched-chain amino acid supplementation during prolonged exercise in a warm environment
3.1 – Abstract

Eight healthy males were recruited to examine the influence of BCAA ingestion on prolonged exercise capacity in a warm environment. Subjects entered the lab in the morning following an overnight fast. 500 mL of a BCAA+CHO solution (12 g.L\(^{-1}\) BCAA, 65 g.L\(^{-1}\) CHO) or CHO placebo (65 g.L\(^{-1}\) CHO) was ingested 45 minutes prior to exercise. Subjects remained seated in a comfortable environment for 45 minutes, before cycling to volitional exhaustion at 60 % $\dot{V}_{\text{O}}_{2}$ peak in climatic chamber maintained at 35.0 °C. An additional 250 mL of the appropriate solution was consumed at 15 minute intervals throughout exercise. No difference in time to exhaustion was apparent between the BCAA (62.8 ± 15.7 min) and placebo (60.8 ± 18.8 min) trials ($P = 0.583$). The addition of BCAA to the ingested solutions produced a marked increase in plasma BCAA immediately prior to exercise ($+843 \pm 229 \mu \text{mol.L}^{-1}; P < 0.001$), with this difference maintained throughout. Consequently, a significantly lower f-TRP:BCAA was observed during the BCAA trial compared with the placebo ($P < 0.001$). Core temperature was similar throughout both trials ($P = 0.855$), with temperatures of 39.2 ± 0.6 °C and 39.1 ± 0.6 °C respectively recorded at exhaustion. Weighted mean skin temperature was also unaffected by the supplementation ($P = 0.522$). The subjects’ RPE ($P = 0.496$) and perceived thermal stress ($P = 0.668$) was not different between trials. No effect of BCAA supplementation on blood glucose ($P = 0.063$) or lactate ($P = 0.498$) concentrations was apparent during the trials. These results suggest that ingestion of a BCAA+CHO solution does not enhance the capacity to perform prolonged exercise in a warm environment over that of a CHO placebo.

3.2 – Introduction

It has been suggested that changes to the synthesis and metabolism of central neurotransmitters may contribute to the genesis of fatigue during prolonged exercise (Newsholme et al., 1987). While changes in the central synthesis of $5\cdot \text{HT}$ have long been associated with alterations to mood, arousal, lethargy and sensory perception, Newsholme and colleagues (1987) were first to propose that fatigue during exercise may result from changes in the activity of serotonergic neurons. The authors suggested
that the shift in substrate utilisation that occurs as exercise progresses would result in a
greater cerebral uptake of f-TRP, with the increased delivery of this amino acid
precursor thought to be directly responsible for determining the rate of 5-HT synthesis
(Fernstrom, 1983). See Chapter 1 for a detailed description of mechanism behind
Newsholme’s central fatigue hypothesis.

The acute administration of pharmacological agents thought to manipulate extracellular
5-HT concentrations has provided some support for this hypothesis (Davis et al., 1993;
Struder et al., 1998; Wilson and Maughan, 1992), but nutritional manipulation of
plasma amino acids have produced inconsistent results despite a good rationale. The
positive findings of an early field study conducted by (Blomstrand et al., 1991)
supporting an ergogenic benefit of BCAA have not been reproduced in apparently well-
controlled laboratory studies conducted in temperate environmental conditions
(Blomstrand et al., 1995; Madsen et al., 1996; Struder et al., 1998; van Hall et al.,
1995). Although evidence in humans is sparse, rodent studies have demonstrated
BCAA administration to be effective at attenuating 5-HT synthesis during exercise
(Gomez-Merino et al., 2001) and prolonging exercise capacity (Calders et al., 1999;
Calders et al., 1997).

It is widely accepted that the capacity to perform prolonged exercise is significantly
reduced in a warm environment (Galloway and Maughan, 1997; Parkin et al., 1999),
but the mechanism for the deleterious effects of heat stress remain unclear. While it
appears that performance is primarily limited by thermoregulatory and fluid balance
factors (Hargreaves and Febbraio, 1998), it has been proposed that hyperthermia may
result in an acceleration of central fatigue (Bruck and Olschewski, 1987; Nielsen,
1992). Subsequent work has provided support for this proposal, with elevated core
temperature demonstrated to impair maximal muscle activation (Nybo and Nielsen,
2001a), alter brain activity (Nielsen et al., 2001) and increase perceived exertion (Nybo
and Nielsen, 2001c). These recent findings suggest that the CNS is important in the
development of fatigue during prolonged exercise in a warm environment, but the
underlying neurobiological mechanism(s) behind these responses are currently
unknown.
Work conducted by Mittleman and colleagues (1998) has provided some evidence in support of the importance of serotonergic neurotransmission in the development of central fatigue during exercise in a warm environment (34.4 °C). BCAA supplementation was reported to improve exercise capacity by 14% when compared to a polydextrose placebo (BCAA 153.1 ± 13.3 min, placebo 137.0 ± 12.2 min; P = 0.04). The ingestion of the BCAA solution resulted in a marked elevation in circulating BCAA and consequently a 50% reduction in the plasma concentration ratio of f-TRP to BCAA. The authors reported no differences in cardiovascular, thermoregulatory or substrate responses between trials, suggesting that the supplementation regimen may have been successful at attenuating the development of serotonin-mediated central fatigue. It is worth highlighting that a relatively low exercise workload was employed during this investigation (40% \( \dot{V}O_2 \) peak). Additionally, as a consequence of the low exercise intensity, the subjects’ core temperature at fatigue (37.3 – 37.7 °C) was considerably lower than the critical core temperature suggested to be important in the acceleration of central fatigue in a warm environment (~40 °C; Nielsen et al., 1993).

The aim of the present investigation was to re-examine the findings of Mittleman et al. (1998), by determining the effects of BCAA ingestion on exercise capacity during prolonged exercise in a warm environment. The present investigation employed a workload corresponding to 60% \( \dot{V}O_2 \) peak, which is closer to the intensity of exercise typically undertaken during athletic training and would elicit whole-body hyperthermia. In order to allow comparisons with the work of Mittleman et al. (1998) a similar supplementation regimen was followed, with BCAA administered at a similar time prior to the start of exercise and in similar quantities.

3.3 – Methods

Subjects. Eight healthy male subjects were recruited (age 30.9 ± 8.6 y; height 1.77 ± 0.05 m; mass 77.4 ± 8.4 kg; \( \dot{V}O_2 \) peak 4.3 ± 0.3 L.min\(^{-1}\)). All volunteers were recreationally active and regularly participated in endurance exercise, but were not accustomed to exercise in a warm environment at the time of the study. Prior to the start of the investigation all subjects received written details outlining the nature and
Chapter 3

purpose of the study, after which a statement of informed consent was signed. The protocol received ethical approval from the Grampian Research Ethics Committee.

Experimental protocol. All subjects completed an incremental exercise test to determine $\dot{V}O_2$ peak, a familiarisation trial and two experimental trials. The incremental test is described in Chapter 2, and was used to determine the exercise intensities undertaken by each subject during the remainder of the study. Approximately one week later, the subjects completed a familiarisation trial that mirrored exactly the protocol employed in the experimental trials. This was included to ensure the subjects were accustomed to the exercise protocol and environmental conditions and to minimise any learning or anxiety effects which may serve to confound the results of the study. Subjects received an appropriate quantity of the placebo solution during the familiarisation trial (see below), but were unaware of which solution was being administered. The standardisation of pre-trial conditions is outlined in Chapter 2.

Experimental trials were randomised and undertaken in a double-blind, crossover manner. Subjects reported to the laboratory in the morning (~9 am), following an overnight fast. Upon arrival, post-void, nude body mass was recorded before a rectal thermister was inserted 10 cm beyond the anal sphincter. Surface skin thermisters were placed at four sites (upper arm, chest, thigh and calf), and a heart rate monitor was positioned. Whilst seated in a comfortable environment (25.9 ± 0.2 °C), a 21 g butterfly cannula was introduced into a superficial forearm vein and remained in place throughout the experimental period to allow blood sampling whilst at rest and during exercise.

Following the collection of a 10 mL baseline blood sample (~45 minute), 500 mL of either a placebo (65 g.L⁻¹ CHO) or BCAA solution (65 g.L⁻¹ CHO, 6 g.L⁻¹ leucine, 3 g.L⁻¹ isoleucine, 3 g.L⁻¹ valine) was ingested. Both solutions comprised a commercially available sports drink powder (Gatorade Thirst Quencher, Chicago, IL), prepared to the manufacturers’ guidelines, with or without the addition of the BCAA. During the rest period heart rate, rectal and skin temperatures were recorded at 5 minute intervals. A second blood sample was then drawn 45 minutes following the baseline collection.
Subjects then entered a climatic chamber, maintained at 35.0 ± 0.3 °C (initial relative humidity 30 ± 3 %), and commenced cycle exercise at a workload corresponding to 60 ± 2 % $\overline{V}O_2$ peak. The experimental trials required the subject to continue to volitional exhaustion, defined as an inability to maintain a pedal cadence of ≥ 60 rev.min$^{-1}$ despite verbal encouragement from the experimenters. Measurements of core and skin temperatures, as well as heart rate, were recorded at 5 minute intervals throughout the exercise period. Ratings of perceived exertion and thermal stress were assessed at 10 minute intervals. Venous blood samples were drawn every 15 minutes during the first hour of exercise, and at the point of exhaustion. In addition to the initial bolus of fluid given whilst at rest, subjects also received 250 mL of appropriate solution (placebo or BCAA) in sports drink bottles every 15 minutes during exercise.

Immediately following the cessation of exercise, subject were removed from the climatic chamber into a comfortable environment, where the heart rate monitor, skin and rectal temperature probes and cannula were removed. A second nude body mass was then recorded to enable the estimation of the sweat losses incurred during exercise.

**Blood handling and analysis.** The 10 mL blood samples collected throughout the protocol were drawn into dry syringes and immediately dispensed into tubes containing an anticoagulant. 2.5 mL was dispensed into a tubes containing $K_2$EDTA, 2.5 mL was added to tubes containing ice-cold fluoride oxalate, with the remaining 5 mL placed into a tube containing lithium heparin. Samples treated with fluoride oxalate or lithium heparin were centrifuged to yield plasma, which was stored at −20 °C until analysis. Duplicate 100 μL aliquots of EDTA-treated whole blood were rapidly deproteinised in 1 mL of ice-cold 0.3 N perchloric acid. These were centrifuged and the resulting supernatant used for determination of blood glucose and lactate concentrations. Whole blood was also used for the determination of haemoglobin (Hb) and packed cell volume (PCV), allowing percentage changes in blood, plasma and red cell volumes relative to the first resting sample to be estimated (Dill and Costill, 1974). f-TRP was separated from albumin bound TRP, as described in chapter 2. Plasma and ultrafiltrate samples were then kept frozen a −20 °C ready for the determination of FFA, BCAA and f-TRP.
3.4 – Results

The subjects' individual times to exhaustion in each trial are presented in Figure 3.1A. The ingestion of a BCAA + CHO solution resulted in no change to exercise capacity when compared to the CHO placebo, with mean times of 62.8 ± 15.7 min and 60.8 ± 18.8 min recorded respectively (P = 0.583). This represents a 3.1 ± 13.0 % difference between trials, but there was no apparent trend in the change in exercise capacity following the ingestion of BCAA (Figure 3.1B). There was no evidence of an order effect during the experimental trials (Wk1 62.7 ± 16.7 min, Wk2 61.0 ± 17.9 min; P = 0.637).

![Figure 3.1](image_url)

**Figure 3.1**: Individual exercise times to exhaustion (A) and percent changes in exercise capacity (B) between the placebo and BCAA trials. The dark lines represent the mean changes in exercise capacity.

The ingestion of BCAA prior to the start of exercise resulted in a elevation in plasma BCAA concentrations (+843 ± 229 μmol.L⁻¹; P < 0.001), with this level maintained throughout exercise (Figure 3.2). During the placebo trial there was a tendency for a reduction in circulating BCAA concentration as time progressed, but this failed to reach significance (P = 0.111).
Figure 3.2: Plasma BCAA concentration at rest and during exercise. a and b denote a significant difference from −45 min time point in the placebo and BCAA trials respectively (P < 0.05). * (P < 0.05) and ** (P < 0.01) denote a significant difference between the BCAA trial and the corresponding time point on the placebo trial.

Table 3.1: Changes in plasma f-TRP concentrations (μmol.L⁻¹) and the plasma concentration ratio of f-TRP to BCAA (no units). a and b denote a significant difference from the −45 time point in the placebo and BCAA trials respectively (P < 0.05), * denotes a significant difference from the corresponding time-point (P < 0.01).

<table>
<thead>
<tr>
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<th>Ex</th>
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<tr>
<td>f-TRP:</td>
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<td></td>
<td></td>
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<tr>
<td>Placebo</td>
<td>6.8 ± 2.5</td>
<td>5.5 ± 1.2</td>
<td>7.5 ± 2.0</td>
</tr>
<tr>
<td>BCAA</td>
<td>6.0 ± 3.7</td>
<td>5.5 ± 2.6</td>
<td>8.6 ± 5.0</td>
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<td>f-TRP:</td>
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<td></td>
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</tr>
<tr>
<td>Placebo</td>
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<td>0.014 ± 0.003</td>
<td>0.022 ± 0.006</td>
</tr>
<tr>
<td>BCAA</td>
<td>0.014 ± 0.010</td>
<td>0.005 ± 0.002</td>
<td>0.009 ± 0.007</td>
</tr>
</tbody>
</table>

Changes in plasma f-TRP and the plasma concentration ratio of f-TRP to BCAA are presented in Table 3.1. No difference in circulating f-TRP was apparent between experimental trials (P = 0.906). Baseline (-45 min) f-TRP:BCAA values were similar...
(\(P = 0.846\)), with a marked reduction in this ratio observed immediately prior to exercise and at the point of exhaustion following the ingestion of the BCAA+CHO solution (\(P < 0.001\)). At the point of exhaustion f-TRP:BCAA was elevated over the -45 minute time point in the placebo trial.

**Figure 3.3**: Change in core (A) and weighted mean skin (B) temperature at rest and during exercise. a and b denote a significant difference from the pre-exercise value in the placebo and BCAA trials respectively (\(P < 0.05\)).
Core temperature was not different during the two trials ($P = 0.855$) with a progressive rise observed throughout the exercise period (Figure 3.3A). At 35 minutes of exercise, and beyond, core temperature was significantly elevated over resting values ($P < 0.05$). Core temperature increased similarly during both trials reaching $39.1 \pm 0.6 \, ^\circ C$ and $39.2 \pm 0.6 \, ^\circ C$ at the point of exhaustion in the placebo and BCAA trials respectively ($P = 0.534$). Weighted mean skin temperature was not different between trials whilst seated at rest prior to commencing exercise ($P = 0.194$). Skin temperature increased rapidly in both trials during the initial 15 minutes of exercise, after which the temperature continued to rise at a slower rate (Figure 3.3B).

![Graph](image)

**Figure 3.4:** The heart rate response at rest and during exercise. a and b denote a significant difference from the pre-exercise value in the placebo and BCAA trials respectively ($P < 0.05$).

Heart rate was significantly elevated above resting values throughout the exercise bout in both trials ($P < 0.001$; Figure 3.4), but BCAA ingestion produced no change whilst at rest or during exercise ($P = 0.823$). From the onset of exercise RPE progressively increased throughout both trials, with no detectable difference between the BCAA and placebo ($P = 0.496$; Figure 3.5A). There was a tendency for an elevated RPE at the
point of exhaustion during the placebo trial ($P = 0.078$). No difference in thermal stress was observed between the two trials ($P = 0.668$; Figure 3.5B). The perceived sensation of thermal stress was significantly elevated above the initial exercise time point following 40 minutes of exercise in both experimental trials.

**Figure 3.5**: Change in RPE (A) and thermal stress (B) during the placebo and BCAA trials. a and b denote a significant difference from the 10 minute time point in the placebo and BCAA trials respectively ($P < 0.05$).
Figure 3.6: Blood glucose (A) and lactate (B) concentrations at rest and during exercise. a and b denote a significant difference from the -45 minute time point in the placebo and BCAA trials respectively (P < 0.05).

There was a clear tendency for a difference in blood glucose concentration between trials, but this was not significant (P = 0.063). Following a small rise in glucose post-ingestion, a transient fall was apparent during the initial 15 minutes of exercise in both
the placebo ($P = 0.023$) and BCAA ($P < 0.001$) trials. Blood glucose concentrations were restored to resting levels as exercise progressed. BCAA supplementation produced no effect on the blood lactate concentration ($P = 0.498$; Figure 3.6B). Lactate levels increased progressively during both trials, reaching peak values of $4.7 \pm 2.4$ mmol.L$^{-1}$ and $4.5 \pm 2.0$ mmol.L$^{-1}$ at exhaustion in the placebo and BCAA trials respectively. Plasma FFA were not different between trials, but tended to be lower following BCAA ingestion ($P = 0.264$). Ingestion of both placebo and BCAA solutions produced a marked reduction in FFA immediately prior to commencing exercise, increasing to $0.38 \pm 0.10$ mmol.L$^{-1}$ and $0.32 \pm 0.10$ mmol.L$^{-1}$ at exhaustion. To determine the relationship between changes in plasma FFA and plasma f-TRP observed in the placebo and BCAA trials, the difference between baseline and exhaustion values were correlated. The correlation coefficients were calculated as $-0.22$ ($P = 0.596$) for the placebo and $-0.04$ ($P = 0.927$) for BCAA trial.

Table 3.2: Percentage changes in blood, plasma and red cell volumes relative to the -45 minute sample at rest and during exercise. $a$ and $b$ denote a significant difference from the -45 minute time point in the placebo and BCAA trials respectively ($P < 0.05$).

<table>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>2.4 ± 2.3</td>
<td>-5.3 ± 2.0$^a$</td>
<td>-5.8 ± 2.5$^a$</td>
<td>-5.5 ± 3.0$^a$</td>
</tr>
<tr>
<td>BCAA</td>
<td>2.4 ± 3.7</td>
<td>-4.0 ± 2.2$^b$</td>
<td>-4.2 ± 3.4$^b$</td>
<td>-3.9 ± 4.5$^b$</td>
</tr>
<tr>
<td>Plasma Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>3.5 ± 3.4</td>
<td>-9.2 ± 3.6$^a$</td>
<td>-7.1 ± 9.0$^a$</td>
<td>-9.1 ± 5.8$^a$</td>
</tr>
<tr>
<td>BCAA</td>
<td>3.8 ± 5.4</td>
<td>-7.4 ± 3.2$^b$</td>
<td>-9.0 ± 3.7$^b$</td>
<td>-9.3 ± 3.6$^b$</td>
</tr>
<tr>
<td>Cell Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.9 ± 1.5</td>
<td>-0.4 ± 1.1</td>
<td>-0.6 ± 1.6</td>
<td>-0.5 ± 1.9</td>
</tr>
<tr>
<td>BCAA</td>
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<td>0.4 ± 2.3</td>
<td>0.2 ± 2.6</td>
<td>0.3 ± 3.0</td>
</tr>
</tbody>
</table>

Percentage changes in blood, plasma and red cell volumes relative to the first resting sample are presented in Table 3.2. Reductions in blood ($P < 0.001$) and plasma ($P < 0.001$) volumes were observed during exercise, but there was no difference between experimental trials apparent at any point during the protocol.
3.5 - Discussion

There appears to be an inverse relationship between exercise time to fatigue and ambient temperature (Galloway and Maughan, 1997; Parkin et al., 1999). Limited evidence suggests that central 5-HT activity may be an important factor in the early onset of fatigue during prolonged exercise in the heat. The aim of this investigation was to further investigate the effects of BCAA supplementation on exercise capacity during prolonged exercise in a warm environment.

The results of the present study demonstrate that the ingestion of a solution containing BCAA and CHO offers no additional ergogenic benefit over the ingestion of a CHO placebo during prolonged exercise performed in a warm environment (Figure 3.1; \( P = 0.583 \)). These findings fail to support previous data from Mittleman and co-workers (1998) reporting a marked (14 %; \( P = 0.04 \)) improvement in time to exhaustion when cycle exercise was performed at 40 % VO\(_2\) peak in a warm environment following the ingestion of BCAA. While the authors were cautious in attributing a mechanism behind this positive observation, the main focus of the study was directed at the use of BCAA in limiting the development of serotonin-mediated central fatigue.

Although a number of studies investigating the efficacy of BCAA supplementation on exercise capacity in temperate conditions (~20 °C) have failed to identify an ergogenic benefit, despite a marked reduction in the plasma concentration ratio of f-TRP to BCAA (Blomstrand et al., 1995; Madsen et al., 1996; Struder et al., 1998; van Hall et al., 1995), it has been suggested that central fatigue may be accelerated by the development of hyperthermia. This may go some way to explaining the marked reduction in exercise capacity observed in a warm environment. While recent work undertaken by Nielsen's research group in Copenhagen have established that hyperthermia results in a reduction in maximal muscle activation (Nybo and Nielsen, 2001a), altered brain activity (Nielsen et al., 2001) and increased perceived exertion (Nybo and Nielsen, 2001c), the biochemical mechanism(s) underlying these changes remains unknown at present.

The measured changes in plasma BCAA, f-TRP and the plasma concentration ratio of f-TRP to BCAA apparent in the present investigation were comparable to those
reported by Mittleman and colleagues (1998) and other studies examining the effect of BCAA supplementation. In accordance with the recommendations of Newsholme and Castell (2000) and to mirror the supplementation protocol employed by Mittleman et al. (1998), BCAA were administered as a number of small bolii, before and during exercise, rather than a single pre-exercise dose. The ingestion of 6g BCAA prior to the start of exercise resulted in a marked elevation in plasma BCAA concentrations (+843 ± 229 μmol.L⁻¹; P < 0.001), with this level maintained throughout the exercise bout through the repeated consumption of small boluses (3 g / 15 min). Subjects ingested between 6 and 18 g BCAA during exercise, due to variations in exercise duration. During the placebo trial the mean circulating BCAA concentration decreased progressively throughout exercise, although failing to reach significance. While there was no difference in f-TRP levels between trials, a marked reduction in the ratio of f-TRP to BCAA was observed during the BCAA trial due to the presence of exogenous BCAA.

It has been proposed that changes in the availability of amino acid precursors and competing large neutral amino acids of the magnitude reported in this, and other BCAA supplementation studies, may be insufficient to produce functional changes in central monoamine synthesis (Fernstrom, 2000). While a 13- to 26-fold increase in the plasma concentration ratio of TRP to BCAA has been reported to be necessary to induce a change in the tissue content of 5-HT and its metabolite 5-HIAA in the brain stem of monkeys (Leathwood and Fernstrom, 1990), recent brain microdialysis studies do not support this suggestion. Both Meeusen et al. (1996) and Gomez-Merino et al. (2001) have reported marked changes in extracellular 5-HT in the hippocampus following TRP or valine infusion. These changes were apparent following a similar alteration to plasma amino acid concentrations as observed during the present investigation.

Core temperature at the point of fatigue during the placebo and BCAA trials was 39.1 ± 0.6 °C and 39.2 ± 0.6 °C respectively (P = 0.534). These mean values were around 0.5 °C lower than the critical core temperature of 39.7 ± 0.5 °C proposed by Nielsen et al., 1993), thought to result in an acceleration of central fatigue. While the concept of a critical core temperature has been subsequently supported by both human (Gonzalez-Alonso et al., 1999) and animal (Walters et al., 2000) based investigations, there are
reported instances where fatigue during prolonged exercise in the heat occurs at lower core temperatures (Sawka et al., 1992). If the attainment of a critical core temperature does act to accelerate central fatigue, potentially serving as a protective mechanism, the underlying cause of fatigue remain unclear at present. Although it has recently been demonstrated that exercise-heat induced hyperthermia results in altered brain activity, with a shift from higher frequencies (β-bands) to slower (α) wave bands (Nielsen et al., 2001). This increase in the α/β index late in exercise was proposed to represent a reduced state of arousal, which may contribute to the development of fatigue.

It has been demonstrated that the permeability of the blood-brain barrier (BBB) may become temporarily compromised during exposure to sub-lethal hyperthermia (Sharma and Dey, 1987; Wijsman and Shivers, 1993), potentially altering the transport kinetics of amino acid uptake into the CNS. It is possible to speculate that during the present investigation the whole body hyperthermia observed towards the end of exercise may have induced disturbance to the permeability of the BBB potentially comprising the efficacy of BCAA supplementation. During the Mittleman et al. (1998) study the core temperatures of the subjects were not elevated above 38 °C, largely due to the low-moderate exercise intensity employed. The influence of exercise on BBB function, using a proposed peripheral marker of permeability, is explored in Chapter 7.

A reduction in perceived exertion has been previously reported with the ingestion of BCAA (Blomstrand et al., 1997), and might be expected as changes in central 5-HT activity have been associated with feelings of reduced arousal, tiredness and lethargy. This response was not observed in the present investigation, although a difference between trials became more apparent as the exercise progressed and approached significance at the point of exhaustion (Figure 3.5A; P = 0.078). No difference in perceived thermal stress was recorded throughout the study. Previous investigations have also failed to observe changes in RPE or thermal stress despite a marked change in core temperature following the acute pharmacological manipulation of central 5-HT levels with pizotifen (Strachan, 2002). It is possible that the sensitivity of these perception scales may not be sufficient to detect subtle changes in exertion or thermal stress resulting from manipulation of central monoamine synthesis or that no such differences exist.
During both treatments, cardiovascular and substrate responses were similar at rest and during exercise. As previously demonstrated in a number of investigations (Blomstrand et al., 1995; Madsen et al., 1996; Mittleman et al., 1998; van Hall et al., 1995), the ingestion of BCAA produced no difference in the glucose and lactate response to exercise when compared to the placebo (Figure 4.5). In the present investigation there was no evidence that carbohydrate availability was limiting, with blood glucose concentrations maintained throughout exercise following an initial transient fall. Additionally, while it is accepted that CHO oxidation is elevated during exercise in a warm environment (Febbraio et al., 1994), fatigue during prolonged exercise does not appear to correspond with a depletion of muscle glycogen stores (Parkin et al., 1999).

As a result of the CHO content of the ingested solutions, FFA and consequently f-TRP concentrations were not significantly elevated during exercise. It is widely reported that a strong positive relationship exists between plasma FFA concentrations and levels of circulating f-TRP (Curzon et al., 1974). No such relationship was apparent in the present investigation, but marked increase in plasma FFA is thought to be required before TRP is displaced from albumin, and perhaps a threshold exists before a significant change in f-TRP can be measured (Struder and Weicker, 2001).

The work conducted by Mittleman and colleagues (1998) remains to date the only convincing published investigation to observe an ergogenic benefit of BCAA supplementation. This study employed low-intensity exercise (40% \( \dot{V}O_2 \text{max} \)) and consequently time to exhaustion was between 130 and 170 minutes, whereas other investigations undertaken to investigate the efficacy of BCAA supplementation have prescribed exercise at higher relative intensities (e.g. 60 – 80% \( \dot{V}O_2 \text{max} \)). It has been proposed that the development of serotonin-mediated central fatigue may become important only after 2 – 3 hours of exercise have passed (Nybo et al., 2003b). This hypothesis is supported by animal studies employing \textit{in vivo} microdialysis techniques to monitor changes in extracellular 5-HT (Bequet et al., 2001). The results of these investigations demonstrated that there appears to be a delayed elevation in extracellular 5-HT, with no change in hippocampus and cortical 5-HT concentrations apparent until 60 minutes of exercise had passed. This may explain the failure to observe an ergogenic effect in the present study where the exercise time was only around 60 minutes.
It is possible that a failure to observe a benefit from BCAA ingestion may arise from a disturbance in catecholaminergic neurotransmission. The rationale behind BCAA supplementation is to block the uptake of TRP, but the possibility that BCAA ingestion will limit the transport of TYR into the CNS and consequently attenuate central DA and NA synthesis has not been considered. As central catecholamines are thought to be important mediators of motivation and arousal during exercise (Davis and Bailey, 1997) limiting central TYR availability may adversely impact on exercise performance.

In a number of investigations where no change in exercise capacity has been reported, BCAA supplements have been administered along with CHO (Galiano et al., 1991; Madsen et al., 1996; van Hall et al., 1995). It is possible to suggest that in the present investigation, any potential ergogenic effect of BCAA may have been masked by the administration of BCAA and CHO together. This was recently demonstrated in rats with performance enhanced following the infusion of either CHO or BCAA, but no further improvement was apparent when both were administered together (Calders et al., 1999). While the role of BCAA in limiting f-TRP transport across the BBB is accepted as important, this is secondary to the role of FFA. Liberation of FFA from the adipose tissue and the subsequent displacement of TRP from albumin forms the core of the central fatigue hypothesis proposed by Newsholme et al. (1987). The insulinogenic effect of CHO ingestion has been reported to attenuate the increase in the plasma concentration ratio of f-TRP to BCAA during prolonged exercise in a dose dependent manner (Davis et al., 1992). In addition to its widely reported peripheral benefits (Coyle, 1992) and potential effect on central glucose metabolism (Nybo et al., 2003a), CHO appears to be a far more attractive ergogenic supplement for prolonged exercise.

The results of the present investigation fail to support the positive findings of Mittleman et al. (1998). Despite a large increase in plasma BCAA and consequently a marked reduction in the plasma concentration ratio of f-TRP to BCAA, supplementation did not influence exercise capacity in a warm environment compared to a CHO placebo. Failure to observe an ergogenic effect may have been caused by the administration of BCAA along with CHO or the relatively short duration of exercise completed by the subjects.
Chapter 4

Branched-chain amino acid supplementation during prolonged exercise in a warm environment after a regimen designed to reduce muscle glycogen
4.1 – Abstract

Eight healthy males were recruited to examine the effect of BCAA supplementation on exercise capacity in a warm environment following a regimen designed to reduce glycogen availability. Subjects remained seated for 2 hours, before cycling to volitional exhaustion at 50% V\textsubscript{O} peak in a warm environment (30.0 ± 0.2 °C). 250 mL of a 12 g.L\textsuperscript{-1} BCAA solution or placebo was ingested at 30 minutes intervals prior to exercise, with an additional 150 mL consumed every 15 minutes throughout exercise. BCAA ingestion (111.0 ± 29.2 min) had no effect on exercise capacity (placebo 103.9 ± 26.9 min; P = 0.129). No difference in HR (P = 0.345), core temperature (P = 0.628), or weighted mean skin (P = 0.114) temperatures was apparent between the trials. The ingestion of the BCAA solution produced a marked increase in plasma BCAA immediately prior to exercise (+1126 ± 158 µmol.L\textsuperscript{-1}; P < 0.001) with this difference maintained throughout. Consequently, a significant reduction in f-TRP:BCAA was observed during the BCAA trial when compared to the placebo (P < 0.001). Plasma ammonia was significantly elevated during exercise throughout the BCAA trial (P < 0.001), with no difference apparent during the placebo (P = 0.608). Blood glucose (P = 0.114) and lactate (P = 0.836) concentrations were not different between trials. Ingestion of a BCAA solution prior to, and during, prolonged exercise did not significantly influence exercise capacity in a warm environment, but evidence of variations in the individual sensitivity to the supplementation regimen was apparent.

4.2 – Introduction

It is clear that an individual’s capacity to perform prolonged exercise is significantly reduced when exercise is undertaken in warm conditions (Galloway and Maughan, 1997). While it appears that performance is primarily limited by thermoregulatory and fluid balance factors (Hargreaves and Febbraio, 1998), hyperthermia may also result in an acceleration of fatigue processes residing in the central nervous system (CNS) leading to a reduced drive to continue exercise (Nielsen, 1992).
Changes in the synthesis and metabolism of central neurotransmitters, in particular serotonin (5-HT), have been suggested to contribute to the genesis of fatigue during prolonged exercise (Newsholme et al., 1987). See Chapter 1 for a detailed description of Newsholme’s central fatigue hypothesis. Oral supplementation of the BCAA has been suggested to limit the entry of f-TRP into the CNS, reducing the rate of 5-HT synthesis and potentially enhancing exercise capacity (Blomstrand et al., 1991). In rats, valine administration has been demonstrated to be effective in limiting TRP uptake into the CNS and consequently attenuating the increase in extracellular 5-HT observed during prolonged exercise (Gomez-Merino et al., 2001). These findings, considered in conjunction with animal studies demonstrating a clear improvement in exercise capacity when BCAA were infused (Calders et al., 1999; Calders et al., 1997), provide good evidence for the central fatigue hypothesis and an ergogenic benefit of BCAA supplements.

Attempts to enhance exercise capacity in humans through BCAA supplementation have been largely inconclusive when exercise has been performed in temperate conditions (Blomstrand et al., 1991; Madsen et al., 1996; Struder et al., 1998; van Hall et al., 1995). However, an apparently well-controlled laboratory study reported a marked improvement in exercise capacity (+14 %) when low-moderate intensity exercise was performed in a warm environment (34 °C) following the ingestion of BCAA (Mittleman et al., 1998). These data potentially support the premise that 5-HT mediated central fatigue is accelerated when performing exercise in warm conditions. Furthermore, there is indirect evidence that central serotonergic activity may be upregulated during combined exercise and heat stress (Pitsiladis et al., 2002). However, this data may be misleading as prolactin release from the pituitary gland is governed through an complex interaction between a number of neurotransmitters rather than by serotonin alone (Meeusen et al., 2001; see Chapter 1).

The aim of the present investigation was to develop the work described in Chapter 3 to further examine the effect of BCAA ingestion on exercise capacity in a warm environment. Treatments were administered without the addition of CHO and trials were undertaken following a regimen designed to reduce glycogen availability. This was intended to simulate the metabolic conditions experienced during the latter stages of prolonged exercise, and effectively accelerate exercise-induced changes in
serotonergic neurotransmission. Exercise was performed at a low-moderate intensity with an extended pre-exercise supplementation period to allow sufficient time for changes in peripheral amino acids to significantly alter CNS concentrations and influence central neurotransmission.

4.3 – Methods

Subjects. Eight healthy male subjects were recruited (age 28.5 ± 8.2 y; height 1.76 ± 0.04 m; mass 74.5 ± 6.7 kg; \( \text{VO}_2 \text{peak} \) 4.1 ± 0.5 L.min\(^{-1}\)). All subjects took part in regular endurance exercise, but were not accustomed to exercise in a warm environment at the time of the study. Prior to the start of the investigation all subjects received written details outlining the content of the study. Following any questions regarding the protocol, a written statement of consent was signed. The protocol employed received ethical approval from the Grampian Research Ethics Committee.

Experimental protocol. To calculate the workloads used during the investigation, each subject first completed an incremental cycle ergometer test to determine \( \text{VO}_2 \text{peak} \), as described in chapter 2. Prior to the start of the experimental trials, two preliminary trials were undertaken. During the first preliminary trial subjects completed up to two hours of cycle exercise in the environmental conditions employed throughout the investigation, with the second preliminary trial mirroring the complete experimental protocol. Each subject then completed 2 experimental trials (placebo and BCAA), randomised and administered in a double-blind crossover manner, separated by at least 7 days. Standardisation of pre-trial conditions is outlined in Chapter 2.

All experimental trials commenced in the evening (5 – 6 pm) following a 4 hour fast, other than the ingestion of 500 mL of plain water 2 hours before the start of each trial. Upon arrival in the laboratory, post-void nude body mass was measured, and subjects then completed a bout of exercise on a cycle ergometer (Gould Corival 300, Groningen, Holland) corresponding to approximately 75 % \( \text{VO}_2 \text{peak} \) for 60 minutes in temperate conditions (18 – 20 °C). During exercise, expired gas was collected and
analysed at 15 minute intervals. These data were used to estimate rates of substrate oxidation and energy expenditure (Peronnet and Massicotte, 1991). Following the cessation of exercise, subjects showered and were re-weighed. Subjects then consumed 50 kJ.kg$^{-1}$ body mass of a low carbohydrate, high fat meal (cheese omelette and pork pie; 6 % CHO, 73 % fat, 21 % protein), along with a quantity of sugar-free fruit drink with electrolytes (Robinsons Special R, Chelmsford, UK) equal to 150 % of the body mass lost, to help ensure the adequate replacement of sweat losses (Shirreffs et al., 1996). A similar protocol has been employed previously to produce a significant reduction in muscle glycogen stores (Lemon and Mullin, 1980).

The next morning at 8 am, following an overnight fast other than ad libitum intake of plain water, subjects returned to the laboratory. Post-void nude body mass was recorded, and subjects were then asked to insert a rectal thermister 10 cm beyond the anal sphincter for the measurement of core temperature. Surface skin temperature probes were attached to four sites (chest, upper arm, thigh and calf) and a heart rate monitor (Polar Beat, Kempele, Finland) was positioned. Subjects were then seated in a comfortable environment (25 – 26 °C), and a 21 g cannula was inserted into a superficial forearm vein. A baseline blood sample (7.5 mL) was drawn, after which subjects consumed 250 mL of either a placebo (sugar-free fruit drink) or BCAA (sugar-free fruit drink with 6 g.L$^{-1}$ leucine, 3 g.L$^{-1}$ isoleucine, 3 g.L$^{-1}$ valine; SHS International Ltd, Liverpool, UK) solution. A further 250 mL of the appropriate drink was ingested at 30 minute intervals throughout the 2 hour rest period. Heart rate and core and skin temperatures were recorded at 15 minute intervals at rest with two further blood samples drawn at 60 and 120 minutes.

Subjects then entered a climatic chamber, maintained at 30 ± 0.2 °C (relative humidity 38 ± 8 %), and commenced cycle exercise at a power output corresponding to 50 % $\dot{V}O_2$peak. During exercise, heart rate, skin and core temperatures, and RPE and perceived thermal stress were recorded every 10 minutes. In addition, 7.5 mL blood samples were drawn at 15 minute intervals, as well as at the point of exhaustion. 150 mL of the appropriate solution (placebo or BCAA) was ingested by the subjects every 15 minutes during exercise. Exercise continued until volitional exhaustion, defined as an inability to maintain a pedal cadence of ≥ 60 rev.min$^{-1}$ despite verbal
encouragement from the experimenters. Following the cessation of exercise, the subject returned to a comfortable environment where all probes and the cannula were removed. Subjects were then asked to shower, and were reweighed to allow the estimation of sweat losses occurring during exercise.

**Blood handling and analysis.** Blood samples collected throughout the experimental protocol were drawn into dry syringes with 2.5 mL dispensed into tubes containing K$_2$EDTA. Duplicate 100 μL aliquots of whole blood were rapidly deproteinised in 1 mL of ice-cold 0.3N perchloric acid. These were centrifuged, and the resulting supernatant was used for determination of blood glucose and lactate concentrations. 1.5 mL of EDTA treated whole blood was immediately extracted and spun in a microcentrifuge before the plasma was transferred to a plain tube and frozen in liquid nitrogen. Samples were kept in liquid nitrogen ready for analysis of ammonia (NH$_3$; Sigma Diagnostics, Poole, UK) and free fatty acids using commercially available assay kits. Whole blood was also used for the determination of haemoglobin (Hb) and packed cell volume (PCV), allowing percentage changes in blood, plasma and red cell volumes relative to the first resting sample to be estimated (Dill and Costill, 1974). The remaining 5 mL was added to a tube containing lithium heparin and centrifuged to obtain plasma. f-TRP was separated from albumin bound TRP, as described in Chapter 2. Plasma and ultrafiltrate samples were then kept frozen at -20 °C ready for the determination of BCAA and f-TRP.

### 4.4 – Results

The data collected during the glycogen depletion protocol are displayed in Table 4.1. During the 60 minute cycle exercise subjects expended approximately 3.6 ± 0.5 MJ and 3.7 ± 0.5 MJ on the placebo and BCAA trials respectively (P > 0.05). Approximately 82% of the total energy demand on each of the trials was derived from CHO oxidation, corresponding to the oxidation of around 178 g of CHO. An increase in oxygen uptake was observed in both trials as the exercise bout progressed, but no difference in VO$_2$ was apparent between trials (P > 0.05). The respiratory exchange ratio fell progressively throughout exercise, becoming significantly lower
than the corresponding 15 minute time point after 30 minutes in the BCAA trial and 45 minutes in the placebo. Again, no difference in RER was apparent between trials ($P = 0.578$). Both heart rate ($P < 0.001$) and RPE ($P < 0.001$) increased significantly during the 60 minutes of exercise in both experimental trials.

Table 4.1: Summary of the glycogen depletion exercise results (mean ± SD). a and b denote a significant difference from the 15 minute time point in the placebo and BCAA trials respectively ($P < 0.05$).

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<th>30</th>
<th>45</th>
<th>60</th>
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<tbody>
<tr>
<td>$\dot{V}O_2$ (L.min$^{-1}$)</td>
<td></td>
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<tr>
<td>Placebo</td>
<td>2.77 ± 0.34</td>
<td>2.90 ± 0.36$^a$</td>
<td>2.98 ± 0.37$^a$</td>
<td>2.97 ± 0.32$^a$</td>
</tr>
<tr>
<td>BCAA</td>
<td>2.84 ± 0.37</td>
<td>2.90 ± 0.33</td>
<td>3.00 ± 0.39$^b$</td>
<td>3.02 ± 0.35$^b$</td>
</tr>
<tr>
<td>$\dot{V}CO_2$ (L.min$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>2.68 ± 0.31</td>
<td>2.68 ± 0.30</td>
<td>2.75 ± 0.31</td>
<td>2.69 ± 0.25</td>
</tr>
<tr>
<td>BCAA</td>
<td>2.79 ± 0.33</td>
<td>2.68 ± 0.31</td>
<td>2.78 ± 0.31</td>
<td>2.77 ± 0.27</td>
</tr>
<tr>
<td>RER (no units)</td>
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<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.97 ± 0.05</td>
<td>0.93 ± 0.03</td>
<td>0.93 ± 0.04$^a$</td>
<td>0.91 ± 0.05$^a$</td>
</tr>
<tr>
<td>BCAA</td>
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<td>0.93 ± 0.03$^b$</td>
<td>0.93 ± 0.03</td>
<td>0.92 ± 0.03$^b$</td>
</tr>
<tr>
<td>HR (beat.min$^{-1}$)</td>
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<td></td>
<td></td>
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<td>Placebo</td>
<td>149 ± 10</td>
<td>156 ± 10$^a$</td>
<td>160 ± 9$^a$</td>
<td>165 ± 9$^a$</td>
</tr>
<tr>
<td>BCAA</td>
<td>147 ± 7</td>
<td>152 ± 6$^b$</td>
<td>158 ± 7$^b$</td>
<td>162 ± 7$^b$</td>
</tr>
<tr>
<td>RPE (no units)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>14 ± 2</td>
<td>15 ± 2</td>
<td>15 ± 2</td>
<td>16 ± 2$^a$</td>
</tr>
<tr>
<td>BCAA</td>
<td>14 ± 1</td>
<td>14 ± 2</td>
<td>15 ± 2$^a$</td>
<td>16 ± 2$^b$</td>
</tr>
</tbody>
</table>

Mean exercise time to exhaustion was 111.0 ± 29.2 min on the BCAA trial and 103.9 ± 26.9 min on the placebo trial ($P = 0.129$). This represents a mean difference of 7.1 ± 11.6 min, or 6.4 ± 12.9 %, between trials, with 4 subjects demonstrating a clear improvement in exercise capacity with BCAA, 3 subjects with little or no difference between trials and 1 subject clearly exercising for longer on the placebo trial (Figure 4.1 A + B). No treatment order effect was apparent over the course of the investigation ($P = 0.844$).
Figure 4.1: Individual exercise times to exhaustion (A) and percent changes in exercise capacity (B) between the placebo and BCAA trials. The dark lines represent the mean changes in exercise capacity.

All subjects received 1 litre of test solution (250 mL/30 min) during the 2 hour rest period prior to exercise. This provided 12.0 g BCAA during the BCAA trial. Ingestion of this quantity of BCAA resulted in a mean increase in circulating plasma BCAA concentration of 1126 ± 158 μmol.L⁻¹ (Figure 4.2A; P < 0.001). The quantity of BCAA ingested during exercise ranged between 5.4 g and 18.0 g due to variations in performance time. Following an initial reduction in BCAA concentration during the first 15 minutes of exercise, a progressive increase in plasma BCAA was apparent, returning to pre-exercise levels at the point of exhaustion. During the placebo trial, plasma BCAA remained stable with no change observed during either rest or exercise (P > 0.964).
Figure 4.2: Plasma BCAA (A; mean ± SD) and f-TRP (B; median, range omitted for clarity, see Appendix I) concentrations at rest and during exercise. a and b denote a significant difference from the -120 min time point in the placebo and BCAA trials respectively (P < 0.05). * (P < 0.05) and ** (P < 0.01) denote a significant difference between the BCAA trial and the corresponding time point on the placebo trial.
Ingestion of the BCAA solution resulted in consistently lower f-TRP concentrations when compared to the placebo trial (Figure 4.2B; \( P < 0.003 \)). A progressive increase in f-TRP was observed on both trials, but this reached significance over baseline only at the point of exhaustion (\( P < 0.05 \)). The median plasma concentration ratio of f-TRP to BCAA was similar during the placebo (0.0086 range 0.0055 - 0.0105) and BCAA trials (0.0104 range 0.0047 - 0.0153) prior to the ingestion of first bolus of solution (Figure 4.3). BCAA ingestion resulted in a 4-fold reduction in the f-TRP:BCAA immediately prior to the start of exercise (\( P < 0.001 \)), with no change apparent whilst at rest in the placebo trial. This difference was maintained throughout exercise, with median values at exhaustion of 0.0267 (0.0163 - 0.0527) on the placebo trial and 0.0069 (0.0060 - 0.0119) on the BCAA trial (\( P < 0.001 \)).

**Figure 4.3:** Changes in the plasma concentration ratio of f-TRP to BCAA (median ± range) at rest and during exercise. a and b denote a significant difference from the -120 min time point in the placebo and BCAA trials respectively (\( P < 0.05 \)). * (\( P < 0.05 \)) and ** (\( P < 0.01 \)) denote a significant difference between the BCAA trial and the corresponding time point on the placebo trial.
Figure 4.4: Change in core (A) and weighted mean skin (B) temperature at rest and during exercise. a and b denote a significant difference from the pre-exercise time point in the placebo and BCAA trials respectively (P < 0.05).

No difference in basal core or weighted mean skin temperature was apparent between trials (Figure 4.4A + B). The drink treatment had no effect on core temperature during the experimental trials (P = 0.628). Core temperature progressively increased
throughout exercise to 38.9 ± 0.5 °C on the placebo trial and 38.9 ± 0.5 °C on the BCAA trial at the point of exhaustion (P = 0.785). Following an increase during the initial 20 minutes of exercise, mean skin temperature remained relatively stable through to the point of exhaustion.

![Heart rate graph]

**Figure 4.5:** Heart rate at rest and during exercise. a and b denote a significant difference from the pre-ingestion time point in the placebo and BCAA trials respectively (P < 0.05).

Heart rate was elevated from the onset of exercise, with a steady increase apparent throughout the exercise period (Figure 4.5). At exhaustion heart rates of 156 ± 15 beat.min\(^{-1}\) (placebo) and 159 ± 13 beat.min\(^{-1}\) (BCAA) were recorded (P = 0.122). No difference was observed between the experimental trials (P = 0.345).
Figure 4.6: Rating of perceived exertion (A) and thermal stress (B) during exercise. a and b denote a significant difference from the 10 minute time point in the placebo and BCAA trials respectively (P < 0.05).
A progressive increase in RPE was recorded during both experimental trials, but this was significantly greater than the initial measurement only after 50 minutes of exercise and at the point of exhaustion (Figure 4.6A). Perceived exertion was not influenced by the drink treatment, with no difference between trials at any time point ($P = 0.840$). Perceived thermal stress also tended to increase throughout exercise, becoming significantly elevated over the 10 minute time point following 50 minutes of exercise in the placebo trial and only at exhaustion in the BCAA trial (Figure 4B). Again, no difference in perceived thermal stress between trials was apparent ($P = 0.351$).

Changes in blood ($P = 0.464$) and plasma ($P = 0.547$) volumes were not different between trials (Table 4.2). Plasma volume was significantly reduced throughout exercise on both trials than at rest ($P < 0.05$), but remained stable throughout the exercise period. The ingestion of BCAA produced no change in red cell volume ($P = 0.134$), with no significant difference apparent during exercise in either trial ($P > 0.05$).

**Table 4.2:** Percentage changes in blood (BV), plasma (PV) and red cell (CV) volumes relative to the -120 min value at rest and during exercise. a and b denote a significant difference from the -120 min time point in the placebo and BCAA trials respectively ($P < 0.05$).

<table>
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<th>-60</th>
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<th>30</th>
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<tr>
<td><strong>BV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Placebo</td>
<td>1.4 ±</td>
<td>-1.1 ±</td>
<td>-4.1 ±</td>
<td>-3.3 ±</td>
<td>-3.8 ±</td>
<td>-4.2 ±</td>
<td>-5.4 ±</td>
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<tr>
<td>1.6</td>
<td>1.5</td>
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<td>3.0</td>
<td></td>
</tr>
<tr>
<td>BCAA</td>
<td>0.4 ±</td>
<td>0.1 ±</td>
<td>-3.4 ±</td>
<td>-3.5 ±</td>
<td>-3.2 ±</td>
<td>-3.6 ±</td>
<td>-5.0 ±</td>
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<tr>
<td>1.8</td>
<td>1.9</td>
<td>1.7$^b$</td>
<td>1.5$^b$</td>
<td>1.1$^b$</td>
<td>2.0$^b$</td>
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<tr>
<td><strong>PV</strong></td>
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<tr>
<td>Placebo</td>
<td>2.2 ±</td>
<td>-1.4 ±</td>
<td>-6.3 ±</td>
<td>-4.7 ±</td>
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<td>-7.8 ±</td>
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<tr>
<td>3.3</td>
<td>2.3</td>
<td>2.4$^a$</td>
<td>2.7$^a$</td>
<td>2.2</td>
<td>2.3$^b$</td>
<td>4.3$^a$</td>
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<tr>
<td>BCAA</td>
<td>0.4 ±</td>
<td>-0.8 ±</td>
<td>-5.9 ±</td>
<td>-5.6 ±</td>
<td>-4.9 ±</td>
<td>-5.6 ±</td>
<td>-8.0 ±</td>
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<tr>
<td>2.5</td>
<td>2.6</td>
<td>2.7$^b$</td>
<td>2.2$^b$</td>
<td>1.9$^b$</td>
<td>2.8$^b$</td>
<td>4.3$^b$</td>
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<td><strong>CV</strong></td>
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<tr>
<td>Placebo</td>
<td>0.3 ±</td>
<td>-0.7 ±</td>
<td>-1.2 ±</td>
<td>-1.6 ±</td>
<td>-1.9 ±</td>
<td>-1.4 ±</td>
<td>-2.3 ±</td>
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<tr>
<td>1.5</td>
<td>1.1</td>
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<td>1.8</td>
<td>1.7</td>
<td>2.3</td>
<td>3.6</td>
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<tr>
<td>BCAA</td>
<td>0.9 ±</td>
<td>1.3 ±</td>
<td>0.0 ±</td>
<td>-0.6 ±</td>
<td>-0.8 ±</td>
<td>-1.0 ±</td>
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<tr>
<td>1.8</td>
<td>2.7</td>
<td>1.9</td>
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TABLE 4.3: Blood and plasma metabolite data at rest and during exercise in the placebo and BCAA trials. a and b denote a significant difference from the -120 min time point in the placebo and BCAA trials respectively (P < 0.05). * (P < 0.05) and ** (P<0.01) denote a significant difference between the BCAA trial and the corresponding time point in the placebo trial.

<table>
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<th>-120</th>
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<th>30</th>
<th>45</th>
<th>60</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Placebo</td>
<td>4.1 ± 3.9 ± 4.3 ± 4.5 ± 4.4 ± 4.2 ± 4.1 ±</td>
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<tr>
<td></td>
<td>(mmol.L⁻¹)</td>
<td>0.8 ± 0.7 ± 0.8 ± 0.8 ± 0.8 ± 1.0 ± 1.0 ±</td>
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<tr>
<td></td>
<td>BCAA</td>
<td>4.0 ± 3.5 ± 4.0 ± 4.2 ± 4.2 ± 4.0 ± 4.1 ±</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>0.4 ± 0.6 ± 0.4 ± 0.5 ± 0.6 ± 0.6 ± 0.6 ±</td>
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<tr>
<td>Lactate</td>
<td>Placebo</td>
<td>0.53 ± 0.78 ± 0.67 ± 0.66 ± 0.68 ± 0.79 ± 1.51 ±</td>
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<tr>
<td></td>
<td>(mmol.L⁻¹)</td>
<td>0.23 ± 0.36 ± 0.25 ± 0.23 ± 0.22 ± 0.30 ± 0.59 ±</td>
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<tr>
<td></td>
<td>BCAA</td>
<td>0.59 ± 0.77 ± 0.66 ± 0.65 ± 0.67 ± 0.72 ± 1.53 ±</td>
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<td></td>
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<td>0.25 ± 0.25 ± 0.26 ± 0.26 ± 0.24 ± 0.29 ± 0.65 ±</td>
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<td>FFA</td>
<td>Placebo</td>
<td>0.58 ± 0.58 ± 0.61 ± 0.80 ± 0.81 ± 0.86 ± 1.27 ±</td>
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<tr>
<td></td>
<td>(mmol.L⁻¹)</td>
<td>0.09 ± 0.14 ± 0.13 ± 0.18 ± 0.15 ± 0.22 ± 0.39 ±</td>
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<tr>
<td></td>
<td>BCAA</td>
<td>0.47 ± 0.32 ± 0.45 ± 0.66 ± 0.70 ± 0.70 ± 1.17 ±</td>
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<td>0.08 ± 0.10** ± 0.14* ± 0.17* ± 0.16* ± 0.19 ± 0.29 ±</td>
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</tbody>
</table>

Blood glucose and lactate and plasma free-fatty acid data are presented in Table 4.3. The pre-exercise ingestion of BCAA resulted in a progressive non-significant reduction in the blood glucose concentration prior to start of exercise. Circulating blood glucose concentrations were stable throughout exercise, with no difference between trials recorded (P = 0.114). Blood lactate concentrations were unaffected by the ingestion of BCAA (P = 0.836). Plasma FFA concentration was reduced following BCAA ingestion, with levels consistently lower throughout exercise than during the placebo trial (P < 0.01). Figure 4.7 illustrates the relationship between changes in plasma FFA and plasma f-TRP observed in the placebo and BCAA trials. The correlation coefficients were calculated as 0.82 (P = 0.012) for the placebo and 0.59 (P = 0.120) for BCAA trial.
No difference in baseline plasma NH$_3$ concentrations (n = 6) was apparent between experimental trials (Figure 4.8; P = 0.512), with ingestion of BCAA prior to exercise resulting in a slight, but non-significant, increase in the plasma NH$_3$ concentration (P = 0.179). The BCAA solution resulted in a significant elevation in plasma NH$_3$ throughout exercise when compared to the placebo treatment (P < 0.001). Values at the point of exhaustion were 94.7 ± 17.7 μmol.L$^{-1}$ and 134.7 ± 6.8μmol.L$^{-1}$ for the placebo and BCAA trials respectively. Circulating plasma prolactin was significantly elevated above resting values at the point of exhaustion in both trials (P = 0.004; Figure 4.9). No difference between trials was apparent at rest before (P = 0.485) or at the point of exhaustion (P = 0.578).

**Figure 4.7:** The relationship between the change in plasma FFA and plasma f-TRP during the placebo and BCAA trials.
Figure 4.8: Plasma NH$_3$ concentration during the placebo and BCAA trials. a and b denote a significant difference from the -120 min time point in the placebo and BCAA trials respectively. * (P < 0.05) and ** (P < 0.01) denote a significant difference between the BCAA trial and the corresponding time point on the placebo trial.

Figure 4.9: Plasma prolactin concentration at rest and at the point of exhaustion during the placebo and BCAA trials. a and b denote a significant difference from the pre-ingestion time point in the placebo and BCAA trials respectively.
4.5 – Discussion

Accelerated central fatigue resulting in a reduced drive to continue exercise has been proposed as an important mechanism mediating the reduction in exercise capacity observed when exercising in warm environmental conditions (Nielsen, 1992). Recent data go some way to supporting this suggestion: high core temperatures have been demonstrated to impair maximal muscle activation (Nybo and Nielsen, 2001a), alter EEG brain activity (Nielsen et al., 2001) and increase perceived exertion (Nybo and Nielsen, 2001b), but the underlying neurobiological mechanism(s) behind these responses are not clear at present. Indirect support for the hypothesis that the early onset of fatigue in the heat results from an increased activity of central serotonergic neurones comes from evidence that BCAA supplementation can prolong exercise capacity when low-moderate intensity exercise is performed in the heat (Mittleman et al., 1998). The purpose of the present study was to develop the work described in chapter 3 to further investigate the effect of BCAA supplementation on exercise capacity in a warm environment following an exercise and diet regimen designed to reduced glycogen availability.

Despite a 4-fold reduction in the plasma concentration ratio of f-TRP to BCAA, exercise capacity, determined by time to volitional exhaustion, was not influenced by ingestion of a BCAA solution (111.0 ± 29.2 min) when compared to a placebo treatment (103.9 ± 26.9 min). Although the findings of Mittleman and colleagues (1998) support an effect of BCAA ingestion on exercise performance, the available evidence is not entirely convincing. A number of investigations have failed to demonstrate an improvement in exercise capacity following BCAA supplementation under temperate environmental conditions (Blomstrand et al., 1995; Blomstrand et al., 1997; Madsen et al., 1996; Struder et al., 1998; van Hall et al., 1995).

Despite a 4-fold reduction in the plasma concentration ratio of f-TRP to BCAA, exercise capacity was not influenced by ingestion of BCAA, but there was a large inter-individual variation in the subjects' response to the supplementation regimen (see Figure 1). This apparent variation may be explained by recent data demonstrating that net uptake of tryptophan across the brain was increased in only 50% of subjects.
following 2 hours moderate-intensity exercise, despite a marked elevation in plasma f-TRP (Nybo et al. 2003). Additionally, a number of studies employing different markers of 5-HT receptor function suggest that differences in the sensitivity of the 5-HT system to manipulation may exist between individuals. Marked differences in platelet 5-HT transporter density (Strachan and Maughan, 1998; Struder et al., 1999) and in the prolactin response to a buspirone challenge (Bridge et al., 2003; Jakeman et al., 1994) are reported in the published literature. Additionally, patients suffering from chronic fatigue syndrome appear to possess an altered sensitivity of central serotonergic receptors (Bakheit et al., 1992). This variability may also extend to other neurotransmitter systems implicated in the development of fatigue (e.g. dopamine, noradrenaline), but evidence for this is not clear at present.

Studies conducted by Blomstrand et al. (1991) and Mittleman et al. (1998), both of which involved exercise of a long duration performed at a low-moderate intensity, are the only published human investigations to date reporting an ergogenic effect of BCAA supplementation in humans. It is possible that central fatigue, resulting from increased serotonergic neurotransmission, may become important only after a significant duration of exercise has passed (Nybo et al., 2003). This may go some way to explaining a failure to observe an ergogenic benefit in studies where the exercise time was substantially less than 180 minutes (Blomstrand et al., 1995; Madsen et al., 1996; van Hall et al., 1995). The present study employed a glycogen depletion protocol completed the evening prior to the main trials in an attempt to simulate metabolic conditions found later in exercise, therefore potentially accelerating the onset of central fatigue. The relatively low blood glucose concentrations and elevated plasma FFA whilst at rest suggest the regimen was successful in reducing CHO availability, although no direct measure of changes in muscle glycogen was made. Therefore, the duration of exercise in the present investigation should be have been sufficient for serotonin-mediated fatigue to become important, as suggested by Nybo and co-workers (2003).

As reported in previous studies investigating BCAA ingestion during exercise in a warm environment (Mittleman et al., 1998), the ingestion of BCAA produced no difference in heart rate or in blood glucose or lactate concentrations at rest and during
exercise compared with the placebo trial. The circulating plasma FFA concentration was significantly reduced at the end of the seated rest period and during the initial 30 minutes of exercise, presumably due to the insulinogenic effect of BCAA ingestion. Subjective ratings of exertion ($P = 0.840$) were also unaffected by the treatment (Figure 4A). If the supplementation regimen had resulted in an altered rate of 5-HT synthesis, and the central fatigue hypothesis holds true, a reduction in RPE may be expected. A previous study conducted by (Blomstrand et al., 1997) reported a significant reduction in RPE during exercise at 70% $\dot{V}O_2\text{max}$, but work by Mittleman and colleagues (1998) failed to identify such a difference despite a marked improvement in exercise capacity. It is also possible that the standard 15-point Borg RPE scale (Borg, 1982) may be not sensitive enough to detect subtle differences in perceived exertion resulting from alterations in central neurotransmission or that no such differences resulted from BCAA feeding.

Oral ingestion of BCAA also failed to produce any clear difference in measures of thermoregulation during both rest and exercise, including core ($P = 0.628$) and weighted mean skin ($P = 0.114$) temperature as well as perceived thermal stress ($P = 0.351$). Such changes may have been expected as central neurotransmitter systems including 5-HT and dopamine are intrinsically involved in the regulation of body temperature (Hasegawa et al., 2000; Lee et al., 1985; Lin et al., 1998). Pharmacologically-induced alterations in central 5-HT neurotransmission may modify thermoregulatory function in rats (Lin et al., 1998) and humans (Strachan, 2002). Therefore if the supplementation regimen was indeed successful at limiting 5-HT synthesis, a change in core temperature might have been expected. Core temperature at exhaustion in the present study was below the 39.7 °C threshold considered to be limiting (Gonzalez-Alonso et al., 1999; Figure 3A). This suggests that the thermoregulatory stress encountered may not have been sufficient to accelerate central fatigue, but this is in marked contrast to the improvement in exercise capacity demonstrated by Mittleman et al (1998) despite lower core temperatures observed at fatigue (37.3 – 37.7 °C).

Elevated production of ammonia ($NH_3$) is widely cited as a possible explanation for the failure of BCAA supplements to attenuate the development of central fatigue and
consequently enhance exercise capacity (Davis and Bailey, 1997; Wagenmakers and van Hall, 1996). In the present investigation a marked increase in plasma NH$_3$ concentration was evident throughout exercise following the ingestion of BCAA (Figure 5; $P < 0.001$). During the low-moderate intensity exercise undertaken in this study, increased production of NH$_3$ is thought to result primarily from the oxidation of BCAA (Gibala, 2001). Taken together with the reduction in circulating BCAA observed from the onset of exercise, it seems probable that BCAA oxidation was elevated, but no measurement of BCAA utilisation was undertaken to confirm or refute this suggestion.

It is possible that a failure to observe a benefit from BCAA ingestion may arise from a disturbance in catecholaminergic neurotransmission. The rationale behind BCAA supplementation is to block the uptake of TRP, but the possibility that BCAA ingestion will limit the transport of TYR into the CNS and consequently attenuate central DA and NA synthesis has not been considered. As the catecholaminergic neurotransmitters are thought to be important mediators of motivation and arousal during exercise (Davis and Bailey, 1997) limiting central TYR availability may adversely impact on exercise performance and negate any possible benefit of attenuating the exercise-induced increase in central 5-HT.

Wagenmakers and van Hall (1996) suggested that BCAA supplementation under conditions of reduced muscle glycogen availability might result in a reduction in endurance performance in healthy individuals. The authors proposed that elevated ammonia accumulation would result in the depletion of TCA cycle intermediates, but subsequent evidence has demonstrated that exercise under conditions of reduced glycogen availability may have no effect on TCA cycle anaplerosis (Gibala et al., 1999). Elevated circulating NH$_3$ concentrations have also been suggested to negatively affect CNS function leading to impaired motor control and lethargy (Davis and Bailey 1997) and alter the transport characteristics of the blood-brain barrier (Mans et al., 1983) see Chapter 7 for further discussion of the effects of exercise and the blood-brain barrier. It seems likely the levels observed in the present investigation, and other BCAA supplementation studies using healthy human subjects (Madsen et al., 1996; Mittleman et al., 1998; Struder et al., 1998) would be insufficient to produce such an effect (Gibala, 2001).
Circulating plasma prolactin was significantly elevated above resting values at the point of exhaustion ($P = 0.004$). This finding is consistent with a number of previous studies investigating the hormonal response to prolonged exercise in a warm environment (Pitsiladis et al., 2002; Radomski et al., 1998). A curvilinear relationship between plasma prolactin and core temperature has been proposed (Radomski et al., 1998; Strachan, 2002), suggesting the thermal response to exercise may be related to the induction of hormone release during exercise. The failure to observe a difference between trials at exhaustion ($P = 0.578$), raises the question whether the supplementation regimen produced a functional change in 5-HT neurotransmission as prolactin is widely employed as a peripheral marker of central serotonin activity (Struder and Weicker, 2001), but this assumption is not entirely accurate as the regulation of prolactin secretion is also under the control of the dopaminergic system (Checkley, 1980; Meeusen et al., 2001; Strachan and Maughan, 2002).

In conclusion, the results of the present study demonstrate a failure of acute BCAA ingestion to significantly influence exercise capacity in a warm environment, following a regimen designed to reduce glycogen availability. The large variation of exercise capacity change observed between subjects in response to BCAA ingestion raises the question of differences in individual sensitivity to nutritional manipulation of the 5-HT system. It may be that the effect of nutritional supplementation is simply too small to produce a functional change in central neurotransmission in some individuals (Fernstrom, 2000). In contrast to the suggestion of Wagenmakers and van Hall (1996), BCAA ingestion under conditions of low glycogen availability did not produce a reduction in exercise capacity despite a marked elevation in plasma NH$_3$ concentrations. This increase in plasma NH$_3$ observed during exercise following BCAA ingestion is probably not sufficient to result in a marked effect on metabolism or CNS function in healthy individuals.
Chapter 5

Acute changes to the plasma concentration ratio of f-TRP to BCAA following the ingestion of carbohydrate and fat rich meals in man
5.1 - Abstract

Eight healthy volunteers (5 males and 3 females) completed two trials to examine transient changes to the plasma concentration ratio of f-TRP to BCAA at rest following the ingestion of meals. Subjects consumed 34 kJ.kg BM\(^{-1}\) of a fruit flavoured smoothie containing either added CHO or fat. The response to the meals was monitored at 30, 60, 120, 180, 240 minutes after ingestion. The CHO meal produced a significant elevation in blood glucose concentrations during the first 60 minutes post-ingestion (P < 0.001). Plasma FFA was suppressed below pre-ingestion levels after the CHO meal and remained significantly lower than the fat trial throughout the trial (P < 0.001). Plasma f-TRP was not affected by the CHO (P = 0.231) or fat (P = 0.073) meals. Ingestion of the CHO meal resulted in a reduction in plasma BCAA concentrations (P = 0.022), with levels significantly higher in the fat trial (P < 0.05). Despite the change in circulating BCAA, no effect on the plasma concentration ratio of f-TRP to BCAA was apparent following the CHO meal (P = 0.871). The fat meal altered the plasma concentration ratio of f-TRP to BCAA (P = 0.034), with the ratio significantly lower than the CHO trial at 60 minutes after ingestion (P < 0.01). The meals did not influence subjective feelings of tiredness, alertness, concentration and headache during the experimental time-scale. The ingestion of meals containing added CHO and fat resulted in no change to the plasma concentration ratio of f-TRP to BCAA relative to pre-ingestion values over the time-course of the investigation.

5.2 - Introduction

The potential involvement of central monoamines in the development of fatigue during prolonged exercise has been the subject of increasing interest in recent years. One particular model suggests that the shift in substrate mobilisation that occurs during prolonged exercise results in an upregulation of central 5-HT synthesis, producing feelings of tiredness that may contribute to the development of fatigue (Newsholme et al., 1987). The details of this hypothesis are described in Chapter 1. The key relationship in this mechanism of fatigue is the plasma concentration ratio of f-TRP to BCAA. This ratio determines the rate of TRP uptake into the CNS, and
consequently the rate of 5-HT synthesis during exercise (Chaouloff et al., 1985; Chaouloff et al., 1986). A particular attraction of Newsholme’s hypothesis is the potential for nutritional manipulation of neurotransmitter precursors to delay the development of central fatigue. In recent years a number of investigations have been undertaken to alter the ratio of f-TRP:BCAA during exercise through both acute (Blomstrand et al., 1995; Blomstrand et al., 1991; Davis et al., 1992; Madsen et al., 1996; Mittleman et al., 1998; Struder et al., 1998; van Hall et al., 1995) and chronic (Hefler et al., 1995) supplementation of BCAA or CHO.

The purpose of pre-exercise nutritional strategies is to ensure glycogen stores are optimised and euhydration is achieved, while avoiding gastrointestinal distress (Hargreaves, 2001). Many studies have sought to investigate changes in exercise metabolism resulting from the manipulation of the composition and timing of the pre-exercise meal (Coyle et al., 1985; Horowitz and Coyle, 1993; Montain et al., 1991). This work has established that CHO and fat feeding prior to exercise can markedly influence rates of substrate utilisation, and may have profound effects on exercise performance. To date only one study has investigated the influence of pre-exercise meal ingestion on the metabolic indices thought to be important to the development of central fatigue (Paul et al., 1996). This examined changes in the plasma concentration ratio of total-TRP to LNAA in response to relatively small (~1340 kJ) corn, wheat and oat-based meals, containing increasing quantities of protein, ingested 90 minutes before exercise. These cereal-based meals produced no change in TRP:LNAA relative to pre-ingestion values, but this plasma amino acid ratio was lower during exercise and recovery following the corn meal compared to the other treatments. Despite this reduction in circulating amino acids, apparently due to the lower protein content of this meal, performance of a 6.4 km cycle time trial preceded by 60 minutes fixed-intensity submaximal exercise was not different between trials.

While there is considerable debate regarding the availability of plasma TRP for cerebral uptake (see Chapter 1), it appears that brain TRP content is closely related to plasma f-TRP rather than total-TRP whilst at rest (Bloxam et al., 1980) and during exercise (Chaouloff et al., 1985). Paul and colleagues (1996) failed to report changes in f-TRP, despite the ingested meals producing marked changes in circulating FFA. Changes in the plasma concentration ratio of f-TRP to BCAA are thought to more
accurately represent changes in TRP delivery into the CNS and consequent changes in 5-HT synthesis. The aim of this investigation was to examine the magnitude and time-course of changes in the plasma concentration ratio of f-TRP to BCAA following meals containing added CHO or fat. If changes in circulating amino acids in response food ingestion have implications for the development of central fatigue, the timing and composition of the pre-exercise meal may be important for achieving optimum performance during prolonged exercise.

5.3 - Methods

Subjects. To test the hypothesis, eight healthy subjects (5 males, 3 females; age 24.6 ± 4.1 y; height 1.74 ± 0.11 m; mass 78.9 ± 14.9 kg) were recruited. Prior to the start of the investigation all subjects received written details outlining the nature of the study. Following any questions regarding the experimental protocol, a written statement of informed consent was signed. The protocol employed during this investigation received ethical approval from the Grampian Research Ethics Committee.

Experimental protocol. Subjects completed two experimental trials in a randomised order, separated by at least seven days. The standardisation of pre-trial conditions is outlined in chapter 2. Subjects entered the lab in the morning (between 8 and 10am), following an overnight fast. Upon arrival, a post-void body mass was recorded when wearing minimal clothing. Whilst seated in a comfortable environment (24 – 26 °C), a 21g butterfly cannula was introduced into a superficial forearm vein to allow repeated blood sampling throughout the experimental period. After sitting quietly for 15 min to allow equilibrium of body fluids to be reached, two 5 mL baseline blood samples were drawn 5 min apart (-5 and 0 min). Subjects were then given 15 min to ingest 34 kJ.kg BM⁻¹ of a meal containing either added CHO or fat. This quantity was chosen as it was felt that a meal of this energy content might typically be consumed prior to taking part in an athletic event. In order to standardise the nature of the meal administered to the subjects, a fruit flavoured smoothie was prepared to which either sucrose (CHO trial; 86 % CHO, 7 % fat, 7 % protein) or double cream (fat trial; 12 %
CHO, 81 % fat, 7 % protein) was added. The two meals were similar in volume and appearance, although there was a noticeable difference in the taste and texture. The subjects' response to the ingested meals was then monitored over the subsequent four hours, with blood samples taken at 30, 60, 120, 180 and 240 minutes post-ingestion. During the post-ingestion period subjects remained seated except for essential movements. Subjective feelings of tiredness, alertness, concentration and headache were assessed at 60 min intervals using 100 mm visual analogue scales (see Appendix IV).

**Blood handling and analysis.** The 5 mL blood samples collected during the investigation were dispensed into two labelled tubes containing K2EDTA. Deproteinised whole blood was used to determine blood glucose concentration using a commercially available assay kit (Sigma Diagnostics, Poole, UK). Percentage changes in blood, plasma and red cell volumes relative to the first sample were estimated using the methods proposed by Dill and Costill (1974), using haemoglobin and packed cell volume values. The remaining 2.5 mL of whole blood was centrifuged and the plasma transferred into plain eppendorf tubes. Plasma f-TRP was separated from albumin bound TRP as described in Chapter 2. All plasma and ultrafiltrate samples were then kept frozen at -20 °C ready for analysis. Fluorimetric analysis of plasma BCAA (Gleeson and Maughan, 1987) and total- and f-TRP (see Appendix II) were undertaken. Plasma FFA concentrations were determined enzymatically (Half-micro test, Roche Diagnostics, Mannheim, Germany).

**5.4 – Results**

The nutrient content of the ingested meals is presented in Table 5.1. The mean energy content of the meal ingested was 2.7 ± 0.5 MJ (range 1.8 – 3.4 MJ). The relatively large range was due to variation in the subjects' body mass. The experimental meals provided around 2.5 ± 0.6 g of BCAA and 0.2 ± 0.1 g of TRP.
Table 5.1: The nutrient content of the meals ingested by the subjects during the study.

<table>
<thead>
<tr>
<th></th>
<th>EI (MJ)</th>
<th>CHO (g)</th>
<th>Fat (g)</th>
<th>Protein (g)</th>
<th>ΣBCAA (g)</th>
<th>TRP (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>2.7 ± 0.5</td>
<td>136 ± 26</td>
<td>5 ± 1</td>
<td>11 ± 2</td>
<td>2.55 ± 0.62</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>Fat</td>
<td>2.7 ± 0.5</td>
<td>19 ± 4</td>
<td>57 ± 11</td>
<td>11 ± 2</td>
<td>2.51 ± 0.68</td>
<td>0.19 ± 0.06</td>
</tr>
</tbody>
</table>

The blood glucose and plasma free-fatty acid responses to the meals are presented in Table 5.2. The CHO meal produced a marked elevation in circulating blood glucose reaching a peak 30 minutes following meal ingestion (+2.6 ± 0.6 mmol.L⁻¹; \( P < 0.001 \)) and returning to near pre-ingestion levels after 120 minutes. The Fat meal did not alter circulating blood glucose concentrations throughout the 4 hour experimental period (\( P = 0.270 \)). A small increase above pre-ingestion values was apparent at 240 minutes post-ingestion during the fat trial. Glucose was higher during the 2 hour post-ingestion in the CHO trial when compared to the Fat trial (\( P < 0.01 \)). Plasma FFA concentrations were similar prior to the ingestion of the meals (\( P = 0.236 \)), with a marked difference between trials apparent throughout the post-ingestion period (\( P < 0.001 \)). A progressive fall in FFA was witnessed during the initial 120 min of CHO trial, with values failing to return to pre-ingestion levels at 240 min. Following an initial fall, plasma FFA increased following the ingestion of the meal containing added fat, reaching significance at 240 min post-ingestion (\( P < 0.05 \)).
Table 5.2: Resting blood glucose (mmol.L\(^{-1}\)) and plasma free-fatty acid (FFA; mmol.L\(^{-1}\)) responses to the CHO and fat meals. a and b denote a significant difference from the 0 time point in the CHO and fat trials respectively (P < 0.05). * (P<0.05) and ** (P<0.01) denote a significant difference between the CHO trial and the corresponding time point in the fat trial.

<table>
<thead>
<tr>
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<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
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<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CHO</td>
<td>4.2 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>6.8 ± 0.6***</td>
<td>5.4 ± 0.8***</td>
<td>4.9 ± 0.6**</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>Fat</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.3</td>
<td>4.3 ± 0.4</td>
<td>4.1 ± 0.5</td>
<td>4.1 ± 0.4</td>
<td>4.3</td>
</tr>
<tr>
<td>FFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>0.46 ± 0.12</td>
<td>0.24 ± 0.10**</td>
<td>0.13 ± 0.06***</td>
<td>0.08 ± 0.03***</td>
<td>0.11 ± 0.09***</td>
<td>0.28 ± 0.17***</td>
</tr>
<tr>
<td>Fat</td>
<td>0.39 ± 0.07</td>
<td>0.33 ± 0.09</td>
<td>0.25 ± 0.08</td>
<td>0.39 ± 0.14</td>
<td>0.54 ± 0.14</td>
<td>0.64</td>
</tr>
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<td></td>
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</tbody>
</table>

There was no difference in plasma BCAA between trials before the ingestion of the meals (P = 0.581; Figure 5.1A). A reduction in BCAA concentrations was apparent during the CHO trial (P = 0.022), but levels were not significantly different to the pre-ingestion value at any point. The meal with added fat failed to alter BCAA during the 4 h post-ingestion period (P = 0.112). Plasma f-TRP was not influenced by the experimental meals over the time course of the study (CHO, P = 0.231; Fat, P = 0.073), with no difference between trials apparent at any point (P = 0.849).
Figure 5.1: Resting plasma BCAA (A) and f-TRP (B) responses to the CHO and fat meals. * (P < 0.05) and ** (P < 0.01) denote a significant difference between the CHO trial and the corresponding time point in the fat trial.

While the plasma concentration ratio of f-TRP to BCAA did not change after consuming the CHO meal (P = 0.871; Figure 5.2), the fat meal resulted in a modest reduction in f-TRP:BCAA during the 4 h post-ingestion period (P = 0.034). The ratio
of these amino acids was significantly greater during the CHO trial when compared to the fat trial between 60 and 120 minutes after meals ingestion.

Figure 5.2: Changes in the plasma concentration ratio of f-TRP to BCAA in response to the CHO and fat meals. * (P<0.05) and ** (P<0.01) denote a significant difference between the CHO trial and the corresponding time point in the fat trial.

Plasma total-TRP concentrations were not influenced by the CHO (P = 0.777) or fat (P = 0.071) trials (Figure 5.3). The ingestion of the experimental meals produced differing responses in the plasma concentration ratio of total-TRP to BCAA (P = 0.004). While the CHO (P = 0.046) and fat (P = 0.018) meals influenced the total-TRP : BCAA over the time course of the investigation, they failed to significantly alter this ratio relative to pre-ingestion values. Sixty minutes after the meals were consumed total-TRP : BCAA was significantly higher during the CHO trial compared to the fat trial (+0.05 ± 0.03; P = 0.008), with this difference maintained to the end of the 4 h experimental period.
Figure 5.3: Resting plasma total-TRP (A) and changes in the plasma concentration ratio of total-TRP to BCAA (B) in response to the CHO and fat meals. * (P<0.05) and ** (P<0.01) denote a significant difference between the CHO trial and the corresponding time point on the fat trial.
Table 5.3: Percentage changes in blood (BV), plasma (PV) and red cell (CV) volumes relative to the first sample (-5 min).

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV</td>
<td>CHO</td>
<td>1.9 ± 1.5</td>
<td>2.2 ± 2.2</td>
<td>0.3 ± 1.5</td>
<td>0.7 ± 2.0</td>
<td>1.8 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>1.7 ± 1.3</td>
<td>0.3 ± 1.4</td>
<td>0.5 ± 2.2</td>
<td>0.7 ± 2.6</td>
<td>-0.2 ± 2.8</td>
</tr>
<tr>
<td>PV</td>
<td>CHO</td>
<td>3.1 ± 2.5</td>
<td>3.7 ± 3.9</td>
<td>1.5 ± 2.3</td>
<td>2.0 ± 3.0</td>
<td>3.3 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>2.8 ± 2.4</td>
<td>1.1 ± 2.6</td>
<td>1.5 ± 3.5</td>
<td>1.9 ± 3.9</td>
<td>0.5 ± 4.8</td>
</tr>
<tr>
<td>CV</td>
<td>CHO</td>
<td>0.2 ± 1.5</td>
<td>0.4 ± 1.7</td>
<td>-1.0 ± 2.6</td>
<td>-0.8 ± 1.9</td>
<td>-0.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>0.3 ± 0.9</td>
<td>-0.8 ± 1.7</td>
<td>-0.5 ± 1.5</td>
<td>-1.0 ± 1.6</td>
<td>-1.1 ± 1.9</td>
</tr>
</tbody>
</table>

Changes in blood, plasma and red cell volumes are displayed in Table 5.3. There were no main effects for the meal on blood (P = 0.157), plasma (P = 0.170) or red cell (P = 0.138) volumes during the 4 h post-ingestion period.

Subjective feelings of tiredness, alertness, concentration and headache were assessed at 60 min intervals throughout the experimental protocol (Table 5.4). The ingestion of meals containing added CHO or fat did not alter perceived tiredness (P = 0.663). During the 4 h period after the meals, the subjects' reported feelings of alertness (P = 0.425) or concentration (P = 0.172) did not change. There was no difference in subjective feelings reported between trials.
Table 5.4: Changes in subjective feelings of tiredness, alertness, concentration and headache in response to the CHO and fat meals.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiredness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>25 ± 17</td>
<td>43 ± 24</td>
<td>41 ± 26</td>
<td>38 ± 25</td>
<td>29 ± 20</td>
</tr>
<tr>
<td>Fat</td>
<td>39 ± 24</td>
<td>41 ± 19</td>
<td>39 ± 19</td>
<td>32 ± 20</td>
<td>36 ± 26</td>
</tr>
<tr>
<td>Alertness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>72 ± 20</td>
<td>57 ± 21</td>
<td>61 ± 20</td>
<td>67 ± 23</td>
<td>71 ± 10</td>
</tr>
<tr>
<td>Fat</td>
<td>57 ± 26</td>
<td>49 ± 13</td>
<td>59 ± 24</td>
<td>59 ± 25</td>
<td>62 ± 28</td>
</tr>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>71 ± 21</td>
<td>58 ± 25</td>
<td>62 ± 16</td>
<td>67 ± 20</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>Fat</td>
<td>70 ± 23</td>
<td>54 ± 16</td>
<td>66 ± 17</td>
<td>71 ± 21</td>
<td>65 ± 25</td>
</tr>
<tr>
<td>Headache</td>
<td></td>
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<tr>
<td>CHO</td>
<td>17 ± 17</td>
<td>18 ± 18</td>
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<td>Fat</td>
<td>22 ± 24</td>
<td>23 ± 17</td>
<td>24 ± 17</td>
<td>22 ± 18</td>
<td>28 ± 27</td>
</tr>
</tbody>
</table>

5.5 – Discussion

The aim of the present investigation was to examine the time-course and magnitude of changes in plasma amino acids thought to be important to the development of serotonin-mediated central fatigue following the ingestion of meals containing added CHO and fat. Changes in the plasma concentration ratio of f-TRP to BCAA in response to CHO (Fernstrom and Wurtman, 1971) and fat (Fernstrom et al., 1975) meals have been demonstrated to alter brain TRP and 5-HT content and may be important to the development of fatigue during prolonged exercise (Newsholme et al., 1987).

There is a considerable degree of controversy regarding the availability of TRP for uptake into the CNS by the L-transporter (see Chapter 1). Originally it was believed that total TRP was readily available for uptake (Fernstrom et al., 1976; Madras et al., 1974; Pardridge, 1979), but subsequent work has suggested that only plasma f-TRP availability is closely associated with brain TRP tissue content whilst at rest (Bloxam
et al., 1980) and during exercise (Chaouloff et al., 1985; Chaouloff et al., 1986). This premise resulted in the development of Newsholme’s central fatigue hypothesis (Newsholme et al., 1987), and forms the basis of the underlying metabolic mechanism. As the present study examined changes in plasma amino acids at rest, both plasma concentration ratios of f-TRP to BCAA and total-TRP to BCAA were considered due to the relative uncertainty regarding cerebral TRP uptake.

Despite significantly altering circulating concentrations of blood glucose and plasma FFA and a reduction in plasma BCAA in the CHO trial, the meals failed to significantly alter the plasma concentration ratio of f-TRP to BCAA or total-TRP to BCAA compared to pre-ingestion values. Previous work has examined the influence of cereal-based meals on plasma amino acids during prolonged exercise (Paul et al., 1996). The authors reported that the plasma concentration ratio of total-TRP to LNAA was lower during exercise and recovery following the meal with the smallest protein content (the corn meal) than in the other trials, but failed to observe any change relative to pre-ingestion values. The energy content of the meals provided in the Paul study was somewhat small (~1340 kJ) and no reference to the importance of f-TRP in determining the rate of entry of TRP into the CNS was made.

The effect of CHO ingestion on plasma amino acids, brain TRP uptake and central serotonin synthesis has been widely investigated. Original studies conducted by Fernstrom and Wurtman (1971) demonstrated a marked increase in brain 5-HT content following the ingestion of CHO or the peripheral infusion of insulin. Subsequent work established the role of other LNAA in determining the rate of TRP delivery into the CNS, and the influence of insulin secretion on the concentrations of competing amino acids (Fernstrom and Wurtman, 1972). Oral doses of glucose have been reported to produce dose-dependent reductions in the plasma concentration ratio of TRP to LNAA, with a 40% reduction in plasma BCAA following the ingestion of 50 g of glucose (Pan et al., 1982). While it is reasonable to assume that the CHO load (136 ± 26 g) provided in the present investigation produced a marked insulin response, only a modest change in circulating amino acid concentrations and was measured. Although the CHO present in the CHO-containing meal was primarily sucrose, with the hydrolysis of this disaccharide into glucose and fructose required at
the intestinal brush border before absorption can take place. The insulin response to a bolus of sucrose is around half that expected following the ingestion of the same quantity of glucose (Macdonald et al., 1978), suggesting that the amino acid response following this meal would not be as pronounced as observed in previous studies employing a glucose load (Pan et al., 1982).

Following a transient fall, the fat meal increased circulating plasma FFA above pre-ingestion levels, but this was statistically significant only at 4 h post-ingestion. It has been suggested that the ingestion of meals containing fat will raise circulating levels of triacylglycerol to a greater extent than FFA, but work conducted by Fernstrom and co-workers (1975) reported marked dose-dependent increases in plasma FFA soon after the ingestion of fat-containing meals. This animal work demonstrated that the ingestion of meals containing significant quantities of fat would produce a marked increase in f-TRP, consequently reducing the plasma concentration ratio of f-TRP to BCAA (Fernstrom et al., 1975). No such change in plasma amino acids was apparent in the present investigation following the ingestion of a meal containing 81% fat. It is important to note, however, that the increase in serum free tryptophan caused by fat ingestion in the Fernstrom study was not accompanied by significant changes in brain tryptophan content.

Since previous work has clearly demonstrated an effect of CHO and fat ingestion on circulating plasma amino acids (Fernstrom et al., 1975; Fernstrom and Wurtman, 1971; Pan et al., 1982), it is possible to suggest that the protein content of the meals ingested in the present investigation limited such changes. The amino acid content of the meals, whilst relatively small compared to the contributions of CHO and fat (see Table 5.2) and those employed in previous investigations (Fernstrom and Wurtman, 1972; Paul et al., 1996), may have significantly influenced the observed plasma amino acid response. Additionally, it is possible that changes in the plasma concentration ratio of f-TRP to BCAA were limited as the meals did not sufficiently alter circulating FFA and consequently result in changes to f-TRP concentrations. It does not appear that a linear relationship exists between levels of circulating FFA and the liberation of f-TRP from the binding sites on albumin (Struder and Weicker, 2001), with the findings of Chapters 3 and 4 suggesting that a threshold may exist beyond which FFA readily displace TRP.
The ingestion of mixed meals and single macronutrients at rest have been reported to produce transient changes in mood, mental function and perception of fatigue (Craig, 1986; Spring et al., 1986; Young, 1991). The experimental meals failed to alter subjective feelings of tiredness, alertness, concentration and headache assessed using visual analogue scales. This observation may not be surprising since little change to the plasma concentration ratio of both f-TRP and total-TRP to competing amino acids was measured during the post-ingestion period. While these reported changes in mood following meal ingestion have been associated with changes to central serotonergic neurotransmission, evidence is accumulating that alternative mechanisms involving brain glucose availability and interactions with other monoamine systems may be more important (Bequet et al., 2001; Cooper et al., 2003).

In summary, the ingestion of meals containing added CHO and fat resulted in little change to the plasma concentration ratio of f-TRP to BCAA over the time-course of the investigation. It is likely that the changes were not of a sufficient magnitude to influence the development of central fatigue during a subsequent exercise bout. Alterations to circulating plasma amino acids were probably limited by the relatively small quantity of protein present in both experimental meals and the use of sucrose rather than glucose in the CHO trial. In contrast to previous reports, the ingestion of meals produced no effect on subjective feelings of tiredness, alertness and concentration.
Chapter 6

Dopamine / Noradrenaline reuptake inhibition and exercise performance in temperate and warm environments
6.1 - Abstract

Nine healthy endurance-trained males were recruited to examine the effect of a dual dopamine / noradrenaline reuptake inhibitor on performance, thermoregulation and the hormonal responses to exercise. Subjects performed 4 trials, ingesting either a placebo (pla) or 2x300mg bupropion (bup), prior to exercise in temperate (18 °C) or warm (30 °C) conditions. Trials consisted of 60 min cycle exercise at 55% \( W_{\text{max}} \) (Ex 1) immediately followed by a time trial (TT) to measure exercise performance. TT performance in the heat was significantly improved by bupropion (pla 39.8 ± 3.9 min, bup: 36.4 ± 5.7 min; \( P = 0.046 \)), but no difference between treatments was apparent in temperate conditions (pla 30.6 ± 2.2 min, bup 30.6 ± 1.9 min; \( P = 0.954 \)). During Ex1 core and skin temperatures, heart rate, blood lactate, perceived exertion and thermal stress were not influenced by bupropion in either temperate or warm conditions. Time trial power output was not different between treatments at 18 °C. While power output was consistently lower during the TT in warm than in temperate conditions, this decrement was attenuated throughout the bupropion trial in the heat. At the end of the TT both core temperature (pla30 39.7 ± 0.3 °C, bup30 40.0 ± 0.3 °C; \( P = 0.017 \)) and HR (pla30 178 ± 7 beats.min\(^{-1}\), bup30 183 ± 12 beats.min\(^{-1}\); \( P = 0.039 \)), were higher in the bup30 trial than in the pla30. Serum prolactin, cortisol, growth hormone and plasma ACTH concentrations increased throughout exercise in all trials. Plasma ACTH was higher following the ingestion of bupropion (\( P = 0.001 \)). Circulating serum prolactin was elevated above temperate levels during exercise in a warm environment (\( P < 0.001 \)). These data indicate that performance in warm conditions is enhanced by acute administration of a dual DA / NA reuptake inhibitor. No such effect was apparent under temperate conditions. It appears that bupropion enabled subjects to continue to maintain a high power output in the heat, despite the attainment of core temperatures suggested to accelerate central fatigue.

6.2 - Introduction

An individual's capacity to perform prolonged exercise is clearly impaired in high ambient temperatures (Galloway and Maughan, 1997; Parkin et al., 1999; Tatterson et al., 2000). While fatigue during prolonged exercise in temperate conditions is largely
associated with the depletion of muscle glycogen (Hermansen et al., 1967), factors contributing to fatigue when exercising in a warm environment are not entirely clear. While exercise capacity is thought to be primarily limited by thermoregulatory and fluid balance factors (Hargreaves and Febbraio, 1998), it has been suggested that the CNS may become important in the development of fatigue when body temperature is significantly elevated (Bruck and Olschewski, 1987; Nielsen, 1992). Hyperthermia has been proposed to accelerate central fatigue, resulting in a reduction in maximal muscle activation (Nybo and Nielsen, 2001a), altered EEG brain activity (Nielsen et al., 2001) and increased perceived exertion (Nybo and Nielsen, 2001b).

As outlined in Chapter 1, Newsholme’s original central fatigue hypothesis proposed that prolonged exercise results in an increased activity of central serotonergic neurons, producing feelings of tiredness and lethargy that may contribute to the development of fatigue (Newsholme et al., 1987). While early evidence was supportive of this suggestion (Wilson and Maughan, 1992), many subsequent studies designed to manipulate central 5-HT have failed to replicate these positive findings (Meeusen et al., 2001b; Pannier et al., 1995; Strachan, 2002). The relatively small (~1 in every 1 million neurons), but widespread distribution of serotonin throughout the CNS, has led to the suggestion that this monoamine acts largely as a neuro-modulator influencing other neurotransmitter systems within the brain, rather than directly regulating physiological responses (Jacobs and Azmitia, 1992; Trulson and Jacobs, 1979).

The opposite aspect to the serotonin-fatigue hypothesis is the catecholamine-arousal/motivation axis (Davis and Bailey, 1997). Dopamine (DA) and noradrenaline (NA) have been implicated in arousal, motivation, reinforcement and reward, the control of motor behaviour and mechanisms of addiction (Nestler et al., 2001). The depletion of central catecholamines has been linked to CNS fatigue by a number of research groups (Chaouloff, 1989; Davis, 2000; Owasoyo et al., 1992). A series of studies conducted by Bailey and co-workers (1992, 1993 a b) demonstrated that brain 5-HT and DA activity were elevated during exercise, but at the point of exhaustion a marked fall in tissue DA content was apparent. This observation resulted in the suggestion that the ratio of 5-HT to DA activity may be important for the development of central fatigue, with a high ratio of 5-HT to DA associated with
feelings of tiredness and reduced motivation (Davis and Bailey, 1997). Additionally, intracranial stimulation of the ventral tegmental area (VTA), the origin of the dopaminergic projections, motivated rats to run without the need for aversive electric shocks (Burgess et al., 1991; Burgess et al., 1993).

While there is good evidence that amphetamine, which acts as a potent DA releaser, reuptake inhibitor, and MAO inhibitor (Cooper et al., 2003), can enhance capacity to perform prolonged exercise (Gerald, 1978), little evidence from other strategies to manipulate catecholaminergic neurotransmission supports a performance benefit (Chinevere et al., 2002; Meeusen et al., 1997; Piacentini et al., 2002a; Piacentini et al., 2004; Piacentini et al., 2002b; Struder et al., 1998). In particular, Piacentini and colleagues (2004) recently examined the effects of bupropion, a dual DA / NA reuptake inhibitor, on performance and hormonal responses to exercise in temperate conditions. Although bupropion failed to alter exercise performance when compared to a placebo treatment, a marked difference in the hormonal response to exercise was reported. As pituitary hormone release is largely governed through changes in extracellular hypothalamic neurotransmitter concentrations, these hormones have been widely employed as peripheral markers of central neurotransmission (Checkley, 1980; Van de Kar, 1997).

As exercise-induced hyperthermia is thought to result in a reduced drive and a loss of motivation (Bruck and Olschewski, 1987; Nielsen, 1992), maintenance of central catecholamine levels may attenuate the loss of performance apparent when exercise is undertaken in a warm environment. Dopamine has also been implicated in the control of body temperature (Hasegawa et al., 2000; Lee et al., 1985; Lipton and Clark, 1986) and tolerance to exercise in the heat (Bridge et al., 2003). The aim of the present study was to investigate the influence of an acute dose of bupropion, a dual dopaminergic and noradrenergic reuptake inhibitor, on exercise performance and on thermoregulatory and hormonal responses to exercise in temperate and warm environmental conditions.
6.3 – Methods

Subjects. Nine healthy males (age 22.7 ± 4.3 y; Ht 1.83 ± 0.07 m; mass 75.2 ± 6.7 kg; \(W_{\text{max}}\) 372 ± 36 W; \(\dot{V}O_2\) peak 5.1 ± 0.5 L.min\(^{-1}\)) participated in this investigation. All subjects were well-trained cyclists or triathletes, with a long history of endurance-training, but were not accustomed to exercise in a warm environment at the time of the study. Prior to the start of the study all volunteers received written information regarding the nature and purpose of the experimental protocol. Following an opportunity to ask any questions, a written statement of consent was signed. The protocol employed in the present study was approved by the Research Council of the Vrije Universiteit Brussel, Brussels, Belgium.

Experimental protocol. All subjects completed a preliminary maximal exercise test, a familiarisation trial and 4 experimental trials. The preliminary trial is described in Chapter 2, and was used to determine the power output required to elicit 55 % and 75 % \(W_{\text{max}}\). A familiarisation trial was undertaken to ensure the subjects were accustomed to the procedures employed during the investigation and to minimise any potential learning or anxiety effects. This trial was performed in temperate environmental conditions with the placebo treatment administered in a single-blind manner and was identical to the experimental trials in all respects (see below). Experimental trials were undertaken in either temperate (Tmp; 18 °C) or warm (Wrm; 30 °C) conditions, with relative humidity maintained between 50 – 60 % in both conditions. The experimental trials were separated by at least 7 days to minimise the development of heat acclimation and to ensure drug washout (Holm and Spencer, 2000). The standardisation of pre-trial conditions is outlined in Chapter 2.

Subjects entered the laboratory in the morning approximately 90 minutes after consuming a standardised breakfast that included 500 mL of plain water prior to the start of each trial (~150 min before the start of exercise). Nude post-void body mass was measured and an indwelling venous cannula was introduced into a superficial forearm vein to enable repeated blood sampling at rest and during exercise. Subjects inserted a rectal thermister (Gram Corporation LT-8A, Saitama, Japan) 10 cm beyond the anal sphincter for the measurement of core temperature. Surface skin temperature
probes (Gram Corporation LT-8A, Saitama, Japan) were attached to four sites (chest, upper arm, thigh and calf) and a heart rate telemetry band (Polar Accurex plus, Kempele, Finland) was positioned. Subjects were dressed in only cycling shorts, socks and shoes for all trials.

Subjects then entered a climatic chamber maintained at the appropriate environmental conditions and rested in a seated position for 15 minutes. During this period temperatures and heart rate were recorded at 5 minute intervals and a resting venous blood sample was drawn immediately before the start of exercise. The exercise protocol consisted of 60 minutes constant load exercise at a workload corresponding to 55% $W_{\text{max}}$, followed by a time trial (TT) to measure performance. There was a 1 to 2 minute delay between the end of the constant load exercise and the beginning of the TT, to program the ergometer (Lode Excalibur Sport, Groningen, Holland) and computer software. The TT required the subjects to complete a predetermined amount of work equal to 30 minutes at 75% $W_{\text{max}}$ as quickly as possible (Jeukendrup et al., 1996). Subjects began the TT at a workload corresponding to 75% $W_{\text{max}}$, but were free to increase or decrease their power output as desired from the outset. During the TT a computer program displayed a bar indicating the percentage of total work completed to give the subject an indication of their progress. Throughout the protocol no feedback was provided regarding time lapsed, power output, pedal cadence or heart rate. During exercise subjects had *ad libitum* access to plain water.

Core and skin temperatures and heart rate were recorded at 5 minute intervals during exercise. Ratings of perceived exertion and thermal stress were assessed every 15 minutes during the initial 60 minutes and at 10 minute intervals during the TT. Venous and capillary blood samples were drawn after 30 and 60 minutes of constant load exercise and at the end of the TT. Following the completion of the TT, subjects returned to a seated position where recovery was monitored for 15 minutes and a further blood sample was obtained. The probes and cannula were then removed and nude body mass was then re-measured to allow the estimation of sweat losses.

**Drugs.** Subjects ingested 600 mg bupropion (bup: Zyban™, GSK, Middlesex, UK) or a placebo (pla: lactose) spread over two doses: one ingested at night immediately
before sleeping, with the other taken upon waking on the morning of the trial (2 x 300 mg). This is equivalent to the maximum daily therapeutic dose administered during the treatment of depression or to assist in the cessation of cigarette smoking (Holm and Spencer, 2000), and has been demonstrated to alter the hormonal response to prolonged exercise (Piacentini et al., 2004). The treatment was randomised and administered in double-blind crossover manner. Drug and placebo capsules were prepared by an independent pharmacy to appear indistinguishable with regard to dimensions, weight and colour.

**Blood collection and analysis.** Venous blood samples were drawn directly into pre-cooled vacutainer tubes (BD Vacutainer, Plymouth, UK). 10 mL samples were collected into a plain tubes and left to clot for ~1 hour at room temperature before centrifugation. The resulting serum was stored at -20 °C for the determination of prolactin (Roche Diagnostics, Mannheim, Germany), cortisol (Diasorin, Stillwater, USA) and growth hormone (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Samples for plasma adrenocorticotropic hormone (ACTH; Nichols Institute Diagnostics, CA, USA) were collected into 4.5 mL tubes containing K3EDTA. A 0.5 mL aliquot of whole blood was extracted and used for the determination of haemoglobin (Hb) and haematocrit (Hct). Hb and Hct values were used to estimate percent changes in plasma volume relative to the pre-exercise sample using the methods described by Dill and Costill (1974). The remaining EDTA-treated blood was spun, with the resulting plasma stored at -80 °C. Capillary blood (20 μL) drawn from the earlobe was analysed for blood lactate immediately following the completion of each trial (EKF Biosen 5030, Magdeburg, Germany).

**Statistical Analysis.** Data are presented as means ± standard deviation (SD). To evaluate differences in TT performance, 2-factor (temp x drug) repeated measures ANOVA were employed. Data collected over time were analysed using 3-factor (temp x drug x time) ANOVA with repeated measures, Pairwise differences were identified using Tukey's post-hoc test as appropriate. To improve the clarity of figures, differences present between environmental conditions are described in the text.
6.4 – Results

All subjects completed all trials. Some minor side-effects were reported (e.g. mild insomnia, headaches), but these were not consistent with bupropion. Throughout the text the trials are referred to as: placebo trial at 18 °C (pla18), bupropion trial at 18 °C (bup18), placebo trial at 30 °C (pla30) and the bupropion trial at 30 °C (bup30).

Mean time trial performance is displayed in Figure 6.1, with individual percent changes in performance shown in Figure 6.2. Exercise performance was significantly influenced by the ambient temperature, with subjects taking longer to complete the target amount of work in the warm than in the temperate trials ($P < 0.001$). No difference in TT performance was apparent between treatments in the Tmp condition, with times of $30.6 \pm 2.2$ min and $30.6 \pm 1.9$ min recorded in the placebo and bupropion trials respectively ($P = 0.954$). When exercise was performed in a warm environment, subjects completed the TT 9% faster in the bupropion trial ($36.4 \pm 5.7$ min) than in the placebo ($39.8 \pm 3.9$ min; $P = 0.046$).

![Figure 6.1: Time trial performance in the four experimental trials. a, b, c, d denote a significant difference from corresponding values in pla18, bup18, pla30 and bup30 trials respectively (P < 0.05).](image-url)
Figure 6.2: Individual percent changes in exercise performance in the bupropion trial relative to the placebo trial in temperate (A) and warm (B) conditions.

As the TT required the completion of a predetermined amount of work, the time taken to complete the protocol was directly related to the power output maintained throughout this period. TT power output was significantly higher during the Tmp trials ($P = 0.001$; Figure 6.3), with a similar workload maintained throughout the duration of the trial. Mean power output in temperate conditions was $275 \pm 36$ W and $279 \pm 35$ W in the placebo and bupropion trials respectively ($P = 0.531$). Mean power output was greater throughout the bupropion trial in the warm ($234 \pm 35$ W) than during the placebo trial in the same environmental conditions ($211 \pm 23$ W). From the start of the TT in the heat, power output was reduced below the predetermined starting value, but the reduction was not as pronounced in the bupropion trial.
Figure 6.3: Time trial power output in Tmp (A) and Wrm (B) conditions. * (P < 0.05) denote a significant difference between the placebo trial and the corresponding time point on the bupropion trial.
Core temperature at rest was not different between trials ($P = 0.107$; Figure 6.4). Exercise produced a progressive increase in core temperature in all trials ($P < 0.001$). Ambient temperature influenced $T_{core}$ ($P = 0.003$), with values significantly elevated in the warm trials above those recorded in temperate conditions after 50 minutes of Ex1, with this difference maintained throughout the TT and into the recovery. There was no apparent effect of bupropion on $T_{core}$ during Ex1, but core temperature was significantly higher on bupropion during the TT in both temperate and warm conditions. At the end of the performance test core temperature was elevated in bupropion trials in both temperate ($\text{pla18} \ 39.2 \pm 0.2 \ \degree C, \ \text{bup18} \ 39.4 \pm 0.3 \ \degree C; \ P = 0.010$) and warm ($\text{pla30} \ 39.7 \pm 0.3 \ \degree C, \ \text{bup30} \ 40.0 \pm 0.3 \ \degree C; \ P = 0.017$) environmental conditions.

Weighted mean skin temperature increased rapidly from the onset of exercise, reaching a plateau after 20 minutes exercise in all trials (Figure 6.5). Skin temperature was higher throughout both trials at 30 °C than during exercise in 18 °C ($P < 0.001$), but was not influenced by bupropion ($P = 0.386$). There was a tendency for $T_{skin}$ to be higher during the bup30 trial than in the pla30 ($P = 0.099$), but this failed to reach significance at any point.

The change in body mass experienced during exercise, corrected for fluid intake, was significantly greater during trials in the warm ($\text{pla30} \ 3.03 \pm 0.56 \ \text{kg}, \ \text{bup30} \ 3.02 \pm 0.54 \ \text{kg}$) when compared to the temperate conditions ($\text{pla18} \ 2.10 \pm 0.32 \ \text{kg}, \ \text{bup18} \ 2.02 \pm 0.43 \ \text{kg}; \ P < 0.001$).
Figure 6.4: Core temperature responses to the experimental protocol in Tmp (A) and Wrm (B) conditions. * (P < 0.05) and ** (P < 0.01) denote a significant difference between the placebo trial and the corresponding time point on the bupropion trial.
Figure 6.5: Weighted mean skin temperature responses to the experimental protocol in Tmp (A) and Wrm (B) conditions.

Heart rate was higher in the warm when compared to values recorded during the corresponding trial in temperate conditions (P < 0.001). During Ex1 heart rate was not affected by the drug treatment, but values were higher throughout the performance
test with bupropion in the warm (bup30) compared to the same period in the placebo trial (pla30).

Figure 6.6: Heart rate responses to the experimental protocol in Tmp (A) and Wrm (B) conditions. * (P < 0.05) and ** (P < 0.01) denote a significant difference between the placebo trial and the corresponding time point on the bupropion trial.
Figure 6.7: Rating of perceived exertion during exercise in Tmp (A) and Wrm (B) conditions.

Ambient temperature did not influence RPE during exercise, although there was a tendency for perceived exertion to be higher towards the end of Ex1 in 30 °C (P = 0.072; Figure 6.7). Bupropion had no effect on perceived exertion (P = 1.000). The subjects’ rating of perceived thermal stress is shown in Figure 6.8. Compared to the
temperate trials, perceived thermal stress was significantly higher when exercise was performed in warm conditions \( (P < 0.001) \), but during the TT neither RPE nor perceived thermal stress was different between environmental conditions. No difference in the subjects' thermal stress was apparent between drug treatments in either temperate or warm conditions \( (P = 0.785) \).

![Perceived Thermal Stress During Exercise](image)

**Figure 6.8:** Perceived thermal stress during exercise in Tmp (A) and Wrm (B) conditions.
**Table 6.1:** Blood lactate concentrations (mmol.L⁻¹) during exercise during the Tmp and Wrm trials. a, b, c, d denote a significant difference from corresponding values in pla18, bup18, pla30 and bup30 trials respectively (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>TT End</th>
<th>Rec15</th>
</tr>
</thead>
<tbody>
<tr>
<td>pla18</td>
<td>1.18 ± 0.20</td>
<td>1.48 ± 0.52</td>
<td>1.36 ± 0.48</td>
<td>5.17 ± 1.39 cd</td>
<td>2.65 ± 1.35</td>
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<tr>
<td>bup18</td>
<td>1.14 ± 0.20</td>
<td>1.81 ± 0.65</td>
<td>1.72 ± 0.48</td>
<td>5.08 ± 1.47 cd</td>
<td>2.51 ± 0.95</td>
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<tr>
<td>pla30</td>
<td>1.20 ± 0.29</td>
<td>2.04 ± 0.71</td>
<td>2.14 ± 0.79</td>
<td>2.34 ± 0.66 ab</td>
<td>1.33 ± 0.29 d</td>
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<tr>
<td>bup30</td>
<td>1.54 ± 0.58</td>
<td>1.80 ± 0.52</td>
<td>1.81 ± 0.79</td>
<td>3.33 ± 1.56 ab</td>
<td>1.85 ± 0.54 c</td>
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</tbody>
</table>

Blood lactate at rest and during Ex1 was not different between ambient temperatures, but was significantly elevated at the end of the TT in the temperate trials (P < 0.05). Drug ingestion did not influence the blood lactate concentrations (P = 0.225).

**Figure 6.9:** Serum prolactin concentration at rest and during exercise. a, b, c, d denote a significant difference from corresponding values in pla18, bup18, pla30 and bup30 trials respectively (P < 0.05).
There was no difference between trials in serum prolactin concentration at rest ($P = 0.480$; Figure 6.9). Ambient temperature altered the prolactin response to the experimental protocol ($P < 0.001$), with higher circulating concentrations observed at the end of Ex1 and upon completion of the target amount of work in the heat. Serum prolactin was not influenced by bupropion at rest or during exercise ($P = 0.173$). Compared to resting values, no change in circulating prolactin was apparent during Ex1 in temperate conditions, but a marked elevation was measured at the end of the TT and recovery period. Prolactin concentrations were elevated throughout exercise in warm conditions. Ambient temperature did not appear to influence circulating cortisol concentrations ($P = 0.191$; Figure 6.10). There was a difference in resting cortisol measured before the start of exercise between trials ($P = 0.019$), with post-hoc analysis identifying a difference between pla30 and bup30 trials ($P = 0.016$). Cortisol concentrations were not different between trials at any other point. Circulating serum cortisol increased progressively throughout exercise in all trials, with this increase continuing into the recovery ($P < 0.001$).

Figure 6.10: Serum cortisol concentration at rest and during exercise. a, b, c, d denote a significant difference from corresponding values in pla18, bup18, pla30 and bup30 trials respectively ($P < 0.05$).
Figure 6.11: Plasma adrenocorticotropic hormone (ACTH) concentration at rest and during exercise. a, b, c, d denote a significant difference from corresponding values in pla18, bup18, pla30 and bup30 trials respectively (P < 0.05).

Figure 6.12: Serum growth hormone (GH) concentration at rest and during exercise. a, b, c, d denote a significant difference from corresponding values in pla18, bup18, pla30 and bup30 trials respectively (P < 0.05).
Changes in plasma ACTH are presented in Figure 6.11. Plasma ACTH increased progressively throughout exercise in all trials ($P = 0.005$). ACTH concentrations at rest were increased by bupropion ($P = 0.038$). Ambient temperature influenced circulating ACTH, with levels significantly higher during exercise in the heat at the end Ex1. At the end of the performance test plasma ACTH was higher in the bup18 trial ($215 \pm 81 \text{ng.L}^{-1}$) than in the pla18 trial ($155 \pm 61 \text{ng.L}^{-1}$; $P = 0.028$). While serum growth hormone was increased 7 to 20-fold throughout exercise ($P < 0.001$; Figure 6.11), the environmental conditions ($P = 0.131$) and drug treatment ($P = 0.223$) did not influence the growth hormone response to the trial.

Table 6.2: Percentage changes in blood (BV), plasma (PV) and red cell (CV) volumes relative to the 0 time point. a, b, c, d denote a significant difference from corresponding values in pla18, bup18, pla30 and bup30 trials respectively ($P < 0.05$).

<table>
<thead>
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<th>30</th>
<th>60</th>
<th>TT End</th>
<th>Rec15</th>
</tr>
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<td><strong>BV</strong></td>
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<td></td>
<td></td>
</tr>
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<td>pla18</td>
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<td>-0.6 ± 1.3 d</td>
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<tr>
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<td>-6.3 ± 1.4 ab</td>
<td>-6.7 ± 1.7</td>
<td>-2.7 ± 1.0</td>
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<tr>
<td>bup30</td>
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<td>-5.3 ± 2.9</td>
<td>-6.4 ± 1.3</td>
<td>-3.4 ± 1.4 ab</td>
</tr>
<tr>
<td><strong>PV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pla18</td>
<td>-7.6 ± 4.0</td>
<td>-5.8 ± 3.4 c</td>
<td>-10.9 ± 4.0</td>
<td>-1.6 ± 3.4 d</td>
</tr>
<tr>
<td>bup18</td>
<td>-7.2 ± 4.5</td>
<td>-4.9 ± 3.9 c</td>
<td>-9.7 ± 3.5</td>
<td>-0.9 ± 2.4 d</td>
</tr>
<tr>
<td>pla30</td>
<td>-9.6 ± 2.6</td>
<td>-10.4 ± 2.1 ab</td>
<td>-11.5 ± 2.8</td>
<td>-4.1 ± 1.9</td>
</tr>
<tr>
<td>bup30</td>
<td>-7.5 ± 3.9</td>
<td>-8.7 ± 4.4</td>
<td>-10.5 ± 1.9</td>
<td>-5.5 ± 2.2 ab</td>
</tr>
<tr>
<td><strong>CV</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pla18</td>
<td>0.0 ± 1.6</td>
<td>-0.3 ± 1.1</td>
<td>0.7 ± 1.5</td>
<td>0.3 ± 1.5</td>
</tr>
<tr>
<td>bup18</td>
<td>-0.2 ± 1.1</td>
<td>0.0 ± 1.2</td>
<td>0.9 ± 1.8</td>
<td>-0.2 ± 1.2</td>
</tr>
<tr>
<td>pla30</td>
<td>0.2 ± 1.1</td>
<td>-0.4 ± 1.0</td>
<td>0.3 ± 1.2</td>
<td>-0.7 ± 1.0</td>
</tr>
<tr>
<td>bup30</td>
<td>0.3 ± 1.1</td>
<td>-0.2 ± 1.2</td>
<td>-0.3 ± 0.9</td>
<td>0.0 ± 1.6</td>
</tr>
</tbody>
</table>
Compared to resting values, both blood (P < 0.001) and plasma (P < 0.001) volumes were significantly reduced during exercise and after a 15 minute recovery period. There was a difference in blood and plasma (P = 0.005) volume changes between environmental conditions (P = 0.003), with post hoc analysis identifying differences at the end of Ex1 and following the recovery. Bupropion did not influence blood (P = 0.132), plasma (P = 0.080) or red cell (P = 0.897) responses to the experimental protocol.

6.5 – Discussion

The present study investigated the effect of acute administration of a dual dopamine / noradrenaline reuptake inhibitor on performance, thermoregulation and hormonal responses to exercise in temperate and warm environments. While a number of studies have attempted to enhance exercise performance through the manipulation of central catecholaminergic neurotransmission in temperate conditions (Chinevere et al., 2002; Meeusen et al., 1997; Piacentini et al., 2004; Struder et al., 1998), this is the first study to evaluate the effect a dual DA / NA reuptake inhibitor in warm ambient temperatures. Evidence suggests that exercise-induced hyperthermia may reduce motivation and drive to continue exercise, potentially contributing to the reduced capacity to perform prolonged exercise when ambient temperatures are elevated. Additionally, DA and NA have been implicated in the control of thermoregulation (Hasegawa et al., 2000; Lee et al., 1985; Lipton and Clark, 1986).

The results of the present study demonstrate that performance of a relatively short (30 - 45 minutes), pre-loaded TT was improved by bupropion when exercise was performed in warm ambient conditions, but no ergogenic benefit was apparent in a temperate environment. The 9 % increase in performance observed in the warm was accompanied by higher TT heart rate and the attainment of a significantly higher core temperature at the completion of the TT compared to values measured during the placebo trial under the same environmental conditions. Despite these differences no change in blood lactate or subjective feelings of perceived exertion and thermal discomfort were apparent between the pla30 and bup30 trials.
While there is good evidence that amphetamine, a potent DA agonist, can enhance exercise capacity (Chandler and Blair, 1980; Gerald, 1978), the failure to observe a performance effect at 18 °C in the present study supports the findings of recent studies investigating nutritional and pharmacological manipulation of catecholaminergic neurotransmission (Chinevere et al., 2002; Meeusen et al., 1997; Piacentini et al., 2004; Struder et al., 1998). Recently the influence of bupropion on exercise performance and the hormonal response to a 90 minute TT has been investigated (Piacentini et al., 2004). While there were slight differences in the exercise protocol, the drug administration protocol in the present study was identical to that employed by Piacentini et al. (2004). The authors also reported no effect of bupropion on exercise performance in temperate environmental conditions (18 °C).

Bupropion is a relatively weak, but selective, DA and NA reuptake inhibitor, with potency twice as great for DA than NA, while it shows little affinity for the 5-HT system (Holm and Spencer, 2000). Peak plasma concentrations are found 3 hours after ingestion, with the concentration of hydroxybupropion and threohydrobupropion, bupropion’s major metabolites reaching peak concentrations 6 hours post-ingestion. While animal studies suggest that bupropion acts primarily through its effect on DA reuptake (Nomikos et al., 1989), the hormonal responses observed by Piacentini and colleagues (2004) led the authors to suggest that the central action of bupropion in humans was mediated largely through the noradrenergic system. This suggestion is supported by clinical studies (Cooper et al., 1994), and the observation that drugs acting exclusively on DA appear not produce an appreciable antidepressant effect (Nestler et al, 2001). The discrepancy between human and animal data appears to result from differences in drug metabolism, with the metabolite hydroxybupropion not produced in rodents. This metabolite acts primarily on the NA transporter (Piacentini et al., 2003a).

Core temperature was not influenced by the drug treatment during the initial 60 minute period of fixed-intensity exercise (Ex1), but marked differences were evident during the TT under both temperate and warm conditions. It appears that the elevated core temperature towards the end of the TT in the bup30 trial can be explained by an elevated metabolic heat production, occurring as a result of the maintenance of a higher power output throughout the TT. It is interesting to note that only two (of nine)
subjects attained a temperature of 40.0 °C or greater during the pla30 trial, whereas seven breached this value when bupropion was administered (bup30 trial). It is not clear why a difference in core temperature was apparent between drug and placebo trials under temperate conditions, as no difference in power output was measured. Bupropion has recently been reported to possess thermogenic properties (Liu et al., 2002) possibility relating to its amphetamine-like structure (Nestler et al., 2001), and catecholaminergic neurotransmission may influence thermoregulation during exercise (Hasegawa et al., 2000). This may explain the different core temperature response during the TT in temperate conditions, but does not explain why no change in core temperature was measured during Ex1 and in the warm trials.

There is some limited evidence increased dopaminergic activity increases tolerance to exercise in the heat (Bridge et al., 2003). It is possible to suggest that bupropion administration in the heat acted on central DA and NA neurotransmission to maintain motivation and arousal, enabling the subjects to continue to sustain a high power output despite approaching, and in some cases breaching, the critical core temperature proposed by Nielsen et al (1993). Attainment of a critical core temp has been suggested to result in a loss of drive to continue exercise (Nielsen, 1992) and has been associated with increased perception of effort (Nybo and Nielsen, 2001b), altered brain activity (Nielsen et al., 2001) and reduced maximal muscle activation (Nybo and Nielsen, 2001a), all of which have been suggested to contribute to the development of fatigue and may serve as a protective mechanism limiting further heat production (Nielsen, 1992). This could indicate that subjects taking bupropion before exercise in a warm environment were capable of pushing into a potential ‘danger zone’ close to critical core temperature without, or significantly dampened, negative feedback from the CNS.

While Piacentini and co-workers (2004) failed to report a performance effect following bupropion, a marked difference in the hormonal response to exercise was apparent, suggesting that the drug administration produced an effect on central neurotransmission. Increased dopaminergic neurotransmission is associated with an inhibition of pituitary hormone release, with the exception of growth hormone (Checkley, 1980; Van de Kar, 1997). Regulation of hormone release by NA is complicated by the type of adrenoeceptor subtype activated, with conflicting responses.
produced by alpha and beta receptors. As stated earlier, it appears that bupropion acts primarily through changes to NA neurotransmission, making it difficult to predict expected changes to hormone release. Acute bupropion supplementation has been previously reported to increase the ACTH and cortisol response to exercise, while not influencing prolactin and growth hormone concentrations (Piacentini et al., 2004). Although it is difficult to ascertain the effect of acute drug administration due to potential autoreceptor-mediated negative feedback (Artigas et al., 1996), recent animal studies have given an insight into the effect of acute doses on neurotransmission and the central regulation of hormone release (Piacentini et al., 2003a; Piacentini et al., 2003b). The effects and implications of acute vs chronic drug administration are described in detail in Chapter 8.

Changes in circulating prolactin concentrations have been widely employed as a peripheral marker of serotonergic activity (Struder and Weicker, 2001; Van de Kar, 1997), but its reliability has been questioned (Meeusen et al., 2001b; Piacentini, 2003). Interpreting changes in plasma prolactin as an index of 5-HT activity may be misleading as prolactin release from the pituitary gland is governed through an complex interaction between a number of neurotransmitters rather than by serotonin alone (Meeusen et al., 2001a). In the present study, serum prolactin concentrations were significantly elevated during exercise in the heat, but bupropion ingestion did not alter this response. A relationship between core temperature and circulating prolactin has been widely reported (Melin et al., 1988; Pitsiladis et al., 2002; Radomski et al., 1998; Strachan, 2002), and it has been proposed that serotonergic activity may be upregulated during combined exercise and heat stress (Pitsiladis et al., 2002). As changes in core temperature and HPA-hormone secretion are primarily regulated by the hypothalamus, it appears that brain temperature during exercise may be causally related to the release of hormones from the pituitary gland (Radomski et al., 1998).

In contrast to previous reports (Brenner et al., 1997; Strachan, 2002) serum cortisol concentrations were not influenced by the ambient conditions. Brenner and colleagues reported a marked elevation in circulating cortisol concentration when moderate-intensity exercise was performed in warm (40 °C) compared to temperate (23 °C) conditions. A failure to observe a difference in cortisol between environmental
conditions in the present investigation may be due to the attainment of relatively high core temperatures (> 39 °C) in the temperate trial, with clamping of body temperature previously demonstrated to abolish exercise-induced cortisol secretion (Cross et al., 1996). As previously reported (Piacentini et al., 2004) bupropion did not significantly influence the serum cortisol or growth hormone response, whereas plasma ACTH was elevated at the end of the TT in temperate ambient conditions. There was a tendency for ACTH to be higher in the heat with bupropion at this time point, but this difference failed to reach statistical significance due to the large intra-subject variability.

Two important findings arise from this study: 1) subjects completed a pre-loaded time trial 9% faster when bupropion was taken before exercise in a warm environment compared to a placebo treatment. This ergogenic effect was not apparent at 18 °C. 2) Seven (of 9) subjects attained core temperatures equal to, or greater than, 40.0 °C in the BUP trial, compared to only two during the placebo trial. This occurred without any apparent change in the subjects' perceived exertion or thermal sensation. It is possible to suggest that this drug may enable an individual to dampen or override inhibitory signals arising from the CNS to cease exercise due to hyperthermia, and continue to maintain a high power output. The present findings should be noted as the International Olympic Committee (IOC), removed this drug from the list of prohibited substances in January 2003.
Chapter 7

Serum S100b, a proposed marker of blood-brain barrier permeability, and prolonged exercise in temperate and warm environments in man
7.1 – Abstract

Seven active males were recruited to examine changes in serum S100b, a proposed peripheral marker of BBB permeability, following water immersion and prolonged exercise in temperate (Tmp) and warm (Wrm) conditions. Subjects were seated immersed to the neck in water at 35.0 ± 0.1 °C (Tmp) or 39.0 ± 0.1 °C (Wrm) for 30 min. Subjects then entered a room maintained at either 18.3 ± 1.8 °C (Tmp) or 35.0 ± 0.3 °C (Wrm) and completed 60 min cycle exercise at 60 % $\dot{\text{VO}}_2$ peak. Serum S100b was elevated after exercise in the Wrm trial (+0.12 ± 0.10 μg.L$^{-1}$; $P = 0.02$), but not after the Tmp trial ($P = 0.238$). Water immersion and exercise elevated core temperature by 2.1 ± 0.5 °C to 39.5 ± 0.3 °C at the end of exercise in the Wrm trial compared to a 0.9 ± 0.2 °C increase during the Tmp trial ($P < 0.001$). Weighted mean skin temperature was higher throughout the Wrm trial ($P < 0.001$). Body mass loss was significantly influenced by the experimental conditions (Tmp 0.70 ± 0.14 kg, Wrm 1.79 ± 0.35 kg; $P < 0.001$). Heart rate ($P < 0.001$), as well as blood glucose ($P < 0.001$) and lactate ($P < 0.001$), were elevated during exercise in the warm environment. Ratings of perceived exertion ($P < 0.001$) and thermal discomfort ($P < 0.001$) were markedly higher throughout the Wrm trial than in the Tmp trial. The results of this study demonstrate that serum S100b was elevated following water immersion and prolonged exercise in a warm environment, suggesting that BBB permeability may be altered. Changes in BBB integrity may contribute to the development of central fatigue during prolonged exercise by disturbing CNS homeostasis.

7.2 – Introduction

The blood-brain barrier (BBB) is a dynamic structure consisting of microvascular endothelial cells, characterised by the presence of tight junctions and restricted vesicular transport (Kandel et al., 2000). The foot processes of surrounding astrocytes and the presence of pericytes reinforce these tight junctions. The BBB functions through these specialised structures to maintain a stable environment for the CNS by tightly regulating the exchange of molecules between the CNS and the peripheral
circulation. When the BBB is intact diffusion is largely restricted, with transport mediated through a series of selective molecular carrier systems specific to a wide range of substances, including glucose, amino acids, vitamins, electrolytes and peptides (Saunders et al., 1999). Further information regarding the structure and function of the BBB can be found in Chapter 1.

The BBB is largely resistant to changes in permeability, but there are situations where the function of the BBB may become compromised. A number of physiological disturbances have been demonstrated to result in BBB dysfunction, including bacterial and viral infections (Chaudhuri, 2000), brain tumours, stroke and traumatic brain damage (Greenwood, 1991) and the extreme stress of warfare (Hanin, 1996). Either acute or chronic changes to the permeability of this barrier may allow the entry or exit of species that can affect the metabolism of the brain and consequently influence a wide range of homeostatic mechanisms. Additionally, changes in BBB permeability associated with disorders mentioned above may result in secondary neuronal damage, thus further complicating the condition. Paradoxically, the opening of the BBB is desirable in the treatment of some conditions to allow the delivery of therapeutic agents into the CNS that would typically not cross the intact BBB (Rapoport, 2000).

There is some evidence that prolonged exercise may lead to increased BBB permeability. Animal studies have established that the BBB can be widely disrupted following 30 minutes forced swimming exercise (Sharma et al., 1991; Sharma et al., 1996). These changes were found to be relatively acute, with normal BBB function restored 2 hours after the end of exercise. A number of physiological responses occurring during prolonged exercise may potentially lead to a change in BBB function, with the development of hyperthermia (Sharma and Dey, 1987; Wijsman and Shivers, 1993), an upregulation in central serotonin synthesis (Sharma et al., 1991; Sharma et al., 1996), increased circulating ammonia concentrations (Mans et al., 1983) and an increased production of pro-inflammatory cytokines (de Boer and Breimer, 1998) all suggested as possible factors contributing to this response.

At present, it is not clear how an increase in BBB permeability may influence exercise. Some increase in the exchange of substances across the barrier is probably
desirable during exercise, to facilitate the transport of metabolites and other substances into the CNS when cerebral blood flow is elevated. However, a marked disturbance in BBB function during exercise may adversely affect normal brain function and contribute to the development of central fatigue by altering the transport kinetics of neurotransmitter precursors (Chaouloff, 1997) or allowing the accumulation of unwanted molecules in the CNS (Shivers and Wijsman, 1998).

An increase in the permeability of the BBB can result in a detectable leakage of substances from the blood into CNS. This forms the basis of many conventional methods employed to monitor BBB integrity, with the accumulation of plasma proteins (e.g. albumin) in the brain and cerebrospinal fluid (CSF) used as an indicator of changes in BBB permeability using lumbar puncture and tracer-imaging techniques. The possibility that CNS-specific proteins may breach the BBB in areas of disruption and leak into the peripheral circulation has also been recently considered. Kapural and colleagues (2002) proposed that the appearance of CSF-specific proteins, in particular S100b, in the circulation might be used as a relatively non-invasive peripheral marker of BBB function.

S100b is a low molecular weight (21 kDa) calcium-binding protein expressed predominantly within the CNS by astrocytes and Schwann cells. While the precise function of S100b within the CNS is not well understood at present, it is thought to play roles in the modulation of intracellular signal transduction, neuronal development, and cerebral energy metabolism (Zimmer et al., 1995). It is typically found in low concentrations in the serum of healthy individuals, with a CSF to serum concentration ratio of approximately 18 : 1 (Reiber, 2001), thus BBB opening would be expected to significantly elevate serum concentrations (Kapural et al., 2002). S100 proteins are cleared from the peripheral circulation by the kidneys and appear in the urine, with a serum half-life of around two hours (Ali et al., 2000). As brain trauma is known to result in a marked elevation in CSF S100b concentrations and damage to the brain is typically accompanied by a breakdown in barrier function (Marchi et al., 2003), serum S100b has been widely employed in a clinical setting as a peripheral marker of neuronal damage. The application of S100b as a peripheral marker of brain damage and BBB disruption is discussed in detail in Chapter 1.
The aim of the present investigation was to determine whether a combination of water immersion and prolonged exercise in temperate and warm environmental conditions results in a change in circulating concentrations of serum S100b. As the development of whole-body hyperthermia was hypothesised to increase BBB permeability pre-exercise water immersion was included to accelerate the change in core temperature during the warm trial.

7.3 – Methods

Subjects. Seven healthy males (age 25.7 ± 5.0 y, height 1.76 ± 0.08 m, mass 77.1 ± 5.0 kg, \( \dot{V}O_2 \) peak 4.1 ± 0.2 L·min\(^{-1} \)) were recruited to take part in this study. All subjects were taking part in regular endurance exercise, but were not accustomed to exercise in a warm environment at the time of the study. Prior to volunteering to participate all subjects received written details outlining the nature of the study. Following any questions regarding the protocol, a written statement of consent was signed. The protocol described in this Chapter received approval from the Loughborough University Ethics Board.

Experimental protocol. All subjects completed a preliminary test to determine peak oxygen uptake (\( \dot{V}O_2 \) peak), a familiarisation trial and 2 experimental trials. The preliminary test is described in Chapter 2, and was used to determine the power output required to elicit 60 % \( \dot{V}O_2 \) peak. The familiarisation trial mirrored exactly the warm trial and was undertaken to ensure the subjects were accustomed to the protocol and environmental conditions employed throughout the investigation. Experimental trials were completed under either temperate (Tmp) or warm (Wrm) conditions. The experimental trials were administered in a randomised order separated by at least 7 days to minimise the development of heat acclimation. Instructions for the standardisation of pre-trial dietary and physical activity conditions are outlined in Chapter 2.

All experimental trials commenced in the morning following an overnight fast, other than the ingestion of ~500 mL of plain water at least 90 minutes before the start of the
trial. Upon arrival in the laboratory, a rectal thermister was positioned by the subject 10cm beyond the anal sphincter. Temperature thermisters were attached to the skin surface at four locations (chest, tricep, thigh, calf) and a heart rate telemetry band was positioned. Post-void, nude body mass was determined after positioning of the probes to allow ease of measurement following water immersion. Subjects were seated for 15 minutes in a comfortable environment (24 – 26 °C) with one hand immersed in warm water (42 °C) for 10 minutes to enable arterialised venous blood to be drawn. A 21g cannula was introduced into a superficial forearm vein to enable repeated blood sampling whilst at rest and during exercise. Two resting 7.5ml blood samples were drawn (-5 and 0 min), and baseline measurements of temperature and heart rate (HR) were made at 5 minute intervals.

Subjects then entered a water tank and sat for 30 minutes immersed to the neck with the exception of the cannulated forearm. Water temperature was maintained at either 35 ± 0.1 °C or 39 ± 0.1 °C in the temperate and warm trials respectively. Core and skin temperatures and heart rate were recorded at 5 minute intervals during immersion, and the subject’s perceived thermal stress was assessed every 10 minutes. During the final minute of water immersion a blood sample was drawn (Post-Im), after which the subject left the water tank and towelled dry. Nude body mass was then re-measured to determine sweat losses occurring during the period of immersion. Subjects then dressed in shorts and shoes.

Subjects entered a climatic chamber maintained at either 18.3 ± 1.8 °C (Tmp trial) or 35.0 ± 0.3 °C (Wrm trial) and began a bout of cycle exercise (Gould Corival 300, Groningen, Holland) corresponding to 60 % \( \text{V} \text{O}_2 \text{peak} \) for 60 minutes. During both trials the relative humidity was 60 ± 5 %. The time between leaving the water tank and commencing exercise in the climatic chamber was around 5 minutes. Blood samples (7.5 mL) were drawn during exercise at 15 minute intervals. Throughout exercise core and skin temperatures and heart rate were recorded every 5 minutes. Ratings of perceived exertion and thermal stress were obtained at 10 minute intervals. Expired gas was collected and analysed at 20 minute intervals using the Douglas bag method. These data were used to estimate rates of substrate oxidation and energy expenditure. In accordance with local ethics guidelines, trials were prematurely
stopped if a subject’s rectal temperature reached 40.0 °C. Following the cessation of exercise, subjects were returned to a comfortable environment where recovery was monitored for 15 minutes and a post-exercise body mass was measured.

**Blood handling and analysis.** Blood samples collected throughout the experimental protocol were drawn into dry syringes with 2.5 mL dispensed into tubes containing K$_2$EDTA and the remaining 5 mL into plain tubes. Percentage changes in blood, plasma and red cell volumes relative to the second resting sample were determined using haemoglobin and spun packed cell volumes. Deproteinised whole blood was used for determination of blood glucose (God-PAP, Randox, Co.Antrim, UK) and lactate concentrations (Maughan, 1982). The remaining EDTA-treated blood was then spun to yield plasma and stored frozen at −20 °C for the analysis of FFA (Roche Diagnostics, Mannheim, Germany). The 5 mL sample added to a plain tube was kept on ice until the end of each trial before being centrifuged to yield serum. One aliquot of serum was kept frozen at −20 °C for the analysis of S100b using a commercially available enzyme-linked immunosorbant assay (ELISA; Sangtec Medical, Bromma, Sweden). The second aliquot was refrigerated at 4 °C and used for the determination of serum electrolytes: serum Na$^+$ and K$^+$ by flame photometry (Corning 410C, New York, USA), Cl$^-$ concentrations were obtained through coulometric titration (Jenway PCLM 3, Essex, UK) and osmolality by freezing point depression (Gonotec Osmomat 030, YSI, Farnborough, UK) within 7 days of each trial.

### 7.4 -- Results

One subject was prematurely stopped due to a high core temperature after 52 minutes exercise during the Wrm trial in accordance with local ethics guidelines. The final exercise blood sample was drawn at this point and the recovery was monitored as usual. This subject’s data were included in all analyses outlined in this Chapter.

Changes in serum S100b are presented in Figure 7.1. Serum concentration of S100b was not different at rest between trials, with values of 0.06 ± 0.04 μg.L$^{-1}$ and 0.07 ± 0.05 μg.L$^{-1}$ measured in the Tmp and Wrm trials respectively (P = 0.372). A 2.6-fold
increase in S100b was apparent following water immersion and prolonged exercise in the Wrm trial (+0.12 ± 0.10 μg.L⁻¹; P = 0.02), with no change apparent after the Tmp trial (P = 0.238). Two subjects who experienced no change in serum S100b in either trial were primarily responsible for the large variation S100b observed following exercise.

![Graph showing serum S100b at rest (0) and at the end of exercise (Ex60) in the Tmp and Wrm trials.](image)

**Figure 7.1:** Serum S100b at rest (0) and at the end of exercise (Ex60) in the Tmp and Wrm trials. b denotes a significant difference from the 0 time point in the Wrm trial (P < 0.05). * (P<0.05) denotes a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.

Expired gas data collected during exercise are presented in Table 7.1. Exercise \( \dot{V}O_2 \) was elevated in the Wrm trial compared to values during the Tmp trial (P = 0.049), but post-hoc analysis did not identify a difference at any time point. A progressive reduction in RER was apparent during the Tmp trial, reaching significance at the end of exercise (P = 0.042). No change in RER was observed during the Wrm trial (P = 0.567), but it was not different from the Tmp trial at the end of exercise (Tmp 0.87 ±
0.02, Wrm 0.92 ± 0.07; P = 0.151). Rates of CHO (P = 0.294) and fat (P = 0.943) oxidation were not different between trials.

Table 7.1: Oxygen uptake, RER, CHO and fat oxidation during exercise in the Tmp and Wrm trials. a and b denote a significant difference from the 20 minute time point in the Tmp and Wrm trials respectively (P < 0.05).

<table>
<thead>
<tr>
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<th>20</th>
<th>40</th>
<th>60</th>
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<tr>
<td>VO₂ (L.min⁻¹)</td>
<td></td>
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<tr>
<td>Tmp</td>
<td>2.29 ± 0.22</td>
<td>2.35 ± 0.20</td>
<td>2.30 ± 0.20</td>
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<tr>
<td>Wrm</td>
<td>2.36 ± 0.15</td>
<td>2.46 ± 0.13</td>
<td>2.36 ± 0.29</td>
</tr>
<tr>
<td>RER (no units)</td>
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</tr>
<tr>
<td>Tmp</td>
<td>0.91 ± 0.03</td>
<td>0.90 ± 0.03</td>
<td>0.87 ± 0.02 a</td>
</tr>
<tr>
<td>Wrm</td>
<td>0.90 ± 0.01</td>
<td>0.89 ± 0.04</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>CHO Oxidation (g.min⁻¹)</td>
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<tr>
<td>Tmp</td>
<td>2.18 ± 0.38</td>
<td>2.14 ± 0.27</td>
<td>1.75 ± 0.28</td>
</tr>
<tr>
<td>Wrm</td>
<td>2.09 ± 0.16</td>
<td>2.15 ± 0.51</td>
<td>2.33 ± 0.65</td>
</tr>
<tr>
<td>Fat Oxidation (g.min⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tmp</td>
<td>0.33 ± 0.15</td>
<td>0.38 ± 0.14</td>
<td>0.50 ± 0.07</td>
</tr>
<tr>
<td>Wrm</td>
<td>0.40 ± 0.06</td>
<td>0.43 ± 0.16</td>
<td>0.39 ± 0.21</td>
</tr>
</tbody>
</table>

Core temperature at rest was not different between trials (P = 0.253; Figure 7.2A). Water immersion produced an increase in core temperature during the Wrm trial. In contrast, no change in core temperature was observed during water immersion in the Tmp trial. Exercise produced a marked increase in core temperature in both trials, with a plateau apparent after 30 minutes in the Tmp trial compared to a near linear increase in the Wrm trial. Core temperature was elevated by 2.1 ± 0.5 °C to 39.5 ± 0.3 °C at the end of exercise in the Wrm trial compared to a 0.9 ± 0.2 °C increase during the Tmp trial (P < 0.001). There was no apparent association between the change in S100b and the change in core temperature, with correlation coefficients calculated as -0.318 (P = 0.486) for the Tmp trial and -0.021 (P = 0.965) for the Wrm trial. Weighted mean skin temperature was different between trials throughout the period of water immersion, during exercise and the recovery (P < 0.001; Figure 7.2B).
Figure 7.2: Change in core (A) and weighted mean skin (B) temperature at rest, during water immersion and exercise. a and b denote a significant difference from the pre value in the Tmp and Wrm trials respectively (P < 0.05). * (P < 0.05) and ** (P < 0.01) denote a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.

Body mass loss during the experimental protocol was greater in the Wrm than in the Tmp trial (P < 0.001). Thirty minutes immersion in water maintained at 35 °C (Tmp
trial) produced no change to body mass, whereas subjects lost $0.46 \pm 0.17$ kg during Wrm trial ($P = 0.001$). At the end of exercise body mass losses of $0.70 \pm 0.14$ kg and $1.79 \pm 0.35$ kg were recorded in the Tmp and Wrm trials respectively ($P < 0.001$).

**Figure 7.3:** Heart rate at rest, during water immersion and exercise. a and b denote a significant difference from the Pre value in the Tmp and Wrm trials respectively ($P < 0.05$). * ($P < 0.05$) and ** ($P < 0.01$) denote a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.

Heart rate was significantly influenced by the environmental conditions ($P < 0.001$; Figure 7.3). Warm water immersion resulted in a marked elevation in HR above the Tmp trial, with this difference maintained throughout exercise and the recovery. Heart rate at the end of exercise in the Tmp trial was $138 \pm 8$ beat.min$^{-1}$ compared to $174 \pm 11$ beat.min$^{-1}$ during the Wrm trial ($P < 0.001$).
Figure 7.4: Rating of perceived exertion during exercise. a and b denote a significant difference from the Ex10 min value in the Tmp and Wrm trials respectively (P < 0.05). * (P < 0.05) and ** (P < 0.01) denote a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.

Throughout the Wrm trial RPE was elevated above the Tmp trial (P < 0.001; Figure 7.4). Perceived exertion increased with time during both trials, reaching values of 12 ± 0 and 16 ± 2 in the Tmp and Wrm trials respectively. Perceived thermal stress was relatively stable during the Tmp trial, with little change apparent during water immersion or exercise (Figure 7.5). In the Wrm trial the subjects’ perceived thermal discomfort progressively increased and was persistently higher than values obtained in the Tmp trial (P < 0.001).
Figure 7.5: Perceived thermal stress during water immersion and exercise. a and b denote a significant difference from the Im10 value in the Tmp and Wrm trials respectively (P < 0.05). * (P < 0.05) and ** (P < 0.01) denote a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.

The blood glucose and lactate response to the experimental trials are presented in Figure 7.6. Blood glucose concentrations were not different at rest between conditions (P = 0.457) and at no point during either the Tmp (P = 0.097) or Wrm (P = 0.171) trial were blood glucose concentrations different from resting levels. Throughout exercise in the Wrm trial, blood glucose was significantly elevated above corresponding values in the Tmp trial. A 30 minute period of water immersion did not influence blood lactate concentrations. Lactate concentrations were elevated above rest during exercise in both trials and maintained significantly higher during the Wrm trial (P < 0.001).
Figure 7.6: Blood glucose (A) and lactate (B) concentrations at rest, during water immersion and exercise. a and b denote a significant difference from the -5 min value in the Tmp and Wrm trials respectively (P < 0.05). * (P < 0.05) and ** (P < 0.01) denote a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.
Plasma FFA concentrations are shown in Figure 7.7. There was no difference in plasma FFA at rest between trials \((P = 0.459)\). While FFA concentrations did not change significantly during exercise, there was a marked increase apparent after 15 minutes recovery with this response more pronounced in the Wrm trial.

![Graph showing plasma FFA concentrations](image)

**Figure 7.7:** Plasma free-fatty acid concentration at rest, during water immersion and exercise. a and b denote a significant difference from the Im10 value in the Tmp and Wrm trials respectively \((P < 0.05)\). * (\(P < 0.05\)) and ** (\(P < 0.01\)) denote a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.
Table 7.2: Serum sodium (Na⁺; mmol.L⁻¹), potassium (K⁺; mmol.L⁻¹), chloride (Cl⁻; mmol.L⁻¹) and osmolality (Osm; mOsmol.kg⁻¹) at rest, following water immersion and during exercise. * denotes a significant difference from the Pre value in the Tmp and Wrm trials respectively (P < 0.05). ** denotes a significant difference between the Tmp and the corresponding time point in the Wrm trial.

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<th>Ex45</th>
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The environmental conditions did not alter the serum Na⁺, Cl⁻ or osmolality response to the experimental protocol (Table 7.2). Serum K⁺ during exercise in the Wrm trial was elevated above corresponding values in the Tmp trial (P = 0.004). Serum Na⁺ did not change throughout the experimental protocol in either the Tmp or Wrm trial. Serum K⁺ and osmolality were significantly increased above resting values during exercise in both experimental trials.
Table 7.3: Percentage change in blood (BV), red cell (CV) and plasma (PV) volumes relative to the −5 min value. a and b denote a significant difference from the −5 min time point in the Tmp and Wrm trials respectively (P < 0.05). * (P < 0.05) and ** (P < 0.01) denote a significant difference between the Tmp trial and the corresponding time point in the Wrm trial.

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Percentage changes in blood volume were different between trials (P = 0.039), with post-hoc analysis identifying a larger change in the Wrm trial following water immersion and the post-exercise recovery only (Table 7.3). No differences in blood volume were apparent whilst at rest or during exercise. Percentage changes in red cell and plasma volumes were not different between trials. The experimental protocol produced a fall in blood and plasma volumes during both trials, with a marked reduction apparent from the first exercise blood sample (Ex15). There was no further change in blood or plasma volume throughout exercise.
7.5 – Discussion

Exercise-induced hyperthermia has been suggested to result in a reduction in motivation and drive to continue exercise, and this may to some extent explain the reduction in prolonged exercise capacity apparent when ambient temperatures are high (Nielsen, 1992). Recent work has demonstrated that hyperthermia results in a reduction in sustained maximal muscle activation (Nybo and Nielsen, 2001a), altered brain activity (Nielsen et al., 2001) and elevated perceived exertion (Nybo and Nielsen, 2001b), but the underlying neurobiological mechanism(s) responsible for these changes are not clear. The present study examined the possibility that BBB permeability may be altered following water immersion and exercise in warm conditions.

The main observation to arise from the present study was the marked elevation in serum S100b following exercise in a warm environment. This response was not apparent following exercise in temperate conditions. Increased serum concentrations of S100b have been reported following osmotic opening of the BBB (Kapural et al., 2002), with the appearance of this CNS-specific protein in the peripheral circulation up to serum concentrations of 0.34 μg.L⁻¹ proposed as a peripheral marker of BBB permeability (Marchi et al., 2003). A small increase in BBB permeability may be beneficial to brain metabolism during exercise, facilitating the exchange of substances when cerebral blood flow is increased. However, a marked change to BBB integrity may affect normal brain function and influence the development of central fatigue during prolonged exercise by altering the transport kinetics of neurotransmitter precursors (Chaouloff, 1997) or allowing the accumulation of unwanted substances in the CNS (Shivers and Wijsman, 1998).

Previous reports of changes in BBB permeability with exercise have employed a forced swimming model in rats (Sharma et al., 1991; Sharma et al., 1996). The increased permeability reported in these studies was only observed in young rats (< 10 weeks), with no change apparent in older animals (> 30 weeks). The BBB is a dynamic structure capable of adapting to repeated exposure to hyperthermia and other physiological stresses, with conditioning enabling the barrier to behave normally.
(Shivers and Wijsman, 1998). In the present investigation no association was apparent between changes in serum S100b and the age of the subjects ($r = -0.04, P = 0.939$), but this was an homogenous group. Forced swimming places an animal under severe stress and has been associated with marked alterations to serotonin and dopamine metabolism, the appearance of haemorrhagic spots on the wall of the stomach and the excretion of faecal pellets (Sharma et al., 1996). It is important to note that the breakdown in BBB permeability reported in the series of studies by Sharma and colleagues was particularly severe and widespread, with the extravasation of an albumin-tracer complex (Evans blue and $^{131}$I-sodium) into the brain. Any change in BBB permeability in humans occurring during exercise is unlikely to be as dramatic.

While it is accepted that the BBB integrity can be altered by a wide range of physiological disturbances, the molecular and cellular mechanisms behind these changes in vivo are not clear at present (Gloor et al., 2001). Factors that may be important in changes to BBB permeability during exercise in a warm environment, include the development of hyperthermia (Sharma and Hoopes, 2003; Shivers and Wijsman, 1998), changes to central serotonergic neurotransmission (Sharma et al., 1991; Sharma et al., 1996), hyperammonaemia (Mans et al., 1983) and an upregulation in pro-inflammatory cytokine production (de Boer and Breimer, 1998). Prolonged exposure to physiological and psychological stress has been demonstrated to result in widespread BBB disruption in humans (Hanin, 1996). The data collected during the present study (RPE, perceived thermal stress, heart rate) suggest subjects' found the warm trial significantly more difficult to complete. It appears likely that the stress encountered during the temperate trial may have been insufficient to produce a marked change in BBB function.

Whole-body hyperthermia induced by prolonged exposure to passive heat stress has been demonstrated to result in impaired BBB integrity in rats (Sharma and Dey, 1987; Wijsman and Shivers, 1993), but no change was reported in a study using dogs (Katsumura et al., 1995). The apparent difference in BBB response between these species is not clear, but may relate to methodological differences. In the present study the Wrm trial resulted in a marked and sustained increase in core (rectal) temperature, with levels elevated above 39 °C during the final 25 minutes of exercise and throughout the recovery period, compared to a mean core temperature of 38.3 °C at
the end of exercise in the Tmp trial (Figure 7.2A). Additionally, brain temperature, a more representative measure of the thermal load placed on the brain capillary network, is thought to be persistently maintained at least 0.2 °C above core (esophageal) temperature during exercise in a warm environment (Nybo et al., 2002). The failure to observe a significant relationship between changes in S100b and core temperature was unexpected, but was largely due to 2 subjects failing to display any change in serum S100b despite a marked increase in core temperature.

It has been suggested that serotonin may be important in mediating changes in BBB integrity during both exercise and exposure to hyperthermia (Sharma and Hoopes, 2003), with the administration of p-chlorophenylalanine (a tryptophan hydroxylase inhibitor) reported to prevent exercise-induced BBB breakdown (Sharma et al., 1996). Serotonin is a potent vasoactive agent, known to act on both central and peripheral blood vessels (Cohen et al., 1996). While there is some limited evidence that serotonergic neurotransmission is increased during exercise in a warm environment (Pitsiladis et al., 2002), this is not supported by data examining cerebral tryptophan uptake using arterial-venous difference across the brain (Nybo et al., 2003). This does not discount a role for serotonin in mediating exercise-induced changes in BBB function, but it is likely that a complex interaction of neuromodulators is involved in permeability control during exercise.

In contrast to previous work conducted by Galloway and Maughan (1997), \( \dot{V}O_2 \) was significantly elevated during exercise in a warm environment (Table 7.1), but similar findings have been previously reported (Fink et al., 1975). It is widely accepted that exercise in a warm environment is associated with an increased reliance on CHO utilisation, resulting from an increased sympatho-adrenal response (Febbraio, 2001). While rates of CHO oxidation estimated from expired gas failed to support this suggestion, there was a tendency for CHO utilisation to be elevated above the Tmp condition by the end of exercise in the Wrm trial.

The reduced temperature gradient between the skin and the ambient air in the Wrm trial would be expected to result in an increased skin blood flow and a greater dependence on evaporative heat loss (Rowell, 1974). Heart rate was clearly elevated
throughout the period of water immersion and during exercise in the Wrm trial when compared to the Tmp trial, supporting this suggestion. The marked difference in body mass losses experienced between trials indicate sweat production was increased. No differences in blood and plasma volume changes during exercise were apparent between trials despite the marked differences in sweat losses. These data suggest that a greater proportion of the fluids lost during the warm trial were likely to have been derived from other vascular compartments, including the interstitial and intracellular water compartments to maintain blood volume (Costill et al., 1976; Galloway and Maughan, 1997; Nose et al., 1988).

While S100b has been widely employed in a clinical setting as a peripheral marker of brain damage and more recently BBB disruption, it is important to consider the potential limitations of this technique. In a similar manner to the use of plasma prolactin as a marker of serotonergic neurotransmission, the measurement of a peripheral marker is indirect and may not accurately reflect changes occurring within the CNS. Kanner and colleagues (2003) have reported a strong association between the extent and time-course of BBB disruption measured using gadolinium-enhanced MRI and changes in serum S100b, supporting the use of this marker as an effective peripheral marker of BBB permeability. While the beta subunit of this protein is highly specific to the CNS (Ali et al., 2000), it has been found to be expressed in small quantities in peripheral tissues including bone, heart and adipose tissue (Zimmer et al., 1995). Recent work suggests that S100b is liberated into the circulation only when significant trauma occurs to these tissues (Anderson et al., 2001; Pelinka et al., 2003). Additionally, potentially small openings in the BBB may not allow S100b to leak into the peripheral circulation. Thus, it is possible that subtle changes in BBB permeability may not be detected using this technique.

The present study has demonstrated that serum S100b is elevated following prolonged exercise in a warm environment, suggesting that BBB permeability may be altered. Previous animal studies have also observed a marked increase in BBB permeability following forced swimming exercise. The development of hyperthermia, an upregulation in central serotonin synthesis, increased circulating ammonia concentrations and an increased production of pro-inflammatory cytokines has all been suggested as possible factors contributing to this response. These findings may
be important for two reasons: 1) a change in BBB integrity during exercise may disturb normal brain function and contribute to the development of central fatigue. 2) Serum S100b is now being employed as an index of brain trauma in individuals who suffer head injuries during sports (Mussack et al., 2003; Otto et al., 2000). Changes in the permeability of the BBB to this protein marker may give misleading results in exercising individuals.
Chapter 8

General Discussion
8.1 – Background

The capacity to perform prolonged exercise is significantly reduced when exercise is performed in a warm environment (Galloway and Maughan, 1997; Parkin et al., 1999). Additionally, distance covered during a 30 minute cycle time trial is also reduced when ambient temperature was increased from 23 to 32 °C (Tatterson et al., 2000). In contrast to temperate conditions, fatigue during exercise in high ambient temperatures does not appear to be adequately explained by the depletion of muscle glycogen, or by cardiovascular and fluid balance factors (Nielsen and Nybo, 2003; Strachan and Maughan, 2002), suggesting that fatigue may be mediated through mechanisms residing within the CNS. Recent studies have provided evidence that hyperthermia profoundly influences brain function during exercise, resulting in altered brain activity (Nielsen et al., 2001), reduced voluntary activation of muscle during sustained contractions (Nybo and Nielsen, 2001a) and an increased perception of effort (Armada-Da-Silva et al., 2004; Nybo and Nielsen, 2001b). At present the neurobiological mechanism(s) behind these changes are not apparent. There is limited evidence that serotonin may be important to the development of fatigue during prolonged exercise in a warm environment (Mittleman et al., 1998; Pitsiladis et al., 2002), through the mechanism proposed by Newsholme and colleagues (1987). The aim of the work described in this thesis was to examine the possible roles of serotonergic and catecholaminergic neurotransmission and changes in BBB permeability in an attempt to further elucidate the role of the CNS in the development of fatigue during exercise in a warm environment.

8.2 – Nutritional manipulation of serotonin-mediated fatigue during exercise in a warm environment

Since the proposal of the serotonin-based hypothesis of fatigue (Newsholme et al., 1987), a number of investigations have sought to manipulate central neurotransmission during prolonged exercise through the provision of pharmacological agents and dietary supplements. Nutritional strategies employed have included the ingestion of amino acids (BCAA, TRP, TYR) or CHO to alter the cerebral uptake of neurotransmitter precursors. At present evidence for a benefit of
BCAA ingestion in temperate conditions is poor (Blomstrand et al., 1995; Blomstrand et al., 1997; Davis et al., 1999; Hassmen et al., 1994; Madsen et al., 1996; Struder et al., 1998; van Hall et al., 1995). Differences in protocol (Davis and Bailey, 1997), co-ingestion with CHO (Calders et al., 1999) and the timing of pre-exercise BCAA ingestion may partially explain this failure to detect an ergogenic effect, but it is possible that the development of fatigue during prolonged exercise under temperate conditions is adequately explained by peripheral mechanisms of fatigue (e.g. muscle glycogen depletion).

To date, only one published investigation has examined the effect of BCAA ingestion on prolonged exercise in a warm environment. Mittleman and colleagues (1998) reported a 14% increase in exercise capacity following BCAA ingestion when exercise was performed at 34°C. In the absence of any significant peripheral effects, the findings are consistent with the hypothesis that the supplementation regimen limited the transport of TRP across the BBB and consequently attenuated the exercise-induced increase in serotonergic activity. The exercise intensity employed in this study was relatively low (40% \(\text{VO}_2\text{max}\)), resulting in little change to core temperature. It remains to be seen whether these positive findings can be reproduced when exercise is performed at higher intensity, resulting in greater heat production and the attainment of higher core temperatures. The aim of Chapters 3 and 4 was to further examine the effect of acute BCAA ingestion on prolonged exercise capacity in a warm environment.

In contrast to previous findings (Mittleman et al., 1998), but in agreement with a number of studies undertaken in temperate conditions (Blomstrand et al., 1995; Blomstrand et al., 1997; Davis et al., 1999; Galiano et al., 1991; Madsen et al., 1996; Struder et al., 1998), the results of Chapters 3 and 4 fail to support the suggestion that BCAA ingestion delays fatigue during prolonged exercise. This occurred despite a marked reduction in the plasma concentration ratio of f-TRP to BCAA before and during exercise. The magnitude of this plasma amino acid response was similar to (Chapter 3), or greater than (Chapter 4) that observed by Mittleman and colleagues (1998). In accordance with Mittleman’s findings, supplementation produced no effect on cardiovascular and thermoregulatory measures or perception of effort and thermal
stressed. In addition, the ingested amino acids did not markedly alter blood glucose and lactate, or plasma FFA concentrations. Although plasma NH$_3$ concentrations were elevated following BCAA ingestion throughout exercise in Chapter 4, the levels measured were not thought to be high enough to negatively influence cerebral or muscle metabolism (Gibala, 2001). As the first BCAA study (Chapter 3) employed a similar supplementation regimen and exercise was performed under comparable environmental conditions to this previous work, it is possible that the failure to observe any ergogenic benefit in the present study may have been related co-ingestion of BCAA with CHO and / or the relatively short duration of exercise.

The ingestion of CHO has been reported to independently limit the development of central fatigue by attenuating the exercise-induced increase in plasma FFA and f-TRP concentrations, consequently producing a dose-dependent suppression of the plasma concentration ratio of f-TRP to BCAA (Davis et al., 1992). The Mittleman study used a polydextrose (a low-energy sugar substitute) placebo, whereas subjects in the present study ingested between 65 – 130 g CHO depending on exercise time. Despite this apparent difference between protocols, the plasma concentration ratio of f-TRP to BCAA during the placebo trials were remarkably similar, perhaps suggesting that the ingestion of this quantity of CHO was not sufficient to significantly alter circulating plasma amino acids. Although, plasma FFA concentrations at exhaustion in the present study were lower than those reported previously by Mittleman and co-workers (1998), possibly as a result of the shorter exercise duration and the ingestion of CHO.

Furthermore, the relatively short duration of exercise before fatigue (~60 minutes), may have been an important factor in the failure to observe a difference in exercise capacity. Recent evidence suggests that increased activity of the serotonergic neurones may only become important in the development fatigue when the duration of exercise is greater than two hours (Nybo et al., 2003). This suggestion is partially supported by the observation that extracellular 5-HT concentrations were only elevated following 60 minutes treadmill exercise (Gomez-Merino et al., 2001a), but this delayed response was only when the animals were fed prior to exercise (Meeusen et al., 1996). Time to fatigue in the study conducted by Mittleman et al. (1998) was between 130 and 170 minutes, compared to times of between 43 and 101 minutes recorded in Chapter 3. The increased exercise intensity resulted in a elevated heat.
production and a significant elevation in core temperature resulting in a relatively short exercise time.

To further investigate the influence of BCAA ingestion on the capacity to perform prolonged exercise in a warm environment, a second study was undertaken using a modified protocol to further explore the positive findings of Mittleman et al. (1998). As a time delay may exist between changes in peripheral amino acid availability and the cerebral uptake of f-TRP (Curzon and Knott, 1974), the pre-exercise ingestion period was extended from 45 minutes (as employed in Chapter 3) to 120 minutes. By reducing the exercise intensity (to 50 % \( \dot{V}O_2 \) peak) and ambient temperature (to 30 °C) exercise time was significantly extended (placebo 103.9 ± 26.9 min; BCAA 111.0 ± 29.2 min; range 59 to 157 minutes). In addition, exercise was preceded by an exercise and dietary regimen designed to reduce glycogen availability and effectively simulate the metabolic conditions typically found late in exercise to accelerate changes in serotonergic neurotransmission.

Again, the findings of this study fail to support an effect of BCAA ingestion in delaying fatigue during prolonged exercise in a warm environment, but there was some evidence of individual sensitivity to the supplementation. In the light of the results of Mittleman et al. (1998), it is not clear why no significant benefit was apparent in this second BCAA study (Chapter 4). While evidence for the involvement of central 5-HT in the development of fatigue during prolonged exercise in the heat is limited (Strachan, 2002), the results of Chapter 4 suggest that fatigue was delayed in some subjects following the supplementation regimen, whereas others exhibited little or no effect. The enhancement of exercise capacity in these individuals was greater than the calculated day-to-day variability in the exercise protocol (CV = 12.7 %). This may indicate that a degree of individual variation exists in the sensitivity to manipulation of central neurotransmission. Support for this suggestion is provided by recent work examining the cerebral balances of amino acid precursors during exercise (Nybo et al., 2003). A net uptake of TRP across the brain following moderate-intensity exercise was apparent in only 50 % of subjects, despite a marked elevation in plasma f-TRP. Additionally, it is recognised that platelet 5-HT transporter density (Strachan and Maughan, 1998; Struder et al., 1999) and the prolactin response to a
buspirone challenge (Bridge et al., 2003b; Jakeman et al., 1994; Marvin et al., 1998) vary widely between individuals.

A major limitation when interpreting the findings of precursor loading / manipulation studies is the degree of uncertainty regarding the fate of cerebral uptake. This makes it almost impossible to evaluate whether the manipulations have produce the desired effect on the cerebral TRP balance and whether changes in precursor uptake translate into functional alterations to extracellular neurotransmitter release. While recent data from Nybo et al. (2003) failed to detect a difference in TRP uptake between environmental conditions this does not rule out a possible upregulation of the serotonergic system when exercising in a warm environment. The exchange between arterial and venous blood across the brain does not give an indication of rates of synthesis and the balance between monoamine storage and synaptic release, particularly as it is becoming clear that precursor availability is not the sole determinant of neurotransmitter synthesis (Bequet et al., 2002; Cooper et al., 2003). Although this work is novel and informative, it is important to note that these data are several steps removed from the site of action (5-HT neurons) resulting in conclusions based largely on a degree of speculation. This is a common feature of all central fatigue research (Davis and Bailey, 1997).

The work described in Chapter 5 examined the magnitude and time-course of changes in plasma amino acids at rest following meals containing added CHO or fat. In contrast to a number of previous reports (Fernstrom et al., 1975; Fernstrom and Wurtman, 1971; Pan et al., 1982) both meals failed to significantly alter the plasma concentration ratio of f-TRP to BCAA or total-TRP to BCAA compared to pre-ingestion values. It appears that the presence of a small quantity of protein in both meals limited any change to circulating concentrations of BCAA and TRP, although the protein content of the meals was significantly smaller than that administered in investigations examining the addition of protein to meals (Fernstrom and Wurtman, 1972; Paul et al., 1996). The ingestion of CHO-containing meals has been previously reported to alter mood and mental function (Spring et al., 1986; Young, 1991), but in the light of the amino acid data, it is not surprising that the meals failed to alter subjective feelings of tiredness, alertness and concentration.
The effectiveness of nutritional strategies at altering central neurotransmission and consequently mood, mental function and perception of fatigue has been questioned. Fernstrom (2000) has suggested that dietary manipulation is unlikely to produce significant alterations to neurotransmission, particularly compared to the magnitude of change possible following the administration of pharmacological agents, including SSRIs. Feeding studies in monkeys have demonstrated that a 13- to 26-fold change in the plasma concentration ratio of f-TRP to BCAA was necessary to induce a change in central serotonergic activity (Leathwood and Fernstrom, 1990). Clearly changes of this magnitude have not been possible with the strategies employed in Chapters 3, 4 and 5 of this thesis, but it should be noted that these observations were primarily based on brain homogenate preparations. Pre-exercise infusion of valine (Gomez-Merino et al., 2001b) and TRP (Meeusen et al., 1997) has been demonstrated to limit and amplify the exercise-induced increase in extracellular 5-HT concentrations respectively. The apparent discrepancy between these human and animal findings may be potentially explained by differences in the regulation of central neurotransmitter homostasis between species.

8.3 - Importance of catecholaminergic neurotransmission to exercise performance in a warm environment

In a follow up to the studies presented in Chapters 3 and 4, the role of catecholaminergic neurotransmission in the development of fatigue during prolonged exercise was investigated. There appears to be a plethora of evidence accumulating to support the involvement of the catecholaminergic neurotransmitters in the fatigue process. Early animal studies suggest that prolonged exercise (Bailey et al., 1992) and exposure to stress (Palkovits et al., 1975) may partially deplete DA in some brain regions, potentially contributing to the development of fatigue (Davis, 2000). The attainment of high core temperatures has been associated with reduced motivation and drive to continue exercise (Bruck and Olschewski, 1987; Nielsen, 1992; Nielsen and Nybo, 2003) and a relationship between a measure of dopaminergic function and tolerance to exercise in a warm environment has been reported (Bridge et al., 2003b). Additional support for an involvement of catecholamines in the development of
Chapter 6 examined the effect of an acute dose of bupropion, a dual DA / NA reuptake inhibitor, on the performance of a preloaded time trial under temperate (18 °C) and warm (30 °C) ambient conditions. Subjects completed the target amount of work 3.4 minutes quicker following the bupropion administration, representing a 9% improvement in performance. The failure to observe a performance benefit at 18 °C, is consistent with the suggestion that central neurotransmission plays a limited role in the development of fatigue under temperate environmental conditions.

The drug did not alter the cardiovascular or thermoregulatory response to exercise in the heat during the initial 60 minute bout of fixed-load exercise. As the time taken to complete the TT is directly related to the power output maintained during this period, it is clear that the drug enabled subjects to maintain a higher workload with no apparent difference in perceived exertion and thermal stress. This latter point is interesting as core temperature during the bupropion trial was significantly higher towards the end of the TT, almost certainly as a direct consequence of the elevated heat production. At the completion of the TT protocol, two (of nine) subjects attained a rectal temperature of 40.0 °C or greater during the placebo trial in the warm, whereas seven subjects breached this value when bupropion was administered. This could indicate that bupropion enabled subjects to push into a potential ‘danger zone’ without any negative feedback from the CNS. The mechanism behind the difference in core temperature apparent between treatments in temperate conditions is not clear at present.

These findings appear to support the suggestion that increased catecholaminergic activity is important in the tolerance to exercise in a warm environment (Bridge et al., 2003b), and that the maintenance of low central ratio of 5-HT to DA is associated with feelings of arousal and motivation, consequently favouring increased performance (Davis and Bailey, 1997). As serotonergic and catecholaminergic projections innervate areas of the hypothalamus, a decrease in the ratio of 5-HT to DA activity may be expected to delay fatigue when core temperature is elevated (Gandevia, 2001). Bupropion administration in the heat may have acted on central DA...
and NA neurotransmission to maintain motivation and arousal, effectively dampening or overriding inhibitory signals arising from the CNS to cease exercise due to hyperthermia. This enabled the subjects to continue to sustain a high power output despite approaching, and in some cases breaching, the critical core temperature proposed by Nielsen et al (1993). The concept of critical core temperature will be discussed in Section 8.5.

The potential danger of this situation was apparent during the 1967 Tour de France cycle race, where the successful British cyclist Tom Simpson collapsed and died from heat-related illness on the Mont Ventoux climb after taking amphetamines to enhance his performance. This may be important as bupropion has an amphetamine-like structure, acting centrally through similar neurochemical mechanisms (e.g. inhibiting catecholamine reuptake), although at a lower potency.

Exercise in the heat significantly increases plasma prolactin release (Bridge et al., 2003a; Pitsiladis et al., 2002; Radomski et al., 1998; Strachan, 2002), a response that was apparent in Chapters 4 and 6. As plasma prolactin has been widely employed as a peripheral index of central 5-HT activity (Struder and Weicker, 2001; Van de Kar, 1997), that has led to the suggestion that serotonergic activity may be upregulated under conditions of heat stress (Pitsiladis et al., 2002). Recent evidence suggests that the regulation of prolactin release is complex, so this assumption may not be correct (Meeusen et al., 2001; Piacentini, 2003). Given that the hypothalamus is the central regulator of both core temperature and pituitary hormone secretion, it has been suggested that an increase in brain temperature may directly result in the induction of hormone release during exercise (Radomski et al., 1998). The present results appear to support this suggestion, with not only a progressive increase in plasma prolactin apparent throughout exercise at 30 °C, but also a significant increase at the end of the TT at 18 °C when core temperature was also elevated above 39.0 °C. Elevated mean skin temperature has also been proposed as a stimulus for prolactin release, modulated through peripheral temperature receptors (Bridge et al., 2003a). In contrast to these findings plasma prolactin continued to increase throughout exercise under both temperate and warm conditions despite weighted mean skin temperature remaining relatively stable.
Chapter 8

General Discussion

At present the central response to acute doses of drugs influencing central neurotransmission is not clear in humans; this is clearly an issue for all studies employing pharmacological agents to manipulate central neurotransmission. While there is good evidence from animal studies employing brain microdialysis to suggest that extracellular concentrations of both DA and NA are elevated following bupropion administration (Piacentini et al., 2003), the effects in humans may not be so clear cut particularly as the metabolism of bupropion is different between rats and humans (see Chapter 6 for details). While there does not appear to be information regarding the acute effects of agents influencing DA and NA, evidence from clinical studies suggests that an acute dose of a 5-HT reuptake inhibitor (SSRI) may produce little change in extracellular neurotransmitter concentrations, due to a reduction in cell firing rate caused by autorreceptor-mediated feedback inhibition (Artigas et al., 1996). This is reflected by the observation that between 4-6 weeks SSRI administration is typically required to observe a therapeutic benefit in depressive patients. However, at this stage it is not clear how combined exposure to exercise, heat stress and a pharmacological agent influences central neurotransmission through the control of neurotransmitter release, reuptake and metabolism.

8.4 – BBB permeability during exercise

The suggestion that the BBB may be compromised during prolonged exercise is not new. Work conducted by Sharma and colleagues (1991, 1996) demonstrated a transient breakdown in barrier function following 30 minutes of forced swimming in rats. This response was attributed to increased brain 5-HT, with the administration of agents to reduce serotonergic activity found to abolish this response. As strenuous exercise produces a widespread disturbance of physiological homeostasis, there are a number of factors that may contribute to a change in BBB permeability. These include the development of hyperthermia (Sharma and Dey, 1987; Wijsman and Shivers, 1993), increased circulating ammonia (Mans et al., 1983) and adrenaline (Abdul-Rahman et al., 1979) concentrations and the production of pro-inflammatory cytokines (de Boer and Breimer, 1998). At present the importance of changes in BBB permeability during exercise is not clear. A small increase in barrier permeability during exercise may be desirable to facilitate the transport of metabolites, such as
glucose and lactate, and other substances into the CNS when cerebral blood flow is elevated. However, a marked disturbance in BBB function during exercise may adversely affect normal brain function and contribute to the development of central fatigue by increased brain-blood interfacing.

The work presented in Chapter 7 investigated changes in serum S100b following combined water immersion and prolonged exercise in temperate and warm environments. A significant elevation in serum S100b was apparent following exercise in the warm environment, with no such response apparent in the temperate trial. No subject exceeded a serum S100b concentration of 0.34 µg.L⁻¹ at the end of exercise. This value was proposed as the maximal value to suggest BBB opening by Marchi and colleagues (2003), with levels greater than this associated with neuronal damage. In the light of the findings of Kapural and co-workers (2002), these data may indicate that BBB permeability is increased during exercise in the heat, potentially playing an important role in the mechanisms of fatigue that operate in prolonged exercise. Disturbance in BBB function is often associated with impaired brain function, potentially impacting on the operation of the organism as a whole (Sharma and Hoopes, 2003). A marked change in BBB permeability during exercise may limit an individual’s capacity to perform prolonged exercise by modifying the transport kinetics of neurotransmitter precursors and other metabolites or allowing the accumulation of unwanted substances in the CNS.

While serum S100b is now widely employed in a clinical setting as a diagnostic tool for the assessment of brain damage/injury (see Chapter 1) and more recently BBB dysfunction (Marchi et al., 2003), a number of factors must be first be considered when interpreting these data. There is the possibility that changes in serum levels of this protein may have arisen from extra-cerebral sources (e.g. bone and adipose tissue), although this is doubtful as it appears that significant tissue trauma is necessary for S100b to be liberated into the circulation (Anderson et al., 2001; Pelinka et al., 2003). As S100 proteins are cleared from the peripheral circulation by the kidneys, with a serum half-life of around 2 hours (Ali et al., 2000), the change observed following exercise may have resulted from reduced renal clearance. Prolonged exercise, in particular under conditions of heat stress, is associated with a
marked reduction in renal blood flow (Rowell, 1974), but there is some evidence that extraction of protein remains high during strenuous exercise (Castenfors et al., 1967). Follow up studies may benefit from the measurement of urinary S100b at rest and after exercise to confirm the effects of exercise on S100b clearance. Additionally, small openings in the BBB may not allow CNS-specific proteins to leak into the peripheral circulation. Thus, it is possible that subtle changes in BBB permeability may not be detected using this technique (Bailey et al., 2004).

Despite these considerations, serum S100b is now being employed as an index of brain trauma occurring during sporting competition (Mussack et al., 2003; Otto et al., 2000). These results indicate that an increase in serum concentration S100b is apparent following exercise at high ambient temperatures, which could result in an incorrect assessment of brain trauma in exercising individuals.

8.5 – Evidence for critical core temperature

Attainment of a critical core temperature has been suggested to result in a loss of drive to continue exercise, potentially serving as a CNS-mediated protective mechanism limiting further heat production (Nielsen et al., 1993; Nielsen and Nybo, 2003). Work conducted by Gonzalez-Alonso et al. (1999) reported an inverse relationship between core temperature at the outset of exercise and time to exhaustion, with fatigue occurring when oesophageal temperature reached ~40.0 °C. Additionally, exercising goats reduced their speed, or refused to move, when the brain temperature was increased to > 42 °C, when body temperature was maintained at 40 °C (Caputa et al., 1986). While there appears to be good evidence in support of this hypothesis, some individuals fatigue during exercise in the heat long before attaining values of 39.5 °C (Sawka et al., 1992). During the studies reported in Chapters 3 and 4, there was a considerable variation in the rectal temperature at fatigue (38.6 – 40.1 °C) with good agreement apparent between the values attained in the placebo and BCAA trials. The well-trained cyclists recruited in the bupropion study (Chapter 6) reached temperatures of between 39.4 to 40.4 °C at the end of the TT. Some of the variation between studies may be attributed to the intimate knowledge of their upper limits in trained cyclists (Chapter 6) compared to the physically active volunteers studied in
Chapters 3 and 4. Differences in the protocol employed between studies may also account for some of this discrepancy. Motivation to continue exercise is different when there is a defined end-point to focus on (e.g. completion of a TT), compared to an open-ended test such as a fixed-intensity ride to volitional exhaustion.

A wide variety of methods have been employed as an index of core temperature, including rectal, oesophageal, tympanic, and more recently the use of a telemetry pill (Moran and Mendal, 2002; Saltin and Hermansen, 1966). When considering the influence of hyperthermia on the CNS, brain temperature, particularly the temperature of the blood perfusing the anterior hypothalamus, is clearly the most important factor. Venous heat removal from the brain is reduced during exercise with hyperthermia (Nybo et al., 2002). This coupled with an increased brain heat production has been demonstrated to result in an elevation in cerebral heat storage. This response was not altered by cooling of the head, despite this intervention reducing facial skin and tympanic temperatures. The results of this study suggest that brain temperature is thought to be maintained at least 0.2 °C above oesophageal temperature during exercise as a result of inadequate heat release. The exercise studies described in this thesis (Chapters 3, 4, 6 and 7) employed rectal temperature as an index of core temperature. This method is widely employed and has been demonstrated to give an accurate representation of the temperature of the body's core during prolonged exercise, despite a lag in response time (Moran and Mendal, 2002). Rectal temperature during exercise has been reported to be slightly higher (~0.1 °C) than oesophageal measures (Moran and Mendal, 2002; Saltin and Hermansen, 1966) and may therefore give a reasonable representation of brain temperature during sustained constant-load exercise, although the dynamics of change are expected to be markedly different.

8.6 – Summary and conclusions

The balance of evidence suggests that neurobiological mechanisms of central fatigue are not important in trained subjects during prolonged exercise in temperate conditions, with muscle glycogen depletion thought to limit exercise capacity. There is, however, some evidence that serotonin-mediated fatigue may be important in ultra-
endurance events, where exercise is performed at a low intensity for many hours (Nybo et al., 2003). This is not to suggest that the brain has no role in fatigue whilst exercising in temperate conditions. Psychology can influence exercise performance and ultimately an individual has to make a conscious decision to cease exercise (Kayser, 2003). When exercise is performed in high ambient temperatures, the development of central fatigue is thought to be accelerated, leading to a loss of drive to continue (Bruck and Olschewski, 1987; Nielsen, 1992; Nielsen and Nybo, 2003). Exercise-induced hyperthermia has been demonstrated to reduce sustained muscle activation (Nybo and Nielsen, 2001a), alter brain activity (Nielsen et al., 2001) and increase perceived exertion (Armada-Da-Silva et al., 2004; Nybo and Nielsen, 2001b). The studies contained in this thesis attempted to uncover some of neurobiological mechanisms behind these responses and to examine strategies to prolong exercise in a warm environment.

The results of Chapters 3 and 4 fail to support the suggestion that BCAA ingestion before and during exercise can delay the development of fatigue in a warm environment. It is doubtful that a single neurotransmitter system is uniquely responsible for the development of central fatigue as originally proposed by Newsholme and colleagues (1987). While 5-HT is implicated in virtually all physiological and psychological processes, it is often found to be ultimately responsible for nothing (Jacobs and Azmitia, 1992). Its wide distribution throughout the brain and regular discharge pattern suggests that its importance lies in exerting a modulatory influence on other monoamine systems (Cooper et al., 2003; Gandevia, 2001), and in this way it may indirectly influence exercise performance.

Dopamine and noradrenaline play a crucial role in motor control, arousal and motivation and have been proposed to be important in fatigue (Davis and Bailey, 1997) and the tolerance to exercise in warm conditions (Bridge et al., 2003b). The results of chapter 6 suggest that these monoamine systems are important in the development of central fatigue during exercise in the heat, and may dampen inhibitory signals from the CNS to reduce heat production, allowing performance to be maintained. On the basis of the present results and those of others (Bridge et al., 2003b) DA and NA are emerging as important mediators of central fatigue during prolonged exercise in the heat.
While mechanisms of fatigue involving central neurotransmission have been widely investigated in recent years, potential changes in BBB function during exercise have received relatively little attention. The appearance S100b, a CNS-specific protein, in the peripheral circulation has recently been explored as a peripheral marker of BBB integrity. Water immersion and exercise in warm conditions produced a marked increase in serum S100b, suggesting that BBB permeability may be increased (Chapter 7). It is important to recognise the limitations of this peripheral marker, and further work is required to clarify the significance of changes in circulating S100b during exercise.

Attempts to simplify the complexity of the involvement of CNS in behavioural and physiological responses rarely meet with success (Fernstrom et al., 1983). Neurotransmitter systems acting within the CNS are not mutually exclusive and it is probable that a complex interaction between neurotransmitters and changes in BBB function, as well as factors not considered in this thesis such as brain glycogen depletion (Dalsgaard et al., 2002; Nybo et al., 2003), all contribute to the development of fatigue during prolonged exercise in a warm environment.

Key points:

1) In contrast to the findings of Mittleman and co-workers (1998), acute BCAA ingestion does not appear to influence exercise capacity in a warm environment.

2) Acute bupropion administration improved exercise performance in the heat, with no effect evident in temperate conditions. This response was attributed to the maintenance of motivation to continue exercise despite the attainment of high core temperatures.

3) Serum S100b was increased following water immersion and exercise under warm conditions. These findings suggest that BBB permeability is disrupted, although there are a number of factors to consider when interpreting these results.
References


Cooper, B. R., Wang, C. M., Cox, R. F., Norton, R., Shea, V., and Ferris, R. M. (1994): Evidence that the acute behavioral and electrophysiological effects of
bupropion (Wellbutrin) are mediated by a noradrenergic mechanism. Neuropsychopharmacology 11, 133-41.


venous blood: incomplete arterialization and alterations in glucagon responses.


Appendix I – Data contained in figures throughout the thesis

The data contained in this appendix is the numerical data presented in the figures displayed throughout the thesis. All data is presented at mean ± standard deviation unless otherwise stated.

Chapter 3 – Branched-chain amino acid supplementation during prolonged exercise in a warm environment

Figure 3.1: Individual exercise times to exhaustion (minutes) and percent changes in exercise capacity between the placebo and BCAA trials

<table>
<thead>
<tr>
<th>Subject</th>
<th>Placebo</th>
<th>BCAA</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60.6</td>
<td>62.4</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>100.7</td>
<td>90.5</td>
<td>-11.3</td>
</tr>
<tr>
<td>3</td>
<td>51.7</td>
<td>55.0</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>52.8</td>
<td>75.7</td>
<td>30.2</td>
</tr>
<tr>
<td>5</td>
<td>53.0</td>
<td>53.2</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>45.1</td>
<td>49.9</td>
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<tr>
<td>7</td>
<td>75.9</td>
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</tr>
<tr>
<td>8</td>
<td>46.7</td>
<td>43.4</td>
<td>-7.6</td>
</tr>
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</table>

Mean±SD 60.8 ± 18.8 62.8 ± 15.7 3.1 ± 13.0

Figure 3.2: Plasma BCAA concentration (μmol.L⁻¹) at rest and during exercise

<table>
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<tr>
<th></th>
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<th>45</th>
<th>Ex</th>
</tr>
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<tr>
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<td>422 ± 117</td>
<td>370 ± 125</td>
<td>393 ± 120</td>
<td>354 ± 109</td>
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<tr>
<td>BCAA</td>
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<td>1287 ± 252</td>
<td>984 ± 200</td>
<td>1146 ± 399</td>
<td>1231 ± 407</td>
<td>1166 ± 511</td>
</tr>
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</table>
Figure 3.3: Change in core ($T_{core}$) and weighted mean skin ($T_{skin}$) temperature ($^\circ$C) at rest and during exercise.

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<td>$T_{skin}$</td>
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Figure 3.4: The heart rate (b.min$^{-1}$) response at rest and during exercise in a warm environment.

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Figure 3.5: Change in RPE and thermal stress during the placebo and BCAA trials.

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<td></td>
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<td>2 ±1</td>
<td>3 ±1</td>
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</table>

Figure 3.6: Blood glucose (mmol.L$^{-1}$) and lactate (mmol.L$^{-1}$) concentrations at rest and during exercise.

<table>
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<tr>
<td>Glucose</td>
<td>Placebo</td>
<td>5.6 ±0.4</td>
<td>6.3 ±0.8</td>
<td>3.7 ±0.4</td>
<td>5.0 ±0.8</td>
<td>6.0 ±0.9</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>5.5 ±0.4</td>
<td>6.0 ±0.7</td>
<td>3.6 ±0.2</td>
<td>4.9 ±0.4</td>
<td>5.5 ±0.7</td>
</tr>
<tr>
<td>Lactate</td>
<td>Placebo</td>
<td>1.2 ±0.2</td>
<td>2.0 ±0.2</td>
<td>3.3 ±2.0</td>
<td>3.8 ±2.0</td>
<td>3.2 ±0.7</td>
</tr>
<tr>
<td></td>
<td>BCAA</td>
<td>1.0 ±0.5</td>
<td>1.9 ±0.3</td>
<td>3.2 ±2.2</td>
<td>3.6 ±2.2</td>
<td>3.4 ±1.4</td>
</tr>
</tbody>
</table>
Chapter 4 – Branched-chain amino acid supplementation during prolonged exercise in a warm environment after a regimen designed to reduce muscle glycogen

Figure 4.1: Individual exercise times to exhaustion (A; minutes) and percent changes in exercise capacity (B) between the placebo and BCAA trials.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Placebo</th>
<th>BCAA</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>106.7</td>
<td>106.3</td>
<td>-0.4</td>
</tr>
<tr>
<td>2</td>
<td>88.4</td>
<td>109.9</td>
<td>24.3</td>
</tr>
<tr>
<td>3</td>
<td>113.3</td>
<td>103.8</td>
<td>-9.2</td>
</tr>
<tr>
<td>4</td>
<td>80.7</td>
<td>78.4</td>
<td>-2.9</td>
</tr>
<tr>
<td>5</td>
<td>58.1</td>
<td>71.1</td>
<td>22.4</td>
</tr>
<tr>
<td>6</td>
<td>123.6</td>
<td>144.5</td>
<td>16.9</td>
</tr>
<tr>
<td>7</td>
<td>117.3</td>
<td>117.1</td>
<td>-0.2</td>
</tr>
<tr>
<td>8</td>
<td>143.0</td>
<td>156.6</td>
<td>9.5</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>103.9±26.9</td>
<td>111.0±29.2</td>
<td>7.6±12.6</td>
</tr>
</tbody>
</table>

Figure 4.2: Plasma BCAA (mean ± SD; μmol.L⁻¹), f-TRP (median ± range; μmol.L⁻¹) concentrations at rest and during exercise.

<table>
<thead>
<tr>
<th></th>
<th>-120</th>
<th>-60</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>535</td>
<td>492</td>
<td>475</td>
<td>504</td>
<td>496</td>
<td>505</td>
<td>499</td>
<td>497</td>
</tr>
<tr>
<td>± 128</td>
<td>± 91</td>
<td>± 91</td>
<td>± 87</td>
<td>± 104</td>
<td>± 124</td>
<td>± 101</td>
<td>± 84</td>
<td></td>
</tr>
<tr>
<td>BCAA</td>
<td>531</td>
<td>1298</td>
<td>1601</td>
<td>1169</td>
<td>1211</td>
<td>1300</td>
<td>1454</td>
<td>1653</td>
</tr>
<tr>
<td>± 117</td>
<td>± 184</td>
<td>± 185</td>
<td>± 173</td>
<td>± 207</td>
<td>± 151</td>
<td>± 155</td>
<td>± 128</td>
<td></td>
</tr>
<tr>
<td>f-TRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>BCAA</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>12</td>
</tr>
</tbody>
</table>
**Figure 4.3:** Changes in the plasma concentration ratio of f-TRP to BCAA (median ± range) at rest and during exercise.

<table>
<thead>
<tr>
<th></th>
<th>-120</th>
<th>-60</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.009</td>
<td>0.011</td>
<td>0.011</td>
<td>0.010</td>
<td>0.013</td>
<td>0.013</td>
<td>0.016</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>(0.005 - 0.010)</td>
<td>(0.007 - 0.015)</td>
<td>(0.006 - 0.015)</td>
<td>(0.007 - 0.015)</td>
<td>(0.009 - 0.015)</td>
<td>(0.011 - 0.021)</td>
<td>(0.011 - 0.024)</td>
<td>(0.016 - 0.024)</td>
</tr>
<tr>
<td>BCAA</td>
<td>0.010</td>
<td>0.003</td>
<td>0.002</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
<td>0.007</td>
</tr>
</tbody>
</table>
|          | (0.005 - 0.015) | (0.002 - 0.015) | (0.003 - 0.015) | (0.003 - 0.015) | (0.003 - 0.015) | (0.003 - 0.015) | (0.006 - 0.015) | (0.012)

**Figure 4.4:** Change in core (A) and weighted mean skin (B) temperature (°C) at rest and during exercise.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_core</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>37.3</td>
<td>37.4</td>
<td>37.8</td>
<td>38.0</td>
<td>38.2</td>
<td>38.3</td>
<td>38.4</td>
<td>38.9</td>
</tr>
<tr>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.5</td>
</tr>
<tr>
<td>BCAA</td>
<td>37.3</td>
<td>37.4</td>
<td>37.8</td>
<td>38.0</td>
<td>38.2</td>
<td>38.3</td>
<td>38.4</td>
<td>38.9</td>
</tr>
<tr>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.5</td>
</tr>
<tr>
<td>T_skin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>33.0</td>
<td>34.6</td>
<td>35.4</td>
<td>35.6</td>
<td>35.7</td>
<td>35.8</td>
<td>35.9</td>
<td>36.1</td>
</tr>
<tr>
<td>±0.7</td>
<td>±0.6</td>
<td>±0.6</td>
<td>±0.6</td>
<td>±0.5</td>
<td>±0.6</td>
<td>±0.5</td>
<td>±0.4</td>
<td>±0.6</td>
</tr>
<tr>
<td>BCAA</td>
<td>32.9</td>
<td>34.4</td>
<td>35.3</td>
<td>35.6</td>
<td>35.6</td>
<td>35.6</td>
<td>35.6</td>
<td>35.8</td>
</tr>
<tr>
<td>±0.6</td>
<td>±0.4</td>
<td>±0.3</td>
<td>±0.3</td>
<td>±0.4</td>
<td>±0.4</td>
<td>±0.4</td>
<td>±0.4</td>
<td>±0.7</td>
</tr>
</tbody>
</table>

**Figure 4.5:** Heart rate (beats.min⁻¹) at rest and during exercise.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>58 ± 10</td>
<td>126 ± 13</td>
<td>130 ± 10</td>
<td>133 ± 9</td>
<td>137 ± 6</td>
<td>139 ± 9</td>
<td>141 ± 10</td>
<td>156 ± 15</td>
</tr>
<tr>
<td>BCAA</td>
<td>56 ± 8</td>
<td>120 ± 10</td>
<td>127 ± 11</td>
<td>129 ± 10</td>
<td>136 ± 10</td>
<td>138 ± 9</td>
<td>142 ± 11</td>
<td>159 ± 13</td>
</tr>
</tbody>
</table>
Figure 4.6: Rating of perceived exertion (RPE) and thermal stress (TS) during exercise.

<table>
<thead>
<tr>
<th></th>
<th>RPE Placebo</th>
<th>RPE BCAA</th>
<th>TC Placebo</th>
<th>TC BCAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
<td>8 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>20</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
<td>9 ± 1</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>30</td>
<td>13 ± 1</td>
<td>12 ± 1</td>
<td>10 ± 1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>40</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
<td>10 ± 1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>50</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
<td>11 ± 2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>60</td>
<td>19 ± 1</td>
<td>19 ± 1</td>
<td>11 ± 2</td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>

Figure 4.7: The relationship between the change in plasma FFA (mmol.L\(^{-1}\)) and plasma f-TRP (μmol.L\(^{-1}\)) during the placebo and BCAA trials.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Placebo</th>
<th>Placebo</th>
<th>BCAA</th>
<th>BCAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔFFA</td>
<td>Δf-TRP</td>
<td>ΔFFA</td>
<td>Δf-TRP</td>
</tr>
<tr>
<td>1</td>
<td>1.24</td>
<td>21</td>
<td>1.18</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>0.35</td>
<td>6</td>
<td>0.58</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>1.06</td>
<td>11</td>
<td>0.85</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>7</td>
<td>0.58</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>0.45</td>
<td>10</td>
<td>0.33</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>0.39</td>
<td>6</td>
<td>0.44</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>1.06</td>
<td>14</td>
<td>0.87</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>0.66</td>
<td>5</td>
<td>0.71</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 4.8: Plasma NH\(_3\) (mmol.L\(^{-1}\)) during the placebo and BCAA trials.

<table>
<thead>
<tr>
<th></th>
<th>-120</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>69.5</td>
<td>68.5</td>
<td>83.8</td>
<td>85.5</td>
<td>87.6</td>
<td>84.4</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td>±24.3</td>
<td>±19.5</td>
<td>±22.6</td>
<td>±39.1</td>
<td>±33.8</td>
<td>±24.7</td>
<td>±17.7</td>
</tr>
<tr>
<td>BCAA</td>
<td>63.6</td>
<td>84.2</td>
<td>120.0</td>
<td>123.5</td>
<td>126.8</td>
<td>135.2</td>
<td>134.7</td>
</tr>
<tr>
<td></td>
<td>±14.9</td>
<td>±26.2</td>
<td>±27.3</td>
<td>±14.5</td>
<td>±20.2</td>
<td>±13.8</td>
<td>±6.8</td>
</tr>
</tbody>
</table>

Figure 4.9: Plasma prolactin concentration (IU.L\(^{-1}\)) at rest and at the point of exhaustion in the placebo and BCAA trials.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>190.0± 151.2</td>
<td>733.9± 408.6</td>
</tr>
<tr>
<td>BCAA</td>
<td>211.5± 92.1</td>
<td>775± 394.4</td>
</tr>
</tbody>
</table>
Chapter 5 – Acute changes to the plasma concentration ratio of f-TRP to BCAA following the ingestion of carbohydrate and fat rich meals in man

**Figure 5.1:** Resting plasma BCAA (A; μmol.L⁻¹) and f-TRP (B; μmol.L⁻¹) responses to the CHO and fat meals

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCAA</td>
<td>393 ± 58</td>
<td>414 ± 73</td>
<td>335 ± 61</td>
<td>335 ± 63</td>
<td>337 ± 34</td>
<td>342 ± 45</td>
</tr>
<tr>
<td>Fat</td>
<td>404 ± 45</td>
<td>422 ± 69</td>
<td>470 ± 66</td>
<td>434 ± 55</td>
<td>402 ± 43</td>
<td>403 ± 39</td>
</tr>
<tr>
<td>f-TRP</td>
<td>5 ± 2</td>
<td>6 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Fat</td>
<td>5 ± 1</td>
<td>6 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

**Figure 5.2:** Changes in the plasma concentration ratio of f-TRP to BCAA in response to the CHO and fat meals

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>0.013</td>
<td>0.015</td>
<td>0.016</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>± 0.005</td>
<td>± 0.004</td>
<td>± 0.004</td>
<td>± 0.005</td>
<td>± 0.004</td>
<td>± 0.005</td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>0.013</td>
<td>0.015</td>
<td>0.011</td>
<td>0.011</td>
<td>0.012</td>
<td>0.013</td>
</tr>
<tr>
<td>± 0.003</td>
<td>± 0.002</td>
<td>± 0.002</td>
<td>± 0.002</td>
<td>± 0.002</td>
<td>± 0.003</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.3:** Resting plasma total-TRP (A; μmol.L⁻¹) and changes in the plasma concentration ratio of total-TRP to BCAA (B) in response to the CHO and fat meals

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP</td>
<td>54 ± 10</td>
<td>59 ± 11</td>
<td>55 ± 11</td>
<td>53 ± 10</td>
<td>53 ± 9</td>
<td>52 ± 10</td>
</tr>
<tr>
<td>Fat</td>
<td>53 ± 7</td>
<td>59 ± 8</td>
<td>57 ± 6</td>
<td>53 ± 7</td>
<td>50 ± 7</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>TRP:BCAA</td>
<td>0.138</td>
<td>0.144</td>
<td>0.167</td>
<td>0.161</td>
<td>0.159</td>
<td>0.153</td>
</tr>
<tr>
<td>± 0.019</td>
<td>± 0.018</td>
<td>± 0.029</td>
<td>± 0.027</td>
<td>± 0.029</td>
<td>± 0.028</td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>0.132</td>
<td>0.143</td>
<td>0.122</td>
<td>0.122</td>
<td>0.128</td>
<td>0.119</td>
</tr>
<tr>
<td>± 0.008</td>
<td>± 0.019</td>
<td>± 0.009</td>
<td>± 0.018</td>
<td>± 0.019</td>
<td>± 0.012</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 6 – Dopamine / Noradrenalin reuptake inhibition and exercise performance in temperate and warm environments

**Figure 6.1:** Time trial performance (minutes) in the four experimental trials

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo 18 °C</td>
<td>30.6 ± 2.2</td>
</tr>
<tr>
<td>Bupropion 18 °C</td>
<td>30.6 ± 1.9</td>
</tr>
<tr>
<td>Placebo 30 °C</td>
<td>39.8 ± 3.9</td>
</tr>
<tr>
<td>Bupropion 30 °C</td>
<td>36.4 ± 5.7</td>
</tr>
</tbody>
</table>

**Figure 6.2:** Individual percent changes in exercise performance in the bupropion trial relative to the placebo trial in temperate (A) and warm (B) conditions

<table>
<thead>
<tr>
<th>Subject</th>
<th>Temperate</th>
<th>Warm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-2.0</td>
<td>-8.2</td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>4.3</td>
</tr>
<tr>
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**Figure 6.3:** Time trial power output in the Tmp (A) and Wrm (B) trials (Watts)

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Figure 6.4: Core temperature (°C) responses to the experimental protocol in Tmp (A) and Wrm (B) trials

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Figure 6.5: Weighted mean skin temperature (°C) responses to the experimental protocol in Tmp (A) and Wrm (B) conditions

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Figure 6.6: Heart rate (beats.min\(^{-1}\)) responses to the experimental protocol in Tmp (A) and Wrm (B) conditions

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203
Figure 6.7: Rating of perceived exertion during exercise in Tmp (A) and Wrm (B) conditions.

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Figure 6.8: Perceived thermal stress during exercise in Tmp (A) and Wrm (B) conditions.

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Figure 6.9: Serum prolactin concentration (ng.mL⁻¹) at rest and during exercise

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Figure 6.10: Serum cortisol concentration (ng.mL⁻¹) at rest and during exercise

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**Figure 6.11:** Plasma adrenocorticotropic hormone concentration (ng.L⁻¹) at rest and during exercise

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**Figure 6.12:** Serum growth hormone concentration (mIU.L⁻¹) at rest and during exercise

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Chapter 7 – Serum S100b, a proposed marker of blood-brain barrier permeability, and prolonged exercise in temperate and warm environments in man

**Figure 7.1:** Mean serum S100b (μg.L⁻¹) whilst at rest (0) and at the end of exercise (Ex60) in the Tmp and Wrm trials.

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<td>Tmp</td>
<td>0.06 ± 0.04</td>
<td>0.12 ± 0.13</td>
</tr>
<tr>
<td>Wrm</td>
<td>0.07 ± 0.05</td>
<td>0.19 ± 0.12</td>
</tr>
</tbody>
</table>
Figure 7.2: Change in core (A) and weighted mean skin (B) temperature (°C) at rest, during water immersion and exercise

<table>
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<th>Im20</th>
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<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
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Figure 7.3: Heart rate (beats.min<sup>-1</sup>) at rest, during water immersion and exercise

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Figure 7.4: Rating of perceived exertion during exercise

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Figure 7.5: Perceived thermal stress during water immersion and exercise

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<td>±2</td>
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</tbody>
</table>
**Figure 7.6:** Blood glucose (A; mmol.L\(^{-1}\)) and lactate (B; mmol.L\(^{-1}\)) concentrations at rest, during water immersion and exercise

<table>
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<tr>
<th></th>
<th>-5</th>
<th>0</th>
<th>Post-Im</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>Rec15</th>
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<tr>
<td>Glucose Tmp</td>
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<tr>
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<td>0.7</td>
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<td>0.4</td>
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<td>0.5</td>
<td>0.5</td>
<td>5.8 ± 0.5</td>
</tr>
<tr>
<td>Glucose Wrm</td>
<td>5.3 ± 0.7</td>
<td>5.4 ± 0.5</td>
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<td>5.4 ± 0.3</td>
<td>5.5 ± 0.4</td>
<td>5.6 ± 0.4</td>
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<td>5.8 ± 0.5</td>
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<tr>
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<td>0.9</td>
<td>0.8</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td>Lactate Tmp</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>2.5 ± 0.8</td>
<td>2.1 ± 0.7</td>
<td>1.8 ± 0.6</td>
<td>1.4 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Lactate Wrm</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>3.4 ± 0.9</td>
<td>2.9 ± 0.9</td>
<td>2.9 ± 0.9</td>
<td>2.5 ± 0.9</td>
<td>1.5 ± 0.9</td>
</tr>
</tbody>
</table>

**Figure 7.7:** Plasma free-fatty acid concentration (mmol.L\(^{-1}\)) at rest, during water immersion and exercise

<table>
<thead>
<tr>
<th></th>
<th>-5</th>
<th>0</th>
<th>Post-Im</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>Rec15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmp</td>
<td>0.31 ± 0.11</td>
<td>0.31 ± 0.11</td>
<td>0.29 ± 0.12</td>
<td>0.20 ± 0.08</td>
<td>0.24 ± 0.10</td>
<td>0.31 ± 0.14</td>
<td>0.41 ± 0.23</td>
<td>0.72 ± 0.28</td>
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<tr>
<td>Wrm</td>
<td>0.33 ± 0.15</td>
<td>0.33 ± 0.14</td>
<td>0.35 ± 0.20</td>
<td>0.25 ± 0.15</td>
<td>0.32 ± 0.19</td>
<td>0.40 ± 0.22</td>
<td>0.58 ± 0.36</td>
<td>1.13 ± 0.40</td>
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</tbody>
</table>
Appendix II - Fluorimetric determination of tryptophan

1 – Introduction

Tryptophan (TRP) is an essential amino acid found in protein-rich dietary sources (meats, nuts, milk, eggs etc). It is unique amongst amino acids as around 80 – 90% of TRP circulates in the blood loosely bound to albumin (McMenamy and Oncley, 1958), with the remaining fraction circulating in a free form (f-TRP). While tryptophan fulfils a number important functions within the body, its role in the synthesis of the neurotransmitter, serotonin has received the greatest attention. In recent years the determination of circulating concentrations of plasma TRP has been an important step in the understanding of neurochemistry and has helped elucidate the role of serotonin in a number of psychiatric disorders. Many of the methods currently employ to determine plasma TRP typically require access to sophisticated and expensive instruments. Enzymatic methods are often many times cheaper to run, but a number of problems have been encountered with these techniques.

A number of methods have been developed to measure TRP fluorimetrically, through the conversion of TRP to the fluorophore, norharman (Bloxam and Warren, 1974; Denckla and Dewey, 1967; Eccleston, 1973; Wood et al., 1977). This is achieved by heating TRP in acid conditions in the presence of varying concentrations of formaldehyde and ferric chloride. While these assay methods have been previously employed to determine TRP concentrations in a number of previous investigations, we were unable to produce consistent and physiologically valid values in our laboratory environment. This problem has been widely reported (Bloxam and Warren, 1974; Eccleston, 1973; Wood et al., 1977), and is probably the reason for the numerous revisions of the original method proposed by Denckla and Dewey (1967).

The aim of the present method was to adapt the original fluorimetric method (Denckla and Dewey, 1967), in the light of subsequent revisions, to produce consistent (low variability) values for both free and total-TRP in our current laboratory environment.
2 – Methods

**Principle.** The assay is based on the oxidative formation of norharman from tryptophan by Ferric chloride (FeCl₃) in the presence of formaldehyde. The reaction is optimised by the presence of an acid medium and exogenous heat. See Denkla and Dewey (1967) for a detailed overview of the biochemical reaction.

**Reagents and apparatus required.** All chemicals and biochemicals were obtained from Sigma (Poole, UK) or BDH (Poole, UK). A filter fluorimeter (Locarte LF 8-9: Locarte, London, UK) with standard pyrex glass cuvettes was used for the measurements. The excitation (373 nm) and emission (452 nm) wavelengths were isolated using filters.

A tryptophan stock standard (250 μmol.L⁻¹) was prepared by diluting 5.1 mg TRP in 100 mL 0.1N NH₄OH (Denckla and Dewey, 1967). Appropriate working standard solutions were prepared immediately prior to each assay run as described in Table 1.

<table>
<thead>
<tr>
<th>TRP Concentration</th>
<th>5 μmol.L⁻¹</th>
<th>10 μmol.L⁻¹</th>
<th>25 μmol.L⁻¹</th>
<th>50 μmol.L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock sol (mL)</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Diluent (mL 0.1N NH₄OH)</td>
<td>4.9</td>
<td>4.8</td>
<td>4.5</td>
<td>4.0</td>
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</tbody>
</table>

Immediately prior to performing the assay, sufficient reaction mixture to run all standards and samples in duplicate, was prepared as follows;

- per mL of 0.3N PCA: 100 μL formaldehyde, 25 μL 6 mmol FeCl₃

**Sample collection and handling.** Blood samples were collected at rest and during exercise via an indwelling venous cannula placed into a superficial forearm vein. Collected blood was immediately dispensed into tubes containing lithium heparin.
Following centrifugation to obtain plasma, 0.5 mL of plasma was placed into a tubes with a 12 kDa cellulose triacetate filter (Whatman, Maidstone UK) and spun for 2 h at ~400g in a refrigerated (4 °C) fixed angle centrifuge. The resultant ultrafiltrate and the remaining whole plasma was stored at −20 °C for determination of f-TRP and total-TRP respectively.

**f-TRP method.** 2 mL of reaction mix was dispensed into plain pyrex tubes, to which 20 μL of blank, standard or ultrafiltrate was added. The tubes were firmly capped and the thoroughly mixed before incubating at 100 ± 1 °C for 1 h. Samples were then left to cool before decanting into fluorimeter tubes to be read. The exposure of the samples, and in particular the standards, to the FeCl₃ was kept to a minimum prior to incubation (< 10 min), to reduce the loss of TRP (see the discussion).

**Total-TRP method.** 20 μL of whole (unfiltered) plasma was deproteinised in 2 mL of ice-cold reaction mixture, and spun for 10 min in a centrifuge at 4000 rev.min⁻¹. The supernatant was then dispensed into plain pyrex tubes. While the plasma samples were in the centrifuge, the standards were prepared to minimise the exposure of the standards to FeCl₃ prior to incubation. The tubes were then firmly capped and the solution thoroughly mixed before incubating at 100 ± 1 °C for 1 h. Samples were then left to cool before decanting into fluorimeter tubes to be read.

3 – Results and Discussion

The enzymatic determination of plasma free- and total-TRP has been widely employed in both clinical and exercise based literature, yet problems have been reported with existing methods. This has lead to a number of revisions to the original methods. The assay described in this appendix is primarily based on a modification of the methods proposed by Bloxam and Warren (1974) and Eccleston (1973).

Free- and total-TRP concentrations and CVs obtained using the present method were compared to values obtained using the widely employed method proposed by Bloxam and Warren (1974) and Eccleston (1973). A comparison of the results obtained are
presented in Table 2, based on the determination of 20 resting, overnight fasted samples.

Table 2: Mean free- and total-TRP concentrations and variability expressed as coefficient of variation (CV) for the Bloxam and Warren (1974), Ecclestone (1973) and present methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>f-TRP (μmol.L⁻¹)</th>
<th>TRP (μmol.L⁻¹)</th>
<th>CV f-TRP (%)</th>
<th>CV TRP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloxam and Warren</td>
<td>13.6</td>
<td>-</td>
<td>45.9</td>
<td>-</td>
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<tr>
<td>Eccleston</td>
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<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Present method</td>
<td>4.6</td>
<td>43</td>
<td>1.6</td>
<td>1.9</td>
</tr>
</tbody>
</table>

It is clear from the present results that the modification of the original method by Bloxam and Warren (1974) failed to yield values within the normal physiological range. This value may have been influenced by the large variability in the technique, with a meaningful standard curve often difficult to construct. This would also significantly reduce the sensitivity of the assay. Similar observations have been previously reported by other laboratories (Wood et al., 1977), yet this method has been routinely employed in a number of clinical and exercise based studies.

The fluorimetric TRP method originally proposed by Denkla and Dewey (1967) and subsequently modified by Bloxam and Warren (1974) was based on the conversion of TRP to norharman, by heating in acid conditions in the presence of formaldehyde and ferric chloride (FeCl₃). Briefly, samples were deproteinised in trichloroacetic acid (TCA) with formaldehyde and FeCl₃ added to the supernatant before incubation. The major problems encountered with this method relate to the use of TCA as the acid medium and the concentration of FeCl₃ (Eccleston, 1973). During the incubation period, CO₂ is liberated from TCA making it impossible to use stoppers to limit evaporative fluid losses. The original method requires this lost fluid to be replaced at the end of the heating step by adding an appropriate quantity of TCA before reading the fluorescence. This is both inconvenient and an obvious source of error. The
presence of FeCl₃ may cause quenching, causing a progressive loss of TRP (Bloxam and Warren, 1974). This response only appears to occur in the standards (plasma TRP appears to be preserved;) and may result in distortion of the calibration curve. Changes implemented by Eccleston (1973) supplemented TCA with 30% perchloric acid (PCA) added to distilled water, allowing the tubes to be capped during incubation. Thus preventing the evaporative loss and a potential source of error. The final concentration of FeCl₃ was also halved to reduce the breakdown of the TRP present in the standards, yet still producing a linear relationship between TRP concentration and fluorescence.

The present method further develops these previous methods by reducing the final concentration of PCA from 10% to 2.5%. This change in the acid medium did not appear to influence the TRP values obtained, with similar values for free- and total-TRP measured between the present method and Eccleston’s (see Table 2). The use of a more dilute PCA solution would also cut reagent costs. Additionally, a reaction mixture containing the appropriate concentrations of PCA, formaldehyde and FeCl₃ was prepared, to which the ultrafiltrate and whole plasma samples were added. This process significantly reduced the number of pipetting steps and time taken to prepare the assay, limiting the exposure of TRP to FeCl₃ prior to incubation that may influence the values obtained.

The standard curve was found to be linear to 100 μmol.L⁻¹. This assay may also be suitable for the determination of higher plasma concentrations, but these were not considered in the preparation of this method.
Appendix III – Thermal Sensation Scale

-10 Cold impossible to bear
-9
-8 Very cold, shivering hard
-7
-6 Cold, light shivering
-5
-4 Most areas of body feel cold
-3
-2 Some areas of body feel cold
-1
0 Neutral
1
2 Some areas of body feel warm
3
4 Most areas of body feel hot
5
6 Very hot, uncomfortable
7
8 Extremely hot, close to limit
9
10 Heat impossible to bear
Appendix IV - Subjective Feeling Questionnaire

Subject:  Trial:  Time:  Date:

Subjective Feeling

How tired do you feel now?

Not at all tired  Very tired

How alert do you feel now?

Not at all alert  Very alert

How well can you concentrate now?

Not at all well  Very well

How does your head feel now?

Not at all sore  Very sore