Title Over-the-counter drugs and non-febrile thermoregulation: is there cause for concern?

Name Josh Foster

OVER-THE-COUNTER DRUGS AND NON-FEBRILE THERMOREGULATION: IS THERE CAUSE FOR CONCERN?

By

Josh Foster BSc (Hons)

A thesis submitted to the University of Bedfordshire, in partial fulfilment of the requirement for the degree of Doctor of Philosophy

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Academic Thesis: Declaration of Authorship

I, Josh Foster, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

OVER-THE-COUNTER DRUGS AND NON-FEBRILE THERMOREGULATION: IS THERE CAUSE FOR CONCERN?

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ABSTRACT

Core temperature (T_c) regulation is fundamental to mammalian survival, since hypothermia ($T_c \leq$ 35°C) and hyperthermia ($T_c \geq$ 40°C) are major risk factors for health and wellbeing. The purpose of this thesis was to determine if acetaminophen, an analgesic and antipyretic drug, increased the onset of hypothermia or hyperthermia during passive cold and heat stress, respectively. It was later investigated if acetaminophen induced inhibition of cyclooxygenase mediated these side-effects.

In Study 1a, the plasma acetaminophen response to a dose of 20 mg·kg^{-1} of lean body mass was determined through enzyme linked immunosorbent assay. In Study 1b, the effect of acetaminophen administration on internal temperature (rectal; T_{re}) during a passive 2-hour mild cold (20°C, 40% relative humidity) exposure was examined. Study 1a showed that the plasma response was homogenous between subjects, reaching peak concentrations between 80-100 minutes (14 ± 4 μg·ml^{-1}). In Study 1b, acetaminophen reduced T_{re} in all participants compared with baseline, and the average peak reduction was 0.19 ± 0.09°C. In contrast, T_{re} remained stable when participants ingested a sugar placebo. Study 1 is the first experiment which confirms a hypothermic side-effect of acetaminophen in healthy humans.

Study 2 investigated whether acetaminophen augmented the rate of T_{re} rise during exposure to passive dry (45°C, 30% r.h.) and humid (45°C, 70% r.h.) heat stress for 2-hours and 45-minutes, respectively. This study showed that the rate of T_{re} rise in the dry (0.005 vs 0.006°C·min^{-1}) and humid (0.023 vs 0.021 °C·min^{-1}) conditions were similar between the acetaminophen and placebo trials ($p > 0.05$). Study 2 is the first experiment which confirms acetaminophen has no meaningful effect on thermoregulation during passive dry or humid heat exposure. Study 3 determined how the hypothermic effect of acetaminophen changes during exposure to a

thermoneutral (25°C, 40% r.h.) and cold (10°C, 40% r.h.) environment for 2-hours. In summary, there was no hypothermic effect of acetaminophen in a thermoneutral environment ($p > 0.05$), whereas T_{re} fell by 0.40 ± 0.15°C compared with baseline during cold stress ($p < 0.05$). Compared with the placebo, T_{re} was ~0.35°C lower at 120 minutes, but was significantly lower from 70-minutes. Study 3 confirmed that there is a relationship between the level of cold stress and magnitude of the hypothermic effect of acetaminophen.

Study 4 determined whether ibuprofen (400 mg), a cyclooxygenase inhibitor, reduced T_{re} during 2-hour passive cold stress (10°C, 40% r.h.) to a level comparable with acetaminophen. Ibuprofen administration did not influence T_{re}, *vastus medialis* shivering, or energy expenditure compared with a placebo throughout the cold exposure ($p > 0.05$). Taken together, this renders it unlikely that cyclooxygenase activity is required for thermogenesis induced by skin cooling. Study 4 provides evidence that acetaminophen induced hypothermia is not exclusively mediated by cyclooxygenase inhibition.

In Summary, this series of experiments has shown that acetaminophen has a hypothermic side effect in healthy humans, which is amplified during acute cold stress. Ibuprofen had no such effect on thermoregulation during cold exposure, so it is unlikely that cyclooxygenase inhibition mediates the hypothermic side-effect of acetaminophen.

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Thank you.

DEDICATION

To my parents, Kenneth & Deborah Foster**.**

PUBLICATIONS

Published works directly from this thesis are:

- **Foster, J**., Mauger, A., Thomasson, K., White, S., & Taylor, L. 2016. Effect of Acetaminophen Ingestion on Thermoregulation of Normothermic, Non-febrile Humans. *Front Pharmacol*, 7.

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Josh Foster conceived the hypothesis and drafted the manuscript. All authors contributed to manuscript revision.

- **Foster, J.,** Mauger, A.R., Govus, A., Hewson, D., Taylor, L. 2017. Acetaminophen (Paracetamol) Induces Hypothermia During Acute Cold Stress. *Clin Drug Investig*, 37:1055-1065.

Josh Foster, Lee Taylor, and Alexis Mauger contributed to conception and study design. All authors contributed to data interpretation and manuscript revision. Josh Foster collected the data.

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- **Foster, J**., Taylor, L., Chrismas, B. C., Watkins, S. L., & Mauger, A. R. 2014. The influence of acetaminophen on repeated sprint cycling performance. *Euro J App Physiol*, 114:41-48.

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ABBREVIATIONS, ACRONYMS & SYMBOLS

β	Beta
δ	Delta
%	Percentage
~	Approximately
<	Less than
>	Greater than
±	Plus-minus
≤	Equal to or less than
≥	Equal to or more than
°C	Degrees Celsius
µg	Microgram
µl	Microlitre
µm	Micrometres
^{18}FDG	2-[^{18}F]fluoro-2-deoxy-glucose
ACTH	Adrenocorticotropic hormone
AIC	Akaike Information Criterion
APAP	Acetaminophen
ATP	Adenosine triphosphate
AUDIT	Alcohol Use Disorder Identification Test
$b \cdot min^{-1}$	Beats per minute
BAC	Blood alcohol content
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CI	Confidence interval
CL	CL316243, a β3-adrenergic receptor agonist
Clo	Clothing insulation value
cm	Centimetre
COX	Cyclooxygenase
CVC	Cutaneous Vascular Conductance
DMH	Dorso-medial hypothalamus
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMG	Electromyography
g	g-force (centrifugal force)
GABA	Gamma-aminobutyric acid
Hz	Unit frequency
IBU	Ibuprofen
kg	Kilogram
kBq	Kilobecquerel
kJ	Kilojoules
LBP	Lateral parabrachial nucleus
LBPel	Lateral parabrachial nucleus, external sub-region
L-NAME	NG-nitro-L-arginine methyl ester
$m \cdot s^{-1}$	Metres per second
MAP	Mean arterial pressure
mg	Milligram
ml	Millilitres
mmol	Millimole
MnPO	Median pre-optic nucleus
MPO	Medial pre-optic area
MVC	Maximal voluntary contraction
NDMA	N-methyl-D-aspartate receptor
NHS	National Health Service
NSAID	Non-steroidal anti-inflammatory drug
O_2	Oxygen
p	Alpha value in significance testing
PAIS	Paracetamol (acetaminophen) in stroke trial
PCR	Polymerase chain reaction
PET-CT	Positron emission tomography-Computed tomography
pg	Picograms
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2

POX	Peroxidase active site within the COX enzyme
Q_{10}	Temperature coefficient
r.h.	Relative humidity
rMR	Rostral medullary raphe region
ROCK	Rho-A/Rho kinase pathway
sEMG	Surface electromyography
$\text{Shiv}_{\text{peak}}$	Peak shivering intensity
T_{c}	Core temperature
T_{re}	Rectal temperature
TRP	Transient receptor potential
TRPA	Transient receptor potential ancryn
TRPM	Transient receptor potential melastin
TRPV	Transient receptor potential vallinoid
T_{sk}	Skin temperature
TS	Thermal sensation
TXA_2	Thromboxane
UCP-1	Uncoupling protein -1
VO_2	Volume of oxygen
W	Watts
W/m^{-2}	Watts per metre squared

CHAPTER 1: GENERAL INTRODUCTION

Human internal/core temperature (T_c) is tightly regulated at an optimum of 37°C. At the lower extremes of T_c (≤35°C), ion fluxes, increases in membrane fluidity, and decreases in enzyme performance and post-translational protein synthesis can occur. While protein denaturation looms at the higher extreme of T_c (>41°C), an event which can be life threatening. In environments that are not considered thermally stressful (i.e. those that do not acutely change the global temperature of the skin), T_c is regulated entirely through subtle changes in cutaneous blood flow (Romanovsky, 2014). This method incurs virtually no metabolic cost, so is a suitable first line of defence against threats to heat balance. As the ambient temperature falls below 18°C, less fuel-efficient mechanisms are utilised for active thermogenesis (heat production), which are partitioned into shivering (skeletal muscle) and non-shivering (brown adipose tissue and skeletal muscle) mechanisms (Blondin et al., 2014). During heat stress, humans rely on heat loss mechanisms to maintain heat balance. Most notably, *homo-sapiens* are able to evaporate sweat from the skin surface, and raise the skin temperature for convective heat transfer with the environment (Marino 2008a). The autonomic adaptations described above will only arise in the absence of sufficient behavioural adaptations which are mediated if thermal comfort is compromised. These include changing clothing, adjusting the thermostat, and seeking a warmer or cooler environment (Havenith and Fiala 2015). Nevertheless, there are scenarios where these thermoregulatory adjustments are insufficient, noted by an uncompensable increase or decrease in T_c.

Heat stress is seen during exercise, occupational settings, and of course during heat waves. A simple definition may be that heat stress arises when active heat loss mechanisms are required to maintain heat balance, or a T_c of 37°C. Heat stroke, a condition caused when T_c reaches upper

extremes of ~41°C and beyond (Leon and Helwig 2010; Simpson and Abelsohn 2012), is a rare occurrence regarding mortality. In the USA, heat stroke caused 125 deaths per million people (NOAA 2013), yet there are thousands of excess deaths during heat waves (Kenney *et al.* 2014). A poignant example is that of the 2003 Europe heat wave which caused over 15,000 excess deaths in France and 70,000 excess deaths across Europe (Robine *et al.* 2008). Most of these heat wave related deaths occur in elderly people who have pre-existing cardiovascular or respiratory illnesses. For example, the Chicago heat wave in 1995 caused approximately 1,686 excess deaths in the period July 14th to July 20th where the ambient temperature peaked; only 4.7% of these deaths were linked to hyperthermia, while over 93% were due to an underlying cardiovascular cause (Kaiser *et al.* 2007). Occupational health and productivity is also negatively impacted by heat stress. There are many studies which show an increased risk of workplace injuries in people are exposed to excessive heat, and this is primarily due to an inability to concentrate in times where sensory input is dominated by an increased skin temperature and wittedness (Tawatsupa *et al.* 2013). These cognitive responses are also met with reductions in workplace productivity which is perpetuated by a greater frequency in recovery breaks. Such productivity losses have been shown to incur annual losses of ~£5 billion GBP in Australia and ~£618 million GBP in Germany (Hübler *et al.* 2008; Zander *et al.* 2015). Without question, climate change will be, and has been, paralleled by an increased frequency of hot weather events. In the last 20 years, 16 of 20 winters and 19 of 20 summers have been above average in ambient temperature (Hansen *et al.* 2010), and record high temperatures have been documented two-fold more often than record low temperatures since the year 2000 (Meehl *et al.* 2009). Even if Europe achieves their goal of reducing CO_2 emissions to 20% below the level shown in 1990 by 2020, the effects of climate change will persist for at least a further 50 years (Monjon 2016).

On the other hand, exposure to cold carries even greater risk to human health, at least at the time of writing this thesis. In the United Kingdom, the hospital admissions in which hypothermia is a primary or secondary cause outweigh that of hyperthermia. In fact, admission rates seem to be rising in this region. The National Health Service (NHS) hospital episode statistics for 2013/2014 indicate that there were 3,780 admissions to NHS hospitals where hypothermia was the primary diagnosis, and nearly 18,000 where hypothermia was the secondary diagnosis. In 2005/2006, admission numbers were 2,010 and 7,069 for primary and secondary diagnoses, respectively. The very young (0 to 4 years) and the elderly (≥ 65 years) accounted for 80% of these admissions, highlighting an impaired ability of these age groups to regulate their T_c during physiological stressors. Hypothermia is associated with many symptoms depending on the magnitude of T_c decline, which Brown et al. (2012) categorised into four stages [I (35 to 32°C), II (<32 to 28°C), III (<28 to 24°C), IV (<24°C)]. At stage I, the patient is normally responsive with a maximal increase in metabolic rate to fuel shivering thermogenesis, however, consciousness is obscured and blood pressure becomes difficult to read as T_c approaches 32°C (Parsons, 2014). At stages II and beyond, there is a high risk of cardiac abnormalities, particularly ventricular fibrillation. In stage II-IV hypothermic patients, an Osborn wave (positive deflection of the 'J' point) is normally present during echocardiography analysis (Osborn, 1953; Omar, 2016). This is an abnormality closely related to risk of fibrillation (i.e. larger Osborn wave indicates greater cardiac rhythm dysfunction). The risk of cardiovascular failure is positively correlated with decreases in T_c (Filippi et al., 2014), and survival from accidental hypothermia is unlikely when stage IV hypothermia is attained (Parsons, 2014).

There is evidence that the use of some over-the-counter drugs may impair T_c regulation during thermal stress. The Vigibase adverse drug reaction database was founded by the World Health

Organisation in 1975 and is a useful tool for initial inspection of specific drugs and risk of hypothermia or hyperthermia. In this thesis, the drugs in question (acetaminophen and ibuprofen) are known as cyclooxygenase (COX) inhibitors, since their mechanism of action stems from blocking the activity of this enzyme. The sole function of the COX enzyme is to convert arachidonic acid to an intermediate named PGH_2 (Dubois *et al.* 1998). Thereafter, cell specific enzymes convert PGH_2 to a number of prostanoids (TXA_2, PGI_2, PGE_2) which serve as lipid hormone like molecules. For example, TXA_2 synthase is present in the platelet and converts PGH_2 to TXA_2 since it is required for platelet aggregation and thrombus formation (Yokoyama *et al.* 1991). Endothelial cells contain PGI_2 synthase which convert PGH_2 to produce both PGI_2 for vasodilatory effects (Spisni *et al.* 1995). Hypothalamic endothelial cells contain PGE_2 synthase which converts PGH_2 to PGE_2, a hormone which increases neuronal drive for thermogenesis during fever (Wilhelms *et al.* 2014). There are many known roles for prostanoids in human biology since COX is present in almost all cell types; these are only examples which are most relevant to thermoregulation. It should be noted early on that COX inhibitors are used to treat pain, inflammation, and fever (COX is involved in the pathophysiology in each process). However, there exists no conclusive evidence that these drugs impact any aspect of T_c in healthy, non-febrile individuals.

Nonetheless, Vigibase indeed shows an interaction between the use of COX inhibitors and hypo/hyperthermia (Lindquist 2008). Acetaminophen has been associated with 340 cases of hypothermia and 45 cases of hyperthermia, while ibuprofen is linked with 184 cases of hypothermia and 20 cases of hyperthermia. These numbers are low relative to the volume of users worldwide, but there are no studies which have investigated this potentially adverse reaction in humans, at different ambient temperatures, and at non-toxic doses. A drug induced

depression of thermogenesis during cold exposure could lead to accidental hypothermia, particularly within the elderly and new-borns who are commonly administered over-the-counter analgesics and are most vulnerable to cold stress (Koponen *et al.* 2013; Liukas *et al.* 2011; Ohlsson and Shah 2016). Equally, these populations are most at risk during exposure to excessive heat since the very young and elderly make up a large percentage of morbidity and mortality rates during heat waves (Kenney *et al.* 2014). The first goal of this thesis is to determine whether acetaminophen interacts with human T_c during heat stress and cold stress. If a potentially adverse reaction is seen in healthy adults, follow up work will determine whether these effects are at least in part mediated through COX inhibition. In the preliminary experiments, acetaminophen was chosen over ibuprofen or other COX inhibitors since it is the most commonly administered drug in the world and has a stronger evidence base to support an influence on non-febrile thermoregulation. In the final study, Ibuprofen was ingested prior to cold exposure as it is a potent non-selective COX inhibitor that it not known to have any other actions which may affect thermoregulation (Wong et al., 2013).

1.1 Objectives of this thesis

The primary objective of this thesis is to investigate whether two of the most common over-the-counter drugs in the world carry a hypothermic side effect when ingested at therapeutic doses. The secondary objective is to determine if the same dose elicits a greater hypothermic response in severe compared with a mild cold exposure. The final objective is to determine whether COX bioactivity is required for non-febrile thermogenesis.

The purpose of this thesis can be summarised in three experimental aims:

1. To validate whether acetaminophen administered at therapeutic doses has a hypothermic action in healthy humans. This will be achieved through the completion of Study 1 (Chapter 4).
2. To investigate if acetaminophen administration influences thermoregulatory responses to dry and humid passive heat stress. This will be achieved through the completion of Study 2 (Chapter 5).
3. To use the information gained from Study 1 and 2 and elucidate the therapeutic or pathological potential of acetaminophen during extreme heat or cold stress. This will be achieved through Study 3 (Chapter 6).
4. To investigate the pharmacodynamics behind the responses shown in Study 1, 2 and 3 using a non-selective cyclooxygenase inhibitor. This will be achieved through the completion of Study 4 (Chapter 7).

CHAPTER 2: LITERATURE REVIEW

2.1 Methodology and structure

A thorough search of the literature relevant to thermal physiology, and its potential interaction with acetaminophen and cyclooxygenase was undertaken. Key words and key authors were entered into Pubmed, Google Scholar and Web of Science. The reference list of relevant articles were also scanned for the purpose of finding appropriate content for the literature review.

In **section one**, the topic of thermoregulation is introduced. Here, there will be a specific focus on why core T_c is regulated at 37°C, and the biology of how this is achieved. Attention will be paid to the neuronal pathways regulating autonomic heat production and heat loss effectors.

Section two focuses on the COX enzyme and prostanoids. In this section, the biology of the enzyme is discussed in addition to its known roles in the pathophysiology of fever generation. This will be followed by a review of the evidence supporting a novel role for cyclooxygenase in non-febrile thermoregulation (heat and cold responses).

Finally, **section three** details the pharmacokinetics and pharmacodynamics of acetaminophen and ibuprofen. These two over-the-counter drugs were administered in experiments detailed in this thesis and have differential potencies of COX inhibition and tissue distribution.

2.2 Thermoregulation

Thermoregulation is a topic that receives considerable attention in the context of human health and performance. In a thermal neutral zone where active heat loss and heat producing mechanisms are not at play, T_c is regulated solely by subtle changes in vasomotor tone (Romanovsky 2014). Human T_c is used as a proxy to describe the extent an individual is losing or

gaining body heat, and can be measured in tissue such as the rectum (Colin *et al.* 1971), oesophagus (Saltin and Hermansen 1966), tympanic membrane (Brinnel and Cabanac 1989), aural (Cross and Stratton 1974), visceral (O'brien *et al.* 1998), and arterial blood (Pennes 1948). Since most of these sites represent only one tissue location in the body, the arterial blood temperature is considered the gold standard T_c measurement since it reflects a global temperature of the body (Pearson 2012). That-being said, arterial blood temperature is highly invasive and difficult to measure, so the rectal temperature is most often used to reflect T_c. Indeed, rectal temperature is used and an index of T_c in each experiment within this thesis.

Heat balance can be predicted from several known parameters which form the heat balance equation, which reads simply as (Kenny and Jay, 2013):

$$(M - W) = (H_{dry} + H_{evap} + H_{resp}) + S \text{ [Watts]} \quad (1)$$

Where M is the metabolic rate and W is the external work performed. $M - W$ is the efficiency in which the energy produced is used on performing that specific task. The term mechanical efficiency defines the amount of energy expended to do a given amount of external work (Wasserman *et al.* 1967). The human body is inefficient in this manner, and 80 to 100% of any external work performed will be released as heat. The external work (W) depends on the mechanical efficiency of the task. For instance, if an individual cycled at an external workload 200 Watts, metabolic heat production would be ~800 Watts because the gross mechanical efficiency of cycling is 20% (Whipp and Wasserman, 1969). H_{dry} is the rate of dry heat transfer, incorporating heat transfer by convection, conduction and radiation. Convection defines heat transfer from a solid surface to a fluid medium (i.e. water, air and blood), and the rate of heat transfer is dependent on the velocity, heat capacity, and volume of surrounding medium.

Conduction defines diffuse heat transfer through direct contact of the skin surface with another solid surface. In most situations, this is negligible, but is an important parameter when the body is in contact with a highly conductive material for an extended period i.e. the application of ice packs prior to exercise in the heat, warming blankets in the perioperative setting, or when an individual has fallen unconscious on a cold floor (Rollstin and Seifert 2012; Ross *et al.* 2013; Torossian *et al.* 2016). Radiative heat transfer defines heat exchange between bodies with different surface temperatures. The mean radiant temperature can be used to estimate total radiative heat transfer within the heat balance equation. H_{evap} is the rate of evaporative heat transfer from the skin. Evaporation of sweat at the skin surface is a powerful driver of heat loss, but can incur a large degree of total body water loss if fluid replacement is inadequate. H_{resp} is heat transfer from respiratory pathways, describing heat exchange between the respiratory tract and the external environment. Each of these variables are reported in Watts (W) or Watts corrected for body surface area ($W \cdot m^{-2}$). An extended background of each of these components are available below and in recent reviews (Cramer and Jay 2016; Kenny and Jay 2013).

Metabolism always represents a source of heat gain, and the rate of metabolic heat production is determined from the amount of energy released from catabolism of carbohydrates, fat, and amino acids. This process is required for the regeneration of adenosine triphosphate (ATP), an organic chemical which serves as an energy source for all cellular and molecular activities, making it critical for almost all physiological processes. It stands to reason that metabolic rate increases in line with the level of physical activity because ATP turnover is elevated. Metabolic rate is normally expressed in Watts (W) corrected for body surface area (i.e. W/m^{-2}) in the thermal physiology literature (Parsons, 2003), but it can also be expressed in kilojoules per minute (1 kJ/min-1 is ~ 17 W), kilocalories per minute (1 kcal/min-1 is ~ 70W), or metabolic equivalents

(1 MET = 58.2 W/m^{-2}). Typical metabolic rates for various activities are displayed below. The conversion of VO2 values into heat production is as follows: Energy equivalent is calculated as (ISO 8996):

$$EE = (0.23RER + 0.77) \times 5.88$$

Based on this information, metabolic rate can be calculated as (Parsons, 2003):

$$M = EE \times \dot{V}O_2 \times 1/BSA \ [W/m^{-2}]$$

Where $\dot{V}O_2$ is oxygen consumption in l/hour-1, and BSA represents the body surface area in m^2. The rate of metabolic heat production is proportional to the increase in $\dot{V}O_2$, because the demand for O_2 (particularly from skeletal muscle tissue) increases in order to fuel the activity. During cold stress, neuronal drive from the CNS causes autonomic rhythmical skeletal muscle contractions (i.e. increasing M), a process required for the maintenance of T_c if voluntary activity is not possible.

Thermal conduction defines the transfer of heat between solid surfaces in contact with one-another. The rate of heat transfer between solid surfaces is defined by three factors, i) the temperature gradient between the two objects in question i.e. the higher the gradient, the faster rate of heat transfer, ii) the thermal conductivity of the material, and iii) the thickness of the material i.e. heat will transfer slower in thicker materials due to its greater volume for heat storage. In most heat-exposure studies, thermal conduction is considered zero as individuals are

Table 2.1. Heat production values for different tasks and types of activity. W/m^{-2} values are generated for a reference body surface area of 1.7 m^2.

Activity	**Rate of heat production**	
Rest	W	W/m^{-2}
Sleeping	75	44
Seated	85	50
Standing	100	59
Occupational tasks		
Walking at 5 km/h	313	184
Light industrial work	125-453	74-266
Heavy industrial work	132-677	78-398
Very heavy industrial work	293-1108	172-652
Cycling		
150 W external load	600	353

Data compiled from Andersen (1978).

rarely in contact with solid objects that affect their heat balance (Cramer and Jay, 2016). Some medical applications are based on thermal conduction however, such as ice-packs for the treatment of inflammation or warming blankets to prevent accidental hypothermia in neonates (Fallis et al., 2006).

Thermal radiation describes the process of heat exchange in the form of electromagnetic waves, and occurs between bodies of different surface temperature. Waves which form the

electromagnetic spectrum include X-rays, light, and radio-waves (Parsons, 2003). Like conduction, there is a net heat flow from hot to cold, and there are several factors which influence radiative heat transfer, including i) the temperature gradient between the body and surrounding surfaces, ii) the effective surface area of the body which changes in line with the body orientation, iii) clothing properties i.e. insulation level (Clo value) and the amount of radiation it reflects or absorbs (influence primarily by the colour). The level of thermal radiation is normally expressed as the mean radiant temperature; the temperature in the centre of a black globe which absorbs the radiation within the environment (Havenith and Fiala, 2015). If exposed to thermal radiation, the temperature inside the globe will rise, and the radiant temperature will be close to the ambient temperature in environments with minimal radiation sources (Hodder and Parsons, 2008). The sun is the most common source of radiant heat in the outdoor environment, but boilers, furnaces, and radiant warming lamps are common sources of thermal radiation in the indoor environment (Parsons, 2003).

Convection defines heat transfer from a solid surface to a fluid medium (i.e. water, air and blood), and the rate of heat transfer is determined by the velocity, heat capacity, and volume of surrounding medium. It is also heavily influenced by the level of clothing insulation (i.e. higher Clo, lower convection) and the temperature of the skin (Parsons, 2003). For example, humans will gain heat from convection if the temperature of the fluid medium (air or water) exceeds the mean skin temperature. Conversely, convection can provide a strong cooling source if the medium has a lower temperature than the skin temperature. It is also important to consider that each fluid medium has a different capacity to transfer heat. For example, water has a greater specific heat capacity (4.19 vs 1.01 kJ·kg-1·K-1) and thermal conductivity (579 vs 25 mw·m-1·K-1) than air, so water is a more powerful source of convective heat transfer. Consequently,

cold water immersion is employed to rapidly cool individuals suffering from heat stroke (Epstein et al., 2015), and perfusion of warm water through a water perfused suit is used to rapidly increase skin temperature in laboratory studies (Rowell et al., 1969).

Evaporation of sweat from the skin surface to the ambient environment the primary natural source of heat loss in humans. If the ambient temperature exceeds that of the skin (creating a source of convective heat gain), evaporative heat loss is the only avenue by which humans can dissipate heat (Cramer and Jay, 2016). That-being-said, evaporation is dependent on the gradient between the water vapour pressure of the ambient air and the water vapour pressure at the skin. If the water vapour pressure in the air exceeds that at the skin, the environment is fully uncompensable, a situation where humans will gain all heat generated through metabolism. Evaporation involves a phase change of sweat on the skin surface from liquid to a water vapour, which will diffuse into the ambient air (Parsons, 2003). To that-end, it is worth noting that unevaporated sweat (i.e. sweat that drips from the skin) is not a source of heat loss and only serves to decrease total body water. The rate of evaporative heat loss is also influenced by the air flow, evaporative resistance of clothing, wettedness of the skin, and the skin surface area (Jay et al., 2015).

Heat exchange through respiration is the final component of the heat-balance equation, describing thermal exchanges between the respiratory tract and the ambient environment. The respiratory tract heats inspired air close to the T_c and becomes saturated with moisture drawn from the surface of the airway (Parsons, 2003). Like convection, conduction, and radiation, the rate of respiratory heat lost to the environment depends on the temperature gradient between the internal and external environment (Cramer and Jay, 2016). It also has an evaporative component, where moisture content of the expired air is a pathway for evaporative heat loss. To that end,

respiratory heat loss is greatest in a cold dry environment, and becomes negligible when i) there is no temperature gradient (convective component) and ii) when there is no vapour pressure gradient (evaporative component). It should be noted that respiratory heat exchange is small due to the very low thermal conductivity of air. For example, respiratory heat transfer at an air temperature of 0°C provides only 10 W more heat loss compared with an air temperature of 20°C.

2.2.1 Why do we regulate core temperature at 37°C?

Prior to understanding the processes that regulate our T_c, it is first important to address what T_c defines, and some important considerations when using this terminology. Thermal physiologists often use the phrase "T_c" to define the internal temperature of an organism, and the skin temperature (T_{sk}) to define the temperature of the shell (Kenny and Jay, 2013). Each of these components can be incorporated into a two-compartment model which produces a metric known as mean body temperature. Mean body temperature is most commonly used for the estimation of heat storage during exercise, but appears seldom in this thesis due to the limitations associated with using the two compartmental prediction (Kenny and Jay, 2013). Throughout the literature review, T_c is generally used to define the internal temperature of the species under investigation, but the specific tissue site measured in each study (rectal temperature, T_{re}) is used throughout the experimental chapters. Other measurement sites include oesophageal, axillary, tympanic, visceral, arterial blood, and sublingual temperature (Havenith and Fiala, 2015), but the T_{re} was chosen for reasons related to logistics, accuracy, and to allow comparisons with prior studies in which most use T_{re}.

Homeostatic regulation of T_c is a key feature of mammalian survival; reductions in T_c result in dysregulated ion channel function (Milburn *et al.* 1995), enzyme performance (Sizer 2006), and a suppression of protein synthesis (Frerichs *et al.* 1998). In contrast, significant increases in T_c are associated with severe protein denaturation (Lepock 2003) and systemic inflammatory response syndrome (Leon and Helwig 2010). Although subject to considerable variation, the human T_c survival limits are in the region of 27 to 42°C (Epstein and Roberts 2011; Stocks *et al.* 2004; Taylor *et al.* 2008), which represents a 10°C decrease and 5°C increase in basal T_c (37°C), respectively. This shows that in general, humans have an increased T_c survival limit during cold exposure, despite our ancestry arising from Africa. Mechanistically, this is because proteins can begin to denature at temperatures as low as 40°C (Ritchie *et al.* 1994), whereas hypothermia decreases metabolism, and by reducing excitotoxicity, can even be used as a therapeutic tool to improve survival rates after traumatic head injury, provided the patient's vital signs are continuously monitored (Cao *et al.* 2014). As mammalian T_c is such a powerfully regulated variable, deviations of more than ± 2°C are rarely witnessed in healthy individuals capable of normal behavioural and autonomic thermoregulation. However, there are many scenarios in which thermoregulatory mechanisms fail, such as in uncompensable heat and cold stress (Cheung *et al.* 2000; Sloan and Keatinge 1973), severe head injury (Badjatia 2009), stroke (Ginsberg and Busto 1998), and drug overdose (Ritter and Eskin 1998; Rollstin and Seifert 2012; Yapakci *et al.* 2001).

Understanding why humans autonomously regulate their T_c close to 37°C is fundamental for the thermal physiologist. It underpins why thermal homeostasis is critical, and is best illustrated by examining the atomic events that arise following significant deviations from this desired temperature (as in hypothermia, when T_c falls below 35°C). Firstly, any physiological change

mediated by a change in temperature does not occur without prior alteration in the biophysical properties of a molecule. The reaction rate of enzymes depends on molecules effectively colliding together, and the kinetic energy of molecules increases in line with the intracellular or blood temperature (Tattersall *et al.* 2012). Thus, the likelihood of molecules colliding with sufficient energy to react increases as a product of T_c, which explains why hypothermia decreases cellular metabolism (Tupone *et al.* 2016) and hyperthermia increases it (Streffer 1988). Again, decreasing brain temperature following traumatic head injury is protective since it substantially reduces metabolism, and thus excitotoxicity within the brain (Tymianski *et al.* 1998).

Of importance to reaction rates is the activation energy (also known potential energy) of the substrate/reactant molecule, a term which defines how much energy is required for a specific chemical reaction to occur. The Maxwell-Boltzmann distribution shows that the number of molecules that meet the activation energy threshold increases in line with temperature, such that there is a positive linear relationship (Starzak 2010). Diffusion (a physical property) requires a very low activation energy of ~2.5 $kJ \cdot mole^{-1}$, whereas it is roughly 50-90 $kJ \cdot mole^{-1}$ for chemical reactions. An adaptation of the Arrhenius equation is used to calculate the temperature dependence/thermal sensitivity of enzymes, ion channels, and physiological functions (i.e. heart rate, muscle contractility, and oxygen consumption to name but a few). This temperature coefficient is known as the Q_{10} because the equation quantifies the difference in reaction rates for a theoretical 10ºC shift in temperature. This does not describe the *ambient* temperature an organism is exposed to, it describes the temperature of the *internal* environment a molecule, cell, or tissue is exposed to. Therefore, the T_c of an organism must be regulated at an optimal performance temperature for biophysical regulation, which for humans is ~37°C. Because a

thermal physiologist seldom examines temperature dependence in such large internal increments (i.e. every 10°C), the equation is constructed as follows:

$$Q_{10} = (k_2/k_1)^{10/(t2-t1)} \quad (2)$$

Where '*k*' is the theoretical reaction rate and '*t*' is the measured temperature. The equation can be applied to determine the effect of temperature on many molecular and physiological functions. For example, one may be interested in the temperature dependency on heart rate at 37 and 40°C, where the heart rate was 65 and 85 $b \cdot min^{-1}$, respectively. In this case, the equation would read $Q_{10} = (85/65)^{10/(40-37)}$, yielding a Q_{10} value of 2.44, which means there will be a 2.44-fold increase in heart rate for every 10ºC increase in T_c. This is somewhat accurate given cardiac pacemaker cells are temperature sensitive (Haverinen and Vornanen 2007), but heart rate it also influenced by interplay of sympathetic and parasympathetic nerve activity (Brodde 1991; Brodde and Michel 1999). For example, the sympathetic nervous system drives tachycardia when T_c falls to ~35°C (Gurabi *et al.* 2014), so the Q_{10} should be interpreted with caution for whole physiological systems that require input from the nervous system.

The Q_{10} is best utilised *ex-vivo* or *in-vitro* since this eliminates any confounding nervous activity. For example, Burdyga and Wray (2002) extracted guinea pig and rat ureteric smooth muscle to calculate the influence of cooling (transfer from 35 to 21°C) on the force amplitude arising from spontaneous contraction. Using electrophysiological techniques, they demonstrated that 14°C cooling decreased guinea pig urethra overall mechanical activity by 20.5 ± 7.5%, but this was increased by 105 ± 11.5% in the rat ureteric tissue. Use of Q_{10} temperature coefficients revealed that it was the rate of relaxation which contributed to the differences in mechanical activity described above. In the guinea pig, a high Q_{10} of 5.1 markedly decreased the rate of relaxation, whereas a slightly lower Q_{10} of 3.7 in the rat allowed more time for the inflow of calcium ions,

overriding any cooling induced kinetic lag of force development. Here, the lack of any nervous interference renders any Q_{10} change attributable to a direct influence of temperature on physiological outcomes. Although the methods employed are not directly relatable to this Ph.D. project (here, *in-vivo* methods are used), it is a valuable reminder of the temperature sensitivity in various biological pathways.

2.2.2 The thermoneutral zone

The thermoneutral zone is a simple but important concept in thermoregulation. It was first defined as *"the range of ambient temperatures within which metabolic rate is at a minimum, and within which temperature regulation is achieved by nonevaporative physical processes alone"* (Bligh and Johnson 1973). Thus, a thermal neutral zone is not accepted if metabolic pathways are recruited for thermogenesis, or evaporation of sweat is required for heat loss. Subtle vasomotor changes incur virtually no metabolic cost, whereas shivering and non-shivering thermogenesis require significant substrate utilisation and increases in metabolic rate (discussed further in sections 2.6.3 and 2.6.4, respectively). Furthermore, a prolonged increase in T_{sk} (40.5°C for 45 minutes) places significant strain on the cardiovascular system, marked by a 125% increase in cardiac output (Rowell *et al.* 1969). In unclothed humans, an air temperature of 23 to 26°C, or a water temperature of ~34 to 36°C is generally thought to satisfy thermal neutrality, based on the resulting T_{sk}. In a clothed human, the thermal neutral zone would shift to a lower temperature depending on the insulation value of the clothing. In the heat, thermal neutrality cannot be achieved at an air temperature above 26°C unless the skin is cooled artificially. For instance, Kingma *et al.* (2014) produced a thermoregulatory model to delineate what specific combinations of T_c, T_{sk}, and ambient temperature are required for the attainment of a thermal neutral zone. The model demonstrates that the thermal neutral zone is dynamic and

shows a high degree of plasticity. Based on this model, the mean T_{sk} must be in the range of 30.6 to 36.8°C and T_c in the range of 36 to 38°C to satisfy the requirements for a thermal neutral zone.

The detection thresholds required to trigger cold and warm thermal sensations depend on the starting T_{sk} and the body region in question. For example, 70 kg human exposed to an ambient temperature of 24°C, local T_{sk} can range between 30 and 34°C while the subject is in a perceived state of sensory thermal neutrality (Gagge *et al.* 1967). Specifically, this means that there are no prevailing sensations of warm or cold, so the sensation is neutral. In fact, work in primates suggest that there is a balanced firing of both cold and warm thermoreceptors when local T_{sk} is between 30 and 34°C (Hensel and Iggo 1971). Indeed, this thermosensory zone is used as a neutral T_{sk} when diagnosing cutaneous sensory disorders such as hypoesthesia (Rolke *et al.* 2006). Filingeri *et al.* (2017) determined the properties of thermal neutral zone, examining its width and whether this changes as a dynamic function of the starting T_{sk}. Using a circular thermal probe on the palm starting at three distinct temperatures (26, 31, and 36°C), they showed that the thermal neutral zone was dynamic and shifted depending on the starting temperature of the probe. For example, at a starting T_{sk} of 31°C, the threshold for warm sensation was ~+2°C, whereas the cold threshold was found to be ~-0.4°C. At 36°C starting temperature, and compared with the 31°C condition, the threshold for warm sensation was ~1.62°C and the threshold for cold sensation was ~1.4°C larger ($p < 0.05$). For a resting T_{sk} of 26, 31, and 36°C starting temperature, the thermal neutral zone fell within a range of 2.36°C (25.7 to 28°C), 2.47°C (30.6 to 33.1°C), and 2.25°C (34.2 to 36.5°C), respectively. The limitations of this study are that it is only valid in a small skin area (the palm) and may be misleading for whole body thermal neutrality. For instance, a range of mean T_{sk} required to satisfy thermal neutrality have not been as well defined.

As humans, we spend most of our lives in a thermal neutral zone through clothing and perceptually avoiding environments below the thermal neutral zone. However, there are many scenarios in which thermal stress might not be avoided, such as the cold stress of birth, in a poorly insulated home during extreme heat or cold, or during exercise. In situations where the T_{sk} falls below or above the inter threshold zone, active thermogenic or heat loss mechanisms are initiated from within the pre-optic anterior hypothalamus. In the next section, the biology involved in T_c stability during skin cooling is described in detail.

2.3 Mechanism of core temperature stability

The following 3-step feedback loop is required for T_c regulation during cold exposure:

1. Feedforward afferent signals of skin and gut temperature directed towards the central nervous system.
2. Neuronal integration of the signal in the pre-optic anterior hypothalamus.
3. Autonomic thermoregulatory responses.

In the following sections, the neuronal pathways responsible for autonomic thermoregulation are discussed. Prior to this discussion, it is important to address translation issues from animal work to human physiology.

Due to ethical and practical issues associated with human experimentation, there are many references drawn from animal work throughout this thesis. In the present literature review, animal work has provided important mechanistic information related to central mechanisms of thermoregulation, and acetaminophen induced hypothermia. While results from animal studies are often used to explain physiological responses in humans, it is erroneous to assume perfect inter-species translation, especially in clinical trials. Despite successful animal testing, 85% of

novel drugs fail when tested on humans in pre-clinical trials, and those that reach a phase III trial (Arrowsmith, 2011), only 50% become FDA approved (Ledford, 2011). Although there is no alternative to using this approach if the studies cannot be performed in humans, it is important to identify the limitations of animal to human translations, with special reference to thermoregulation.

There are several issues of importance when translating work from animals into humans. Besides general physiological complexity, the most obvious limitation is the enormous difference in body characteristics; the first issue is that of body mass. A commonly used mouse as a model for human physiology is the C57BL/6 mouse sub-strain which has a body mass of ~20 grams, but a reference adult human may have a body mass of 70 kg, 3,500 times heavier than the C57BL/6 mouse. In the heat and at fixed workloads, the rate of heat storage is directly proportional to body mass, where there is a negative association (Havenith et al., 1998). In the cold, the rate of heat loss is also negatively associated with body mass i.e. heat loss is slower in heavier organisms (Taylor et al., 2014). There is also a matter of skin surface area and its relation with body mass (i.e. surface to mass ratio). Rodents have a very large surface area to mass ratio compared with humans, and this ratio has an independent effect on heat stress and cold stress responses and vulnerabilities. In the heat, the rate of heat storage is positively associated with the surface area to mass ratio (Havenith, 2001). In the cold, the rate of heat loss is also positively associated with a high ratio i.e. organisms with a high surface area to mass ratio with lose heat faster than those with a low ratio (Allen, 1877). Based on these principles, it is reasonable to assume that the physiological responses to the same heat and cold stimulus are not the same between different species. Indeed, the hypothermic response to acetaminophen may be

exacerbated in mice due to their high surface area to mass ratio, even if doses are corrected for absolute body mass (Ayoub et al., 2004).

A second characteristic of mice that is problematic for human translation is the reliance on brown adipose tissue for thermogenesis. Although the functional efficiency of BAT is similar between mice and humans (Porter et al., 2016), the relative contribution of BAT to total body mass is far greater in the mouse (Porter et al., 2016). In contrast, adult humans rely more on heat production through shivering thermogenesis during acute cold exposure, and little on BAT thermogenesis (Blondin et al., 2014). A major component of this thesis is an attempt to demonstrate acetaminophen induced hypothermia in humans, and to uncover the underlying mechanisms which mediate this side effect (cyclooxygenase inhibition is one possibility). If acetaminophen's hypothermic action in mice is through suppression of cyclooxygenase and BAT function (for which there is already strong evidence), the effect might not be mirrored in adult human beings i.e. because they do not rely heavily on BAT function for thermogenesis.

Another consideration is the difference in thermal neutral zones between humans and rodents. In nude adult humans, an ambient temperature between 24-26°C is considered thermoneutral i.e. it will not induce metabolic heat loss (increasing skin blood flow) or heat gain (shivering, BAT activation) responses (Kingma et al., 2012). In rodents, the thermal neutral zone is close to 30°C to compensate for their greater surface area to mass ratio (Speakman and Keijer, 2013). Despite this difference in preferred thermal neutral zones, experimental mice are often housed at an ambient temperature which optimises comfort for the researcher i.e. ~18 to 21°C (Speakman and Keijer, 2013). This type of environment is below neutral for a mouse, which means they are in a consistent state of mild to moderate cold stress. The fact that mice may constitutively produce

more heat than if they were in a neutral environment has implications for metabolic studies, especially if they are to be translated to human research.

Repeated exposure to cold ambient temperatures has an impact on the mouse (and human) phenotype and genotype. It is well known that mice rely on BAT formation for Tre maintenance, but it is often ignored that mice may adapt in this way because of long term cold exposure. Attention to this key variable, the ambient temperature, has a broad transformative impact on translation to human trials (Ledford, 2011). Specific to the present thesis, studies inducing hypothermia in mice with acetaminophen should be approached with caution because this effect may be dependent on mice being exposed to cold. Unfortunately, there are no trials which have investigated acetaminophen's hypothermic effect in mice housed in their thermal neutral zone. Such a study would help determine if acetaminophen reduces body temperature by inhibiting heat production or activating heat loss mechanisms.

2.3.1 Skin region and thermal sensitivity

The human skin is a ~2 m^2 large organ that forms a barrier between our internal and external environment. It protects us from exogenous pathogens and allows us to detect noxious temperatures, chemicals and mechanical influences. The human skin is critically involved in thermoregulation, since thermosensory nerve endings project to the brain for us to discriminate and initiate an autonomic response to variable ambient and skin temperatures. As such, human T_{sk} is a useful marker of thermoregulatory stress, either as a global mean or on a specific anatomical region. Rowell *et al.* (1969) used a water perfused garment to raise the T_{sk} of five young males to 40.5ºC for ~40 minutes. They demonstrated that cardiac output increased up to 125%, while hepatic blood flow and peripheral resistance fell up to 60%. In cold exposure

experiments, water perfused suits are commonly used to clamp T_{sk} at a value which yields a reliable increase in resting metabolic rate (Blondin *et al.* 2010; Haman *et al.* 2005). For example, an exposure to mild cold, resulting in a T_{sk} of 27.4°C, elicited a 2.5-fold increase in metabolic rate (Haman *et al.* 2005). When the mean T_{sk} is reduced to 19°C, this is met with a 3.5-fold increase in metabolic rate (Martineau and Jacobs 1989). These responses show the importance of the skin in initiating cold and heat defence responses.

The specific role of the skin in thermoregulation is not homogenous throughout each anatomical region, as reflected by large variations in thermal sensitivity and sweat output. Indeed, the non-hairy skin (palms, soles of the feet, ear and some areas of the face) acts as a radiator because it has a large surface area to volume ratio and contains many arteriovenous anastomoses (Romanovsky 2014). As the name suggests, arteriovenous anastomoses form connections between arterioles and venules, and their innervation by the sympathetic nervous system can greatly influence skin blood flow to that area, regulating heat exchange (Walløe 2016). In response to cold stress, sympathetic innervation by vasoconstrictor nerves can reduce blood flow to almost zero, whereas a lack of innervation (stimulated by heat stress) has been shown to increase finger blood flow by ~500% compared with baseline (Nagasaka *et al.* 1987). In the non-hairy skin, arteriovenous anastomoses can reach diameters of up to 150 µm, whereas a typical capillary is only 10 µm (Elstad et al., 2014). These features permit very high ranges of blood flow requirements which either dump large volumes of heat from the skin to the environment or reduce blood flow to ~0% i.e. in response to skin cooling. The hairy skin contains fewer arteriovenous anastomoses, but a greater concentration of cutaneous thermoreceptors (Romanovsky 2014). For example, Cotter and Taylor (2005) showed that the face shows greater thermal sensitivity to skin cooling and skin warming than the forearm, thigh, leg, and foot.

Interestingly, the authors also showed that the extremities were the least sensitive to local hot and cold exposure, supporting the notion the role of the hand and feet as thermal radiators. Moreover, stimulation of the cold receptors by menthol (discussed in the next section) produced no cooling sensation when applied to the hand, but did produce cold defence responses when applied to the abdomen (Almeida *et al*, unpublished observation).

2.3.2 Thermoreceptors

The ability to discriminate between different skin, core, and brain temperatures is a key feature of the somatosensory nervous system. It allows reactive avoidance of noxious (painful) heat or cold and is crucial for behavioural (voluntary) and autonomic (involuntary) thermoregulatory adaptations. For behavioural thermoregulation (conscious discrimination of a change in temperature), thermosensory inputs are delivered to the somatosensory cortex (Craig 2002), while inputs are delivered to the pre-optic anterior hypothalamus for autonomic responses (Nakamura and Morrison 2008a). Changes in skin and core temperature excites nerves of the trigeminal and dorsal root ganglia (DRG), allowing thermal discrimination from sensation arising from the face and other areas of the body, respectively. When stimulated, action potentials from these nerves synapse with neurons in the spinal cord, which then activate second order relay neurons in the lateral parabrachial nucleus (Nakamura and Morrison 2008a, 2011). Based on the initial thermosensory input (hot or cold), neurons in the lateral parabrachial nucleus activate, or inhibit, distinct neuronal populations in the pre-optic area (Nakamura and Morrison 2010). Based on this signal, mechanisms for heat production or heat loss will be activated. This pathway is described in depth later in section 2.4.3.

There are two types of somatosensory nerve fibres required for thermosensory inputs; thinly myelinated Aδ (A delta) fibres which have moderate conduction velocities, and the non-

myelinated C fibres which exhibit slow conduction velocities (Vriens *et al.* 2014). Each fibre type has a small but consistent action potential firing rate even at a thermoneutral T_{sk} [32-33°C (Fowler *et al.* 1988; Schepers and Ringkamp 2009)]. Early work from Darian-Smith *et al.* (1973) reported that, in monkeys, innocuous cold caused electrical activity in nerves firing in the 5-30 $m \cdot s^{-1}$ range, which is indicative of Aδ fibre activity. Later work by Fowler *et al.* (1988) estimated conduction velocity using reaction times of cold sensation in humans, which yielded slower velocities of 2.1 ± 0.8 $m \cdot s^{-1}$, which is at the lower end of the Aδ range. Later, Campero *et al.* (2001) used microneurography to further identify and define thermosensory afferents. Microneurography is an invasive technique, which involves the insertion of a needle electrode (100-200 µm diameter) into intact nerves in awake human participants. The conduction velocities of the functional nerve units which responded to innocuous cooling included those in the C fibre range (< 2 $m \cdot s^{-1}$), a finding that conflicted with the notion that cold thermosensory inputs were mediated exclusively by Aδ fibres (Fowler et al. 1998). The increased specificity of microneurography when compared with estimates of conduction velocity from reaction times likely explain these findings. The discharge rates of each of these fibres are transient i.e. the action potentials reduce to a steady state level once they have reached a discharge rate proportional to the intensity of the cold stimulus. This type of activity explains the transient nature of some cold sensations i.e. initial submersion in cold water invokes an immediate cold sensation, but this decreases in intensity as the stimulus continues despite no change in water temperature.

Given that T_c is the critically regulated variable in thermoregulation, it follows that there are thermosensory nerves excited by hypothermia and hyperthermia residing in the body core, but there is a lack of evidence in this regard. Ruan *et al.* (2005) recorded the activities of afferent

vagal pulmonary C fibres in anaesthetised rats whose pulmonary temperature was maintained at 37, 38.5, and 41ºC for 3 minutes. They demonstrated that the baseline activity did not increase at 38.5ºC, but was elevated 5-fold when the temperature reached 41ºC. The fact that vagal afferent nerves are more responsive at 41ºC suggest that they are activated closer to the noxious temperature ranges. Gupta *et al.* (1979) studied the responsiveness of left splanchnic nerves to cooling in anaesthetised cats. They exposed single fibre preparations to water temperatures regulated between 12 and 38ºC, and showed that no cold sensitive afferents were found in the vagal nerve, but afferents arising from the stomach were particularly sensitive to 12ºC. In support for visceral thermosensitive afferents, a recent study studied the effect of cold (7ºC), cool (22ºC), neutral (37ºC) and warm (52ºC) fluid ingestion (3 $ml \cdot kg^{-1}$ body mass) in already cold exposed humans (Morris *et al.* 2017). Compared with 37ºC, metabolic rate increased by ~25% with 7 and 22ºC fluid, but fell by ~17% following administration of 52ºC fluid. This demonstrates a role for thermosensitive afferents originating in the stomach which respond to a wide range of temperatures (at least 7 to 52ºC). The water temperatures used in that study are supraphysiological because an internal temperature of 7 or 52ºC would be fatal (Parsons 2014). It remains to be elucidated whether the upper and lower extremes of T_c elicit similar metabolic responses (i.e. 35 to 41ºC).

The channels that regulate in the inflow of cations (positively charged ions) are well described, although the precise mechanisms by which they open in response to cold is still under debate. Transient receptor potential cation channels (TRP channels) are present on the membrane of these thermosensory neurons in the skin, gut, and brain, and regulate the inflow of sodium and calcium ions through the cell membrane. These ions are required for the generation of action potentials in response to changes in temperature, although they are also activated by a variety of

chemical agents. The molecular activation mechanisms of temperature responsive TRP channels (thermoTRPs) are not completely defined, but the notion that there is a temperature mediated shift in the voltage-dependent gating process is now undisputed. The theory that shifts in temperature induce an intrinsic conformational change in the channel is the most likely, since heat and cold sensitive ion channels exhibit the same thermosensitive characteristics following reconstitution into artificial lipid membranes (Zakharian *et al.* 2010). Since thermoTRP channels are highly temperature sensitive, they have Q_{10} values of between 10-50, which is far greater than values of 2-7 seen in other ion channels (Voets 2012). The reader is directed to section 2.3.1 for an overview of the Q_{10} temperature coefficient. The currently known subfamilies which are involved in thermoregulation are melastin (TRPM), acyrin (TRPA), and vanniloid (TRPV). The thermosensory channel activated by innocuous heat is most likely TRPV3. The TRP channel that appears to be activated by innocuous cold temperatures is TRPM8, while TRPV1 and TRPA1 are involved in noxious heat and cold sensations, respectively (Denda and Tsutsumi 2011; Kwan and Corey 2009). TRPM8 was first characterised in 2002 by Peier *et al.* (2002a). The authors used DNA databases to identify potential exons from the *TRPM8* gene (based on the known *TRPV1* gene), and yielded a full-length sequence by using prediction software alongside polymerase chain reaction (PCR) techniques. To determine the role of *TRPM8*, the gene was first transfected into Chinese hamster ovary cells, and intracellular calcium concentrations (indicative of channel opening) were quantified in response to chemical agonists and changes in incubation temperature. For the first time, it was shown that TRPM8 was sensitive to a low ambient temperature (15°C) and the organic compound menthol, indicating that TRPM8 may act as a molecular sensor to subneutral temperatures. Using similar techniques in Chinese hamster ovary cells, Peier *et al.* (2002b) discovered a role for TRPV3 for innocuous heat. They showed that

these cells displayed no calcium influx when exposed to low temperatures, but this was maximal when the temperature was between 33 and 45ºC. This shows that TRPV3 is responsive to innocuous heat sensations, but does not cause calcium influx at low temperature or that above 45ºC. The authors also showed that TRPV3 is expressed in epidermal keratinocytes.

The two methods most commonly used to elucidate the roles of specific TRP families in thermoregulation are knockout mouse models and the use of specific agonists and antagonists. Additionally, some authors have investigated the thermosensory role of TRP channels in the human embryonic kidney cell line, where the genetic insertion of thermoTRPs induces high temperature sensitivity. The knockout mouse model (where the deleted gene is denoted as $^{-/-}$ in most genetic literature) is carefully bred to not express a specific gene of interest, whereas agonists and antagonists chemically activate or deactivate the target protein, respectively. Moqrich *et al.* (2005) demonstrated that TRPV3$^{-/-}$ mice showed an impaired ability to thermoregulate when transferred to a warm environment. In a 2-hour protocol where the time spent in specific temperature zones (15 to 55ºC) was recorded, they showed that wild type mice showed a preference toward the zones where the temperature was 30 to 37ºC, evidenced by a significantly increased time spent within that zone. In contrast, the TRPV3$^{-/-}$ mice showed no preference for the warm zone, indicating that TRPV3 is required for innocuous warm sensations. Camphor is an oil extract from the wood of an Asian tree called camphor laurel, and is a potent TRPV3 agonist (Sherkheli *et al.* 2013). Kotaka *et al.* (2014) applied camphor at various strengths (5, 10, and 20%) to the medial forearm of 9 middle-aged adults in a thermoneutral environment (~25ºC). At all strengths, camphor increase skin blood flow 1.5-fold, reaching a peak only 10 minutes after the application.

The use of TRPM8$^{-/-}$ mice in thermoregulation research has significantly advanced our understanding of the role this ion channel has in innocuous cold thermosensation. Dhaka *et al.* (2007) demonstrated that mice (C57Bl/6 strain) lacking the TRPM8 gene had profound differences in cold ambient temperature (15ºC) avoidance compared with wild-type mice. In that experiment, mice could move freely between different temperature zones ranging from 15-53ºC, and time spent in each zone was used as an index of cold thermosensory function. Wild type mice showed a greater preference for ambient temperatures of 30 to 38ºC (which is their normal preferred temperature range), whereas TRPM8$^{-/-}$ mice spent significantly more time in colder areas (< 30ºC). This indicates that the TRPM8 protein is indispensable for adequate sensation of cold ambient temperatures (15-25ºC). More recently, Tajino *et al.* (2011) demonstrated that wild-type and TRPM8$^{-/-}$ mice exert significantly different T_c and thermogenic responses during exposure to 10ºC, but not at 25ºC. For example, staining of harvested brown adipose tissue (a heat producing organ described in section 2.6.4) after cold exposure indicates that phosphorylated nuclear factor kappa B and uncoupling protein-1 [UCP-1 (markers of brown adipose tissue activation)] are vastly decreased in TRPM8 $^{-/-}$ mice. However, brown adipose tissue heat production was not completely inhibited, suggesting that 1) other pathways are involved in cold thermosensation which are yet to be established or 2) TRPM8 was not fully inhibited. Nonetheless, the inhibitory effects on brown adipose tissue thermogenesis resulted in a significant 1ºC T_c reduction in TRPM8$^{-/-}$ mice when exposed to 10ºC, compared with wild-type mice.

An alternative method used to examine TRPM8 function is through use of menthol as a selective agonist. Major benefits to using menthol application (as opposed to knockout mouse models in combination with cold air) are that it can be applied specifically to thermosensitive regions on

the skin, and it can safely be applied to humans (in spray or gel form). When its thermosensory effects were examined on human skin (Yosipovitch *et al.* 1996), it only produced a cooling sensation in 12 out of 18 participants, and the duration of the cooling ranged from 5-70 minutes (mean, 32 minutes). The use of ethanol as a solvent for menthol may account for the large between subject variation for two reasons. Firstly, ethanol itself is a TRPV1 agonist (Gazzieri *et al.* 2006), and activation of TRPV1 substantially decreases thermogenic responses to environmental cooling in mice (Feketa *et al.* 2013). Secondly, ethanol has an inhibitory effect on TRPM8 function via interaction with the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2). In human embryonic kidney cells, the addition of PIP2 reverses the inhibitory effect of ethanol on TRPM8, whereas the addition of a PIP2 antagonist enhanced the inhibitory effect of ethanol (Benedikt *et al.* 2007). Given that ethanol is not an appropriate solvent in this regard, Valente *et al.* (2015) prepared menthol by mixing 10 mL of warm water and l-menthol crystals (10 mg·kg body mass^{-1}) for five minutes to form a gel which was immediately applied to the neck, right arm and leg. Application of the menthol gel caused a mild but significant increase in T_c (~0.2ºC) and decreased skin blood flow (~20 perfusion units), which is indicative of an acute thermogenic response. These effects appeared to deteriorate 3-hours after application of the menthol gel. For a description of the perfusion unit (an arbitrary unit which indicates blood flow alterations during laser Doppler flowmetry), the reader is directed to the work of Cho *et al.* (2009). Finally, Gillis *et al.* (2015) mapped responses to menthol spray for five days to investigate if its thermogenic effects altered by repeated exposures. The subjects were separated into three independent treatment groups (control, 0.2 and 0.5% menthol spray), where thermogenic responses to each treatment were examined during exercise [45% cycling at peak power output (day 1 and 5)] and at rest (day 2, 3 and 4). At rest, 0.2% menthol spray (upper

body) induced a small but significant increase in T_c across a 60-minutes period when compared with control and 0.5% menthol (0.1°C increase). This effect was not lost on the fourth day of treatment, indicating a lack of habituation effect after repeated exposures to menthol spray. There were no differences in thermoregulation when treatments were administered during cycling exercise, indicating that the heat loss mechanisms activated during exercise outweigh any thermogenic effect of menthol spray.

Despite significant differences in T_c induced by menthol, the scale of the response is small. One would expect that if cold exposure was fully stimulated by menthol in a thermoneutral environment, T_c would rise almost immediately due to the autonomous activation of thermogenesis. One reason why menthol doesn't elicit a strong thermogenic response in humans could be due to the mechanism by which it acts as a TRPM8 agonist, so it is important to consider how menthol influences TRPM8 gating. Until recently, the precise mechanism of TRPM8 agonists were unknown. Using calcium imaging within a human embryonic kidney cell line, Janssens *et al.* (2016) studied how the gating kinetics of TRPM8 change when exposed to menthol and allyl isothiocyanate (mustard oil). They found that menthol 'activates' TRPM8 by preventing the channel from closing, whereas mustard oil causes the channel to open faster than it normally would. More specifically, menthol inhibits normal deactivation because it allows for a more stable "open" conformation of the channel. In contrast, mustard oil amplifies TRPM8 activation by destabilising the "closed" conformation. Given that menthol does not force TRPM8 to open, it is unsurprising that its thermogenic side-effects in humans are unreliable in thermoneutral conditions (Gillis *et al.* 2015; Valente *et al.* 2015; Yosipovitch *et al.* 1996). Of note, mustard oil is not advised as a replacement for menthol because it also activates TRPV1 and TRPA1, the respective sensors for noxious heat and cold.

2.3.3 Interaction of dorsal root ganglia nerves with dorsal horn cells

When DRG nerves are activated by thermal information, a process which may be partly regulated by TRP channels, a signal is projected to the spinal cord, where a synapse occurs between thermosensitive neurons and temperature specific spinal and trigeminal dorsal horn cells (Craig 2002). The use of retrograde neural tracers and c-Fos staining techniques have greatly advanced our understanding of both afferent (ascending) and efferent (descending) neural pathways involved in autonomic thermoregulation. c-Fos is a protein expressed in individual neurons immediately after their activity (Hoffman *et al.* 1993), such that staining c-Fos with an antibody allows researchers to map the neuronal activation pathways in response to cold and warm environmental temperatures. These techniques have been used to map the afferent pathways which project to the pre-optic area during acute cold (4ºC), heat (36ºC) or thermoneutral (26ºC) exposure in mice (Nakamura and Morrison 2008b, 2010, 2011). The authors also use various chemical agents to identify what neurotransmitters mediate these responses (Table 2.1).

The authors revealed that glutamatergic projections from lateral parabrachial nuclei (LPB) transmit thermosensory signals directly to pre-optic area. The LPB acts a relay centre for sensory information (temperature, mechanical, chemical) and thus receives an enormous amount of projections from dorsal horn cells (Cechetto *et al.* 1985). c-Fos staining demonstrated that neurons in the external lateral sub-region of the LPB (LPBel) were activated only in response to cold exposure (Nakamura and Morrison 2011), whereas there was a large c-Fos expression in the dorsal subregion of the LPB (LPBd) during heat exposure (Nakamura and Morrison 2010). When the T_{sk} decreases, thermosensory signals projecting from the LPBel to the pre-optic area are eliminated following nanoinjection of a GABA receptor agonist (muscimol) or a glutamate

receptor antagonist (AP5/CNXQ), demonstrating that dorsal horn cells provide glutamatergic inputs to LPBel neurons in order to regulate T_c (Nakamura and Morrison 2008b). The thermosensory pathway within the pre-optic area which mediates autonomic responses to cold begins with initial neuronal inputs delivered to the median pre-optic nuclei (MnPO) from the LPBel. In a recent experiment, it was demonstrated that the thermogenic responses to LPBel stimulation with NMDA are reversed after nanoinjection of AP5/CNXQ in the MnPO (Nakamura and Morrison 2008b). In addition, direct stimulation of MnPO glutamate receptors with NMDA triggers brown adipose tissue thermogenesis, shivering thermogenesis, and tachycardia (Nakamura and Morrison 2008a).

Table 2.2. Chemicals commonly used to determine the role of specific pre-optic area neurotransmitters in thermoregulation.

Chemical	**Action**
Muscimol	GABA receptor agonist
Bicuculline	GABA receptor antagonist
Methysergide	Serotonin receptor antagonist
AP5/CNXQ	Glutamate receptor antagonists
NMDA	Glutamate receptor agonist

2.3.4 The pre-optic area

The subsequent topic of this literature review concerns the mapping of thermosensory neurons within the pre-optic area. At this point, the afferent pathway involving skin cooling, to dorsal root cells, to the LPB, to the MnPO has been described. But how is this information integrated to form an appropriate physiological response? To answer this question, it is necessary to briefly

skip past the MnPO and describe the function of nuclei within the medial pre-optic area (MPO). A prior explanation of MPO neuronal projections is required when explaining the relationship between the MnPO and the MPO.

During heat and thermoneutral exposure, the MPO plays a crucial part in regulating efferent responses by sending GABAergic inhibitory projections to the dorso-medial hypothalamus (DMH) in warm and thermoneutral environments (Nakamura and Morrison 2010). These inhibitory projections are vital for the regulation (or elimination) of thermogenesis in hot environments, such that nanoinjection of bicuculline (a GABA receptor antagonist) into the MPO results in a substantial increase in brown adipose tissue and shivering thermogenesis (Osaka 2004). This evidence renders it likely that, in response to cooling, the MnPO (described above) transmits its own inhibitory projections to the MPO, which in turn eradicates the tonic inhibition from the MPO to the DMH. In support of this view, activation (with NMDA) or disinhibition (by bicuculline) of MnPO neurons drives cold defensive thermogenesis (Nakamura and Morrison 2011). The view that MPO provide tonic inhibitory projections to the DMH (and thus inhibits thermogenesis) is cemented by evidence that nanoinjection of muscimol (a GABA receptor agonist) into the MPO also results in thermogenic activation (Zaretsky *et al.* 2006). As previously stated, MPO neurons tonically inhibit premotor neurons with the DMH and rostral medullary raphe region (rMR). During cold exposure, it is most likely that inhibited MPO neurons can no longer block glutamatergic DMH neurons to the rMR. For example, activation of neurons in the DMH evokes c-Fos expression in regions of the rMR that mediate cold defence responses (Zaretskaia *et al.* 2008). Additionally, muscimol injection into the DMH blocks brown adipose tissue, shivering, and tachycardia during cold exposure (Nakamura and Morrison 2011).

2.3.5 Efferent neuronal pathways

The rMR contains several pre-motor neuronal populations which are activated when MPO neurons are inhibited by GABAergic projections from the MnPO. These populations include the rostral raphe pallidus nucleus and the raphe magnus nucleus. Given that injection of muscimol into the rMR elicits brown adipose tissue activation, shivering, vasoconstriction and tachycardia, it appears that these pre-motor neurons receive tonic GABAergic projections in thermoneutral conditions (Nakamura and Morrison 2008a, 2011). Equally, thermogenesis is blocked through injection of glutamate receptor antagonists into the rMR following NMDA induced glutamate excitation of neurons in the DMH. These findings support the notion that pre-motor rMR neurons are stimulated directly by glutamatergic projections from the DMH. Although, inhibition of DMH neurons does not block the veno-vasoconstrictor response to skin cooling, indicating that alternative projections to the rMR mediate this response. This neuronal pathway has not been fully elucidated, but there is evidence to suggest that some neurons from the MnPO bypass the MPO and DMH, eliciting thermogenic responses (Tanaka *et al.* 2011).

The rMR directly activates ventral root motor neurons in the spinal cord. We know this not only because the rMR has many projections to preganglionic neurons in the spinal cord (Yoshida *et al.* 2009), but also because antagonising the cells within the spinal cord blocks the thermogenic effects of rMR excitation (Yoshida *et al.* 2003). Moreover, excitation of preganglionic ventral root cells with glutamate stimulates thermogenic responses identical to that of skin cooling. Interestingly, the neurotransmitter serotonin may play a key role in core body temperature defence against the cold. Using mice, Madden et al. (2010) injected a serotonin receptor antagonist (methysergide) in the intermediolateral cell column (T2 to T5) and decreased T_{sk} by ~6°C. Injection of methysergide decreased brown adipose tissue activation by 64% compared with the responses with saline injection. Subsequently, the authors repeated the experiment with

specific antagonists for the serotonin 7 and 1A receptor, and reported decreases in brown adipose tissue sympathetic activation by ~53%, respectively. This work demonstrates that the activity of serotonin is critical for full thermogenesis during cold exposure. The DMH also mediates the release of adrenocorticotropic hormone (ACTH) from the hypothalamic pituitary axis in response to cold stress. ACTH release is stimulated from the paraventricular nucleus within the hypothalamus, such that blockade of neurons in this region blunts any rise in plasma ACTH during cold stress (Hunt *et al.* 2010). The presence of ACTH increases the production of glucocorticoids, hormones that activate lipolysis and the breakdown of glycogen in the liver (Xu *et al.* 2009). These responses enhance substrate availability for brown adipose tissue metabolism and shivering thermogenesis.

In summary, this section has described the neuronal network involved in cold induced autonomic thermogenesis. In the later sections, evidence suggests that acetaminophen, a hypothermic agent, may interact with this pathway to depress cold-defence response. The use of glutamate and GABA agonists and antagonists have greatly advanced our understanding of these pathways during cold exposure. However, as described in section 2.3, the translation from animal work should be interpreted with care. In the next section, the physiological responses to cold stress are described in detail.

2.3.6 Cutaneous vasomotion

Control of skin blood flow is an important facet of the human thermoregulatory system. During thermoneutrality or heat exposure, the MPO provides glutamatergic projections to pre-motor neurons in the DMH and rMR, tonically inhibiting efferent signals which would normally result in veno/vasoconstriction. As described earlier, these pre-motor neurons are “disinhibited” through GABAergic projections from the MnPO to the MPO when T_{sk} is reduced below a

thermoneutral threshold. Venoconstriction occurs more rapidly than vasoconstriction because veins have a greater thermal sensitivity than arteries (Webb-Peploe and Shepherd 1968), and because these changes in vasomotor tone do not require substrates to function efficiently, it is the first line of defence in response to a reduction in T_{sk}, in the aim of preserving thermoneutrality. As previously discussed, the stability of T_c in thermoneutral conditions is solely dependent on changes in vasomotor tone. In thermoneutral conditions (~25°C in a nude, resting human), the skin receives up to 10% of cardiac output, equating to approximately 350 ml·min^{-1} skin blood flow (Romanovsky 2014). Because the mammalian body continuously produces heat from normal metabolic processes, it is not surprising that such a large blood volume is purposely directed to the skin for heat dissipation, even in thermoneutral conditions. During extreme heat stress, cutaneous blood flow can reach ~8 litres·min^{-1}, and entirely cease during extreme cold exposure (Rowell *et al.* 1969). This demonstrates the remarkable capacity of the cutaneous circulation to facilitate a wide range of blood flow requirements, a feature made possible by the arteriovenous anastomoses described earlier in this thesis (section 2.4.1). However, this is not homogenous for all skin regions, evident by a cold-induced vasodilation in areas of the skin with very large surface area to volume ratio (i.e. the palms, fingers, toes, and soles of the feet). Although this response seems paradoxical, cold induced vasodilation in these regions is necessary to delay tissue damage/frostbite and improve dexterity in the extremities (Tyler *et al.* 2015). Vasoconstriction is necessary for the redistribution of heat to the central compartments (preserving organ temperature), and to limit heat transfer from the skin to the environment. There are two arms which mediate cutaneous vasoconstriction in response to skin cooling; reflex (autonomic) and local responses to a fall in T_{sk}.

The human skin is indispensable for proper thermoregulatory function owing to its ability to redistribute heat to the external environment. It can receive almost no blood flow during cold stress and up to 8 litres·min^{-1} during severe heat stress, which equates to ~60 % of cardiac output. In normothermic conditions, the sympathetic nervous system controls skin blood flow through balanced release of vasoconstrictor (norepinephrine, peptide y) and vasodilator (nitric oxide, histamine, prostaglandin) substances, such that it receives ~250 mL·min^{-1} in these conditions (Brengelmann and Savage 1997). In section 2.4.1, evidence that the sympathetic response is primarily dictated by T_{sk} during cold exposure, and the T_c during heat exposure was put forward. The skin blood flow will be almost zero during severe cold stress, even if T_c is well regulated. In contrast, maximal skin blood can only be achieved through a raised T_c (~39.5°C). Cutaneous veno and vasoconstriction is the first line of T_c defence during acute cold stress. The effectiveness of this response is especially apparent when observing an initial increase in T_c shortly after exposure to cold, reflecting a redistribution of warm blood from the skin to the central compartments (Study 3). The complex neuronal pathway from the thermoreceptor to the brain, and from the brain to the cutaneous veins and arteries was described in section 2.5.1.

During heat stress, evaporation of sweat from the skin surface is the main avenue for heat loss (Parsons, 2003). An increase in skin blood flow is required for elevated levels of sweat secretion to the skin, and cutaneous vasodilation mediates this response (Bruning *et al.* 2012). Warm sensitive neurons in the skin and core synapse with neurons in the spinal cord, which project to relay neurons in the LPBd. Activation of neurons from the LPBd project to the MnPO, activating the MPO, an area which send inhibitory projections to premotor neurons within the rMR (Nakamura and Morrison 2008b). When rMR neurons are inhibited, cutaneous arteries relax, allowing an elevation in skin blood flow and thus heat loss. These nerves are cholinergic, in that

they release acetylcholine onto the vasculature to stimulate relaxation of the vessel. In addition to acetylcholine, there are co-factors released from endothelial cells which permit full vasodilation, and the best known vasoactive molecules are nitric oxide and prostacyclin. Skin microdialysis infusion of nitric oxide synthase and cyclooxygenase antagonists have been used to determine the roles of these products in vasodilatory responses to heat stress, where they act in a paracrine manner (Melikian *et al.* 2009). In thermoneutral conditions, Kellogg *et al.* (1998) demonstrated that local antagonism of nitric oxide synthase by NG-nitro-L-arginine methyl ester (L-NAME) reduces the cutaneous vascular conductance (CVC) response to heat stress by ~14% compared with a control. Briefly, CVC is an index of vasodilation, and is relative to the CVC achieved by a potent vasodilator assumed to cause maximal vasodilation (CVC_{max}), normally nitroprusside. In the control trial, $\%CVC_{max}$ rose to ~44% during heat stress (1°C rise in T_c), while CVC reached ~30% max following L-NAME microdialysis. Importantly, there is a greater reliance on nitric oxide for full vasodilation in older subjects. Holowatz et al. (2003) used a water perfused suit to raise the T_c of seven young adult subjects by 1°C. During this level of hyperthermia, CVC rose to ~80 of max in young males, but microdialysis infusion of L-NAME reduced this by ~22%, suggesting that nitric oxide is only partly involved in the vasodilatory response to heat stress. Prostacyclin (PGI_2) is a key vasodilatory mediator, and its role in this regard is explained in section 2.4.3 because it is a product of COX bioactivity.

In a thermal neutral zone, the reflex responses to 2°C skin cooling are so efficient that local mechanisms do not contribute to vasoconstriction (Savage and Brengelmann 1996). Reflex vasoconstriction, triggered by skin thermoreceptors, is mediated by α-adrenergic receptors present on the membrane of endothelial cells. The use of pharmacological methods alongside measurements of cutaneous blood flow have been a useful tool in this field for the identifying the

chemical modulators involved. For instance, Stephens *et al.* (2001) used a combination of intradermal yohimbine (α-adrenergic receptor inhibitor)**,** propranolol (α and β-adrenergic receptor inhibitor), bretylium tosylate (blocks release of noradrenaline from axon terminals) and saline (control) to specify which receptors are involved in cutaneous vasoconstriction in response to skin cooling. Although β-adrenergic receptors are not directly involved in vasoconstriction, their inhibition was necessary to block the potential confounding effects of their receptor activation. When the mean T_{sk} was progressively decreased to 30.5ºC across a 15-minute period, cutaneous vascular conductance (CVC) was reduced by ~50% of baseline CVC in the saline treated sites, whereas it was only reduced to ~82% of baseline CVC in the yohimbine + propranolol treated site. Because intradermal yohimbine partially inhibited the cold induced decrease in CVC, it was clear that α-adrenergic receptors had a role in the reflex responses to cold stress. The combination of yohimbine and propranolol did not completely block cutaneous vasoconstriction in response to skin cooling, but did block a norepinephrine induced vasodilation, so it is likely that co-transmitters are involved in this process. In a later study, the same group investigated if neuropeptide y acted as a co-transmitter in this regard (Stephens *et al.* 2004). In response to graded skin cooling, microdialysis infusion of BIBP-3226 (a neuropeptide Y antagonist) reduced the normal CVC response to skin cooling by ~11% (55 and 66% of baseline CVC). However, when BIBP-3226 was combined with yohimbine, the reduction in CVC was only ~3% from baseline, an almost absent constrictor response. This demonstrates that neuropeptide Y is indispensable for full reflex cutaneous vasoconstriction. As there was no significant reduction in CVC following α-adrenergic receptor and neuropeptide Y blockade, it is unlikely that other co-transmitters are involved in autonomic cutaneous vasoconstriction. However, as T_{sk} was only reduced to a mild degree in the experiments described (Stephens *et al.*

2001; Stephens *et al.* 2004), it may not reflect the processes involved during more severe cold stress. This view is supported by evidence from our group that even a mild cold (20ºC) environment reduces T_{sk} to ~27ºC after 120 minutes of exposure. Because a T_{sk} of 30ºC is unlikely to elicit full vasoconstriction, it is possible that other factors are involved in this pathway. This was confirmed by evidence that inhibition of the Rho-A/Rho kinase (ROCK) signaling pathway blocked the vasoconstrictor response to skin cooling (T_{sk} = 26ºC) by 90% compared with saline in healthy participants. Interestingly, ROCK dependent vasoconstriction was more pronounced in hypertensive individuals, suggesting that ROCK antagonism could be beneficial in the treatment cardiovascular disease (Smith *et al.* 2013).

2.3.7 Shivering thermogenesis

Shivering thermogenesis is the largest contributor to heat production in an unacclimatised, cold exposed adult human (Blondin *et al.* 2014b). The biochemical mechanisms of shivering are homogenous with that of voluntary skeletal muscle contraction, which has been described in great-detail elsewhere (Allen *et al.* 2008; Baylor and Hollingworth 2012). Given that skeletal muscle makes up ~40% total body mass in the average middle aged human and ~25% basal metabolic rate (Rolfe and Brown 1997), it is not surprising that this mode of thermogenesis is employed early in response to a reduced T_{sk}. Surface electromyography (sEMG) is the most commonly used technique to quantify the intensity of shivering muscle in humans, where shivering intensity is expressed as a percentage of maximal voluntary contraction (% MVC). Several authors have used this method in humans (Bell *et al.* 1992; Blondin *et al.* 2010; Gagnon *et al.* 2014; Gosselin and Haman 2013; Haman *et al.* 2004a; Haman *et al.* 2004b; Haman *et al.* 2016; Israel and Pozos 1989; Sessler *et al.* 1988; Tikuisis *et al.* 1991) because it is relatively simple to apply and is non-invasive compared to intramuscular EMG. Electrode fibres for

intramuscular EMG can be inserted into the muscle of the anesthetised animal because they do not show any movement besides shivering during cold stress (Nakamura and Morrison 2011). Prediction equations are also available for peak shivering intensity ($shiv_{peak}$) based on body composition, oxygen consumption and metabolic rate (Eyolfson *et al.* 2001). However, use of this equation is now limited since brown adipose tissue also contributes to the increase in resting metabolic rate during cold exposure (Virtue and Vidal-Puig 2013).

The timing for shivering onset in various muscle groups was examined by Tikuisis *et al.* (1991). The researchers passively exposed 13 young males [7 lean (< 12% body fat), 7 normal (< 23% body fat)] to cold air (10ºC, 42% relative humidity) and analysed sEMG patterns in the muscle groups within the trunk and legs (*pectoralis major, rectus abdominis, bicep femoris, rectus femoris*), and outer limbs (*brachioradialis, gastrocnemius*). In the lean subjects, shivering in the trunk muscles began in under 10 minutes, whereas there was no EMG signal from the outer limbs until ~40 minutes. In the subjects with higher body fat, shivering onset was similar in the pectoralis major (~3 minutes), but was delayed by ~40 minutes in the bicep femoris, ~15 minutes in the rectus abdominis, and ~20 minutes for the rectus femoris, and there was no EMG signal detected in the limbs in 5 of the 7 subjects with higher body fat. This work demonstrates that body fat % is a critical predictor of shivering onset during cold exposure i.e. less body fat insulation results in a faster shivering onset. Additionally, it shows that the pectoralis major is recruited for shivering thermogenesis early into acute cold exposure. In a subsequent paper using identical exposures, the researcher group investigated the relative shivering intensity from each muscle, which was expressed as % MVC (Bell *et al.* 1992). The authors demonstrated a differential shivering intensity between the 'lean' and 'norm' subjects. For example, the *pectoralis major* reached a shivering intensity of ~16% MVC in the 'lean' subjects, whereas this

was only ~4% MVC in the 'norm' group. This effect was not seen in any other muscle group. Differences in T_{sk} between the groups may have accounted for the differences between groups, but this was not reported.

Reductions in mean T_{sk} can serve as a strong predictor of shivering intensity during cold exposure. However, it cannot be used in isolation since BAT thermogenesis may also contribute to thermogenesis during skin cooling (section 2.3.8). Using a liquid perfused suit, Haman *et al.* (2005) compared the thermogenic responses in subjects exposed to 5ºC (n = 8) and 10ºC (n = 6) water, where after 90 minutes, mean T_{sk} decreased by 8.4 and 6.8ºC in the 5 and 10ºC perfusions, respectively. Incorporating a prediction equation for $shiv_{peak}$ (Eyolfson *et al.* 2001), the authors demonstrated that relative shivering intensity was ~42% $shiv_{peak}$ in the 10ºC condition, and over 57% $shiv_{peak}$ in the 5ºC condition. The impact of cold acclimation on the contribution of shivering during acute cold exposure was examined by Blondin *et al.* (2014a). Using a water perfused suit, 6 non-acclimatised males were exposed to 10°C for 2-hours daily for 4-weeks, with any change in brown adipose tissue volume, oxidative capacity, and shivering measured pre-and post-acclimation. Although brown adipose tissue volume and oxidative capacity increased by 45 and 119%, respectively, there was no change in the shivering response to cold stress following acclimation. Thus, a longer period of cold acclimation or a more stressful cold stimulus may be required for brown adipose tissue to be fully recruited in place of shivering, although it is possible that cold acclimation may have reduced shivering activity in deeper muscles not measured by sEMG.

There appear to be two distinct patterns of shivering in response to skin cooling, both of which can be identified by EMG patterns (2-5% and 7-15% of MVC) and unit frequency (8-10 Hz vs 0.1-0.2 Hz), each mediated by oxidative type I and glycolytic type II fibres, respectively (Meigal

et al. 1993). Studies in the 1990's demonstrated this in the pigeon (Hohtola *et al.* 1998) and red-winged blackbird (Olson 1994), where the gastrocnemius produced short high intensity bursts compared to the pectorals, which produce sustained low intensity bursts. Two studies in the 1980's support this by demonstrating that glycogen content of the deltoid (type I, oxidative) and vastus lateralis (type II, glycolytic) was reduced equally by ~100 $mmol \cdot kg^{-1}$ after 60-minutes immersion in 18°C water (Martineau and Jacobs 1988, 1989). This suggests that there is a dynamic pattern of sustained shivering and high intensity bursts during prolonged exposure to cold. If shivering reduces muscle glycogen content, can it be sustained in this state through utilisation of lipids or proteins? If lipids and proteins substitute glucose to fuel heat production, then nutritional status is unlikely to impact T_c maintenance during acute cold exposure. This question was answered through manipulation of glycogen reserves prior to mild (Haman *et al.* 2004a) and moderate (Martineau and Jacobs 1989) cold exposure, eliciting an RMR increase of 2.5 and 3.5 fold, respectively.

Haman and colleagues (2004a) exposed 10 males to cold (5°C water perfusion for 90 minutes) in a glycogen loaded (high carbohydrate diet of 494 g/day for 7 days without exercise) and glycogen depleted (low carbohydrate diet of 68 g/day for 7 days and prior exercise) state. They showed that the total shivering intensity of 8 muscles and unit frequency did not change with glycogen depletion or loading, an effect compensated for by a large shift in substrate utilisation. The changes in fuel utilisation required to sustain shivering thermogenesis for 90 minutes in a glycogen loaded and depleted state are shown in Table 2.2. This finding supports the view that resting nutritional status does not influence shivering parameters during exposure to cold, at least for 90 minutes. However, nutritional status can still confound total heat production during acute cold stress due to the potential influence of diet-induced thermogenesis (Ishii *et al.* 2016).

The substrate utilisation patterns to fuel thermogenesis were recently analysed during a cold survival simulation (Haman *et al.* 2016). Prior to this work, substrates for fuelling thermogenesis was only reported for much shorter durations (< 3 hours), however, subjects (n = 8) were exposed to 7.5°C for 12-24 hours in the survival simulation.

Table 2.3. Relative contribution of carbohydrates, lipids, and proteins for ATP production during shivering thermogenesis. Data taken from Blondin *et al.* (2014b).

	Glycogen loaded	**Glycogen depleted**
Carbohydrates	~65%	~28%
Lipids	~23%	~53%
Proteins	~12 %	~19%

Participants were lightly clothed (~0.61 Clo) and were fed a 1,641kcal survival ration bar every 6-hours, and shivering thermogenesis from the *pectoralis major, trapezius, rectus abdominis* and *rectus femoris* was analysed for one hour at 6, 12, and 24 hours. The average total shivering activity of the four muscles was 4.5% (6 hours), 7.2 (12 hours) and 5% (24 hours) MVC, however there was a striking modification in fuel utilisation as time progressed. Carbohydrates were the preferred fuel in the period 0-6 hours, however lipids accounted for over 80% of total heat production between 12-24 hours, with protein accounting for less than 20% in all time periods. This is reflected by a near complete depletion of muscle glycogen content at 12 hours. These data demonstrates the remarkable capacity of the human body to mobilise lipid stores to maintain shivering thermogenesis, and aid survival during prolonged extreme cold exposure. This proffers that pharmacological activation of thermogenesis may play an important role as an anti-obesity treatment, a notion which has recently gained popularity (Xiao *et al.* 2015).

In summary, if there are sufficient stores of lipids or glycogen, shivering thermogenesis is a significant contributor to heat production regardless of nutritional status. In humans, the most effective method to analyse shivering thermogenesis is the use of sEMG because it is non-invasive and can be applied to multiple muscle groups [researchers should refer to Haman *et al.* (2004a) for an in depth methodological overview of this technique for the analysis of shivering activity]. In the next section, attention is given to the contribution of non-shivering thermogenesis to total heat production during acute and chronic cold exposure. Although brown adipose tissue and skeletal muscle contribute to non-shivering thermogenesis, there is a greater focus on brown adipose tissue in this section due to the vast amount of literature in this area. However, attention is still paid to that of non-shivering thermogenesis from skeletal muscle tissue.

2.3.8 Non-shivering thermogenesis

Brown Adipose Tissue: What is it and how is it measured?

There are two distinct forms of adipose tissue in the human body; white and brown, where the primary functions of each are to store and dissipate nutrient energy, respectively. Brown adipose tissue is found in all mammals (albeit in different volumes and activities), and it can be argued that the development of this organ gave our animal group a distinct advantage from an evolutionary perspective. For example, it supports T_c stability during periods of hibernation, allows neonates to survive the cold stress of birth, and protects against excessive storage of lipids through clearance of fatty acid to fuel thermogenesis. A long-held belief surrounding brown adipose tissue was that it was only found in infants, and was of no physiological significance in the few years following birth i.e. its volume of activity regresses with age. However, this view changed since the use of positron emission tomography (PET), which has allowed researchers to

image brown adipose tissue and identify the anatomical regions of its deposits. It has been demonstrated that, in adults, brown adipose tissue was present in physiologically significant volumes (Cypess *et al.* 2009). In that study, the authors analysed 3640 PET-CT scans from 1972 patients and consistently found substantial depots of brown adipose in the neck, supraclavicular and paravertebral regions. Additionally, the likelihood of brown adipose tissue detection was reduced with increasing age, outdoor temperature, and beta blocker use. In the same year, it was also shown (using the PET-CT method) that brown adipose tissue could be induced by acute cold exposure (van Marken Lichtenbelt *et al.* 2009) and norepinephrine administration (Hwang *et al.* 2015). These studies are described in more detail below.

Brown adipocytes form during embryonic development and are entirely distinct from white adipocytes. To generate heat, brown adipose tissue uses endogenous lipids as fuel source for the generation of NADH and $FADH_2$ in the citric acid cycle. In the mitochondria of non-brown adipocytes, these electrons carriers are used in the respiratory chain for the generation of adenosine triphosphate (ATP). However, there is a protein unique to brown adipocytes that uncouples the respiratory chain, such that the protons in the intermembrane space permeate readily back into the mitochondrial matrix, decreasing the gradient between these two spaces. The first law of thermodynamics states that energy cannot be created or destroyed, only converted from one form to another. Thus, the energy which would normally fuel ATP production is simply converted to a different form, heat. The protein in question is often referred to as thermogenin or uncoupling protein 1 (UCP-1); the nomenclature UCP-1 will be used in this thesis.

Heat production from brown adipose tissue is initiated in line with activation of lipolysis. More specifically, the increased concentration of intracellular free fatty acids is the fuel for UCP-1

mediated thermogenesis. The signalling cascade for lipolytic or glycolytic enzyme activation originates in the CNS. Disinhibited pre-motor neurons in the DMH and rMR send sympathetic efferent projections to brown adipose tissue via the ventral horn.

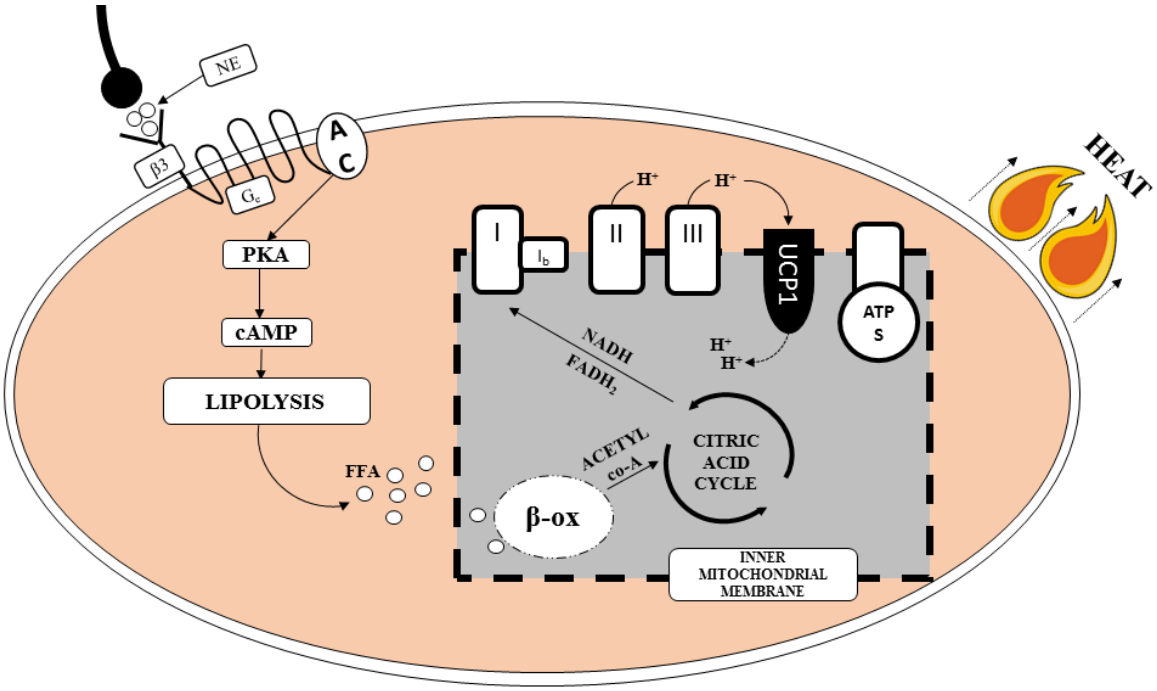

Figure 2.1. Norepinephrine induces lipolysis in brown adipocytes through the cAMP pathway. Free fatty acids diffuse into the mitochondrial membrane and are subject to beta-oxidation, generating acetyl co-A. UCP-1 mediates thermogenesis in a brown adipocyte by uncoupling the respiratory chain. This uncoupling creates a futile cycle in which cellular metabolism remains high due to the constant exchange of H^+ ions in and out of the inner mitochondrial membrane.

As depicted in Figure 2.1, lipolysis in the brown adipocyte is mediated by norepinephrine binding to β3-adrenergic receptors, a process that switches on the cAMP signalling pathway via excitatory G protein-coupled receptors. This pathway phosphorylates enzymes required for breakdown of fats into free fatty acids, which are then utilised in the inner mitochondrial membrane for UCP-1 mediated heat generation.

Because brown adipose tissue dissipates nutrient energy as heat, it is heavily involved in adaptive thermogenesis during cold acclimation, and also acute cold exposure in acclimated

individuals (Blondin *et al.* 2014b). The first conclusive evidence that cold exposure stimulates brown adipose tissue activity was provided by van Marken Lichtenbelt *et al.* (2009), who scanned 24 males (PET-CT) on two occasions; in ambient air temperatures of 22 (thermoneutral) and 16°C (mild cold). They found that cold exposure increased the activity of brown adipose tissue in all but one participant, and body fat percentage was a significant negative predictor ($p < 0.05$) of overall activity (i.e. less fat equals increased activity). In accordance, the lone subject with no detectable ^{18}FDG uptake in brown adipose tissue had the highest percentage of body fat (41.7 %). The mean brown adipose tissue activity [expressed in kiloBequerels (kBq)] in the obese subjects was 102 ± 93 kBq, but 428 ± 394 kBq in the lean subjects. It is also striking that one participant had virtually zero brown adipose tissue activity in thermoneutral conditions (0 kBq), but this increased to 856.6 kBq during cold exposure. More recently, Ouellet (2012) reduced the global T_{sk} of 6 male participants (BMI range, 23.7 to 31 kg·m^2) by 3.8 ± 0.4°C and monitored brown adipose tissue activity using ^{18}FDG (glucose tracer), and ^{18}F-fluoro-thiaheptadecanoic acid [^{18}FTHA (fatty acid tracer)]. There were substantial increases in brown adipose tissue metabolism, as marked by fatty acid [474 ± 66 vs 644 ± 73 μmol·min^{-1}) and glucose (not detectable vs 1,493 ± 460 μmol·min^{-1}) turnover in this tissue. Although these data confirms that acute cold exposure induces brown adipose tissue metabolism, the acclimation status of the participants was not accounted for; prior cold acclimation may explain the minimal contribution of shivering thermogenesis and variable glucose uptake patterns between subjects in prior work (Au-Yong *et al.* 2009; Cypess *et al.* 2009; van Marken Lichtenbelt *et al.* 2009). This notion is supported by many studies showing significantly greater basal brown adipose tissue activity in winter months compared with summer (Au-Yong *et al.* 2009; Ouellet *et al.* 2011; Persichetti *et al.* 2013). Interestingly, a review of studies which used PET-CT to quantify brown

adipose tissue activity revealed that there was a 1% decrease in total activity for every 5°C increase in ambient temperature at the time the experiment took place (Huang *et al.* 2012). Whether or not brown adipose tissue activity is a product of cold acclimation/acclimatisation was later addressed by Blondin *et al.* (2014a). In that study, 6 non-acclimated healthy males were passively exposed to 10°C for 2 hours for 4 weeks (5 $d \cdot wk^{-1}$) using a water perfused suit, and underwent PET-CT scanning at pre-and post-acclimation. The 4-week acclimation period significantly increased brown adipose tissue ^{18}FDG uptake by 45% (66 mL vs 95 mL). The net tissue glucose uptake was also significantly greater in brown adipose tissue compared with a selection of skeletal muscles involved in shivering thermogenesis. The oxidative capacity of brown adipose tissue was analysed using a tracer [^{11}C-acetate (^{11}C)] which is rapidly converted to acetyl-coA (Grassi *et al.* 2012). The area under the curve for ^{11}C radioactivity increased in brown adipose tissue and the *longus colli* muscle only. However, the authors demonstrated that the longus colli retained ^{11}C for non-oxidative metabolism, while it was utilised rapidly in brown adipose tissue for oxidative metabolism.

Beige/brite adipocytes: are they functionally thermogenic?

Besides classical white and brown adipocytes, there is a third fat cell that resides within white adipose tissue depots, yet displays molecular characteristics akin to brown adipocytes i.e. they are dense in mitochondria and express common genes (i.e. *Ucp-1*, *Cidea* and *Pgc1a*). These cells have been named brite (Rosenwald *et al.* 2013) or beige (Lee *et al.* 2014); the beige nomenclature will be used in this literature review. The high concentration of mitochondria within beige cells allow them to be easily characterised within white adipose tissue biopsies through microscopy.

Beige cells differ from brown adipocytes in several ways; i) they derive from different embryonic precursors (Sanchez-Gurmaches *et al.* 2012) ii) they do not express entirely the same genes, despite many commonalities (Harms and Seale 2013) and iii) beige cells do not show a basal expression of *Ucp-1*, rather, it is *induced* through activation of β-adrenergic receptors (Madsen *et al.* 2010). In contrast, classical brown fat cells have a very high basal expression of *Ucp-1* without the need for prior β-adrenergic receptor activation. Unlike classical brown adipocytes, beige cells do not appear to form during embryogenesis. Instead, they arise through activation of precursor cells residing in white fat depots, or transdifferentiation of white fat cells into beige cells during cold exposure or norepinephrine treatment. Whether beige fat cells arise through precursor cells or white fat cell transdifferentiation may depend on the anatomical location of white fat tissue (Harms and Seale 2013). For example, Wang *et al.* (2013) labelled mature white fat cells with the *LacZ* gene to determine if beige cells (induced by cold exposure) were derived from pre-existing white fat cells or precursor cells in subcutaneous fat depots. Because newly formed beige cells did not express the *LacZ* gene, it can be concluded that these cells derive from *de-novo* adipogenesis. It is striking that cold-induced beige cells return to classical white adipocytes after only 5-weeks of exposure to a warm environment (Rosenwald *et al.* 2013). Madsen et al (2010) showed in mice that blocking UCP-1 induction in beige cells during short-term cold exposure (5°C) reduced T_c by up to 2°C after 12 hours. Unfortunately, T_c was only measured at pre, 12, and 24 hours into the exposure, so it is unclear if the beige cell also contributed to thermoregulation in a more acute manner (i.e. in the first few hours). Interestingly, the authors showed that COX activity was indispensable for cold stress mediated UCP-1 induction in this cell type, since this process was inhibited by indomethacin (a non-selective COX inhibitor). This will be expanded upon in section 2.5.3.

The physiological mechanisms regulating thermogenesis have been described thus far, with special attention paid to cutaneous thermosensation, the central nervous system, and the involuntary responses activated by a reduced T_{sk}. In the next section, attention is given to the cyclooxygenase (COX) enzyme, covering its biology, roles in lipopolysaccharide induced fever, and its potential role in cold-induced thermogenesis.

2.4 Cyclooxygenase

2.4.1 Biology and mechanism of action

COX is an enzyme required for production of prostanoids. It uses arachidonic acid as a substrate for the generation of prostaglandin H_2 (PGH_2), a stable intermediate which can then be converted to prostaglandin E_2, I_2, D_2, or thromboxane (TXA_2) by cell specific isomerases and synthases. There are two COX isoforms, known most commonly as COX-1 and COX-2 (the nomenclature PGHS and PTGS are also used). COX-1 and COX-2 have a structural homology of ~60% but differ in their tissue distribution and regulation of expression (Zidar *et al.* 2009). Classic literature suggests that COX-1 is constitutively produced while COX-2 is only induced by cytokines, growth factors, and sheer stress. More specifically, COX-1 is commonly considered a 'housekeeping' enzyme which exhibits a basal production of prostaglandins for normal homeostatic functions. Conversely, COX-2 has long been considered an 'immediate early' enzyme because it only produced prostaglandins when stimulated by mediators such as cytokines, growth factors and sheer stress. However, this approach is simplistic since it suggests that there are no meaningful upstream regulators of *COX-1* gene transcription, and that COX-2 has no constitutive homeostatic function in any cell type. The reaction which allows COX to oxidise arachidonic acid occurs in two steps catalysed by two distinct active sites on the COX

enzyme (Dubois *et al.* 1998). COX enzymes contain two sites of metabolism, coined *cyclooxygenase* and *peroxidase* sites. Arachidonic acid conversion to PGH_2 is mediated through events in both active sites, whereby a tyrosine 385 amino acid be oxidised to form a radical [(Tyr·) (van der Donk *et al.* 2002)]. This is initiated in the *peroxidase* site by reduction of peroxides, yielding a heme ferryl proporphyrin radical [$Fe(IV)PP^{\cdot+}$]. The $Fe(IV)PP^{\cdot+}$ radical can then be transferred to the tyrosine 385 in the *cyclooxygenase* active site, resulting in Tyr· and a reduced form of The $Fe(IV)PP^{\cdot+}$ [Fe(IV)], which is further reduced by 1 electron to regenerate the resting heme [Fe(III)]. This heme reduction can be initiated by many reducing co-substrates within cells, and is essential for starting a new *peroxidase* cycle (Aronoff *et al.* 2006; Boutaud *et al.* 2002). The Tyr· initiates the oxygenation of arachidonic acid through proton abstraction, generating a radical species that is further oxidised by 2 molecules of oxygen (Wu and Tsai 2016). This step yields a PGG_2 radical that is then reduced to PGG_2, regenerating the Tyr· in the process, and once PGH_2 is produced, it is used as a substrate by cell specific isomerases which then generate PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$, or TXA_2 (Mitchell *et al.* 1993).

In 2002, Chandrasekharan and colleagues cloned a third isoform that is derived from the COX-1 gene but retains intron 1. This novel COX isoform, termed COX-1b, is abundantly expressed in canine cerebral cortex and to a lesser extent in human cerebral cortex and aorta (Chandrasekharan *et al.* 2002). Although this protein appeared to be the site of analgesic drugs acetaminophen, antipyrene and aminopyrene (Ayoub *et al.* 2004; Botting and Ayoub 2005) a COX-1 cDNA cloning experiment would later demonstrate that intron 1 is out of frame in humans (Dinchuk *et al.* 2003), and thus would require downstream editing (i.e. ribosomal frame shifting) to yield a functional protein. Interestingly, even when the frame shift is corrected by site-specific mutagenesis, human COX-1b was active but not inhibited by acetaminophen (Shen

et al. 2015). Since then, there has been very few studies investigating the biology and bioactivity of human COX-1b, likely due to a lack of therapeutic potential arising from its selective inhibition.

Each isoform displays a myriad of unique and diverse functions. For example, products derived from COX-1 are crucial for gastrointestinal motility (Josephs *et al.* 1999), platelet aggregation (Huang *et al.* 2016), and parturition (Olson 2003). Selective COX-1 inhibitors such as aspirin are thus considered protective against ischemic events such as heart attack and stroke due to their anti-thromboxane (and thus, anti-clotting) activities (Calonge *et al.* 2009). However, regular use of aspirin and other non-selective COX inhibitors (i.e. ibuprofen) are also implicated in the development of stomach ulcers and other gastrointestinal complications. In contrast, the *COX-2* gene is upregulated in response to inflammatory stimuli (Chang *et al.* 2006), growth factors (Harding *et al.* 2006) and sheer stress (Inoue *et al.* 2002) The COX-2 gene holds binding sites for many transcription factors such as nuclear factor κB, activator protein 1, and cAMP response elements (Appleby *et al.* 1994). Thus, COX-2 is involved in pain (Gangadharan and Kuner 2013) inflammation (Seibert and Masferrer 1994) fever production (Ootsuka *et al.* 2008; Romanovsky *et al.* 1997), and neonatal development (Smith *et al.* 2012). However, more recent evidence has demonstrated that COX-2 is also constitutively expressed in the gastrointestinal tract, brain, thymus, and kidneys (Kirkby *et al.* 2013), and it is also involved in protection against the development of atherosclerotic lesions independently of COX-1 derived PGI_2 (Kirkby *et al.* 2014). Despite putative roles for COX-2 in gastro-intestinal homeostasis, selective COX-2 inhibitors have established efficacy in the treatment of pain and inflammation, while exhibiting no gastrointestinal side effects as shown with non-selective COX inhibitors.

2.4.2 Role of COX in the febrile response to lipopolysaccharide

Fever is a complex physiological response to infection or inflammation, the key feature of which is an increased T_c. This increase in T_c is a fundamental characteristic of fever as it activates and utilises elements of the heat shock response pathway to modify gene expression, cellular signalling and immune cell mobilisation to sites of infection or inflammation (Singh and Hasday 2013). COX-2 is a principal mediator of this febrile response (Engblom *et al.* 2003; Fabricio *et al.* 2006; Ushikubi *et al.* 1998), such that antipyretic (anti-fever) drugs work by inhibition of COX-2 metabolism. After systemic administration of a potent fever inducing compound [lipopolysaccharide (5-100 $\mu g \cdot kg^{-1}$)], the COX-2 gene is upregulated in the brain (endothelial cells), and in liver and lung macrophages (Romanovsky *et al.* 1996). Experimental, lipopolysaccharide induced fever embodies two distinct phases [i.e. T_c peaks (Romanovsky *et al.* 1997)]. The early phase of fever (peaking ~1 h after lipopolysaccharide injection) involves PGE_2 release from lung and liver macrophages to the systemic circulation, which rapidly binds to albumin, and is subsequently delivered to the blood brain barrier (Romanovsky *et al.* 1996). At the blood brain barrier PGE_2 dissociates from albumin and is transported to the pre-optic area, where it exerts potent febrile (thermogenic) effects. In the late phase of fever (peaking ~1 to 6 hours after lipopolysaccharide injection), inflammatory cytokines produced by circulating polymorphonuclear leukocytes (Beeson 1948) act on endothelial cells within the brain vasculature to trigger the production of PGE_2 synthesising enzymes in these cells (Matsumura *et al.* 1998). In addition, PGE_2 may also be released from perivascular microglia and meningeal macrophages throughout various brain regions (Elmquist *et al.* 1997).

Importantly, whether PGE_2 is synthesised in the brain or peripheral organs (a process dependent on which phase of fever is present), thermogenic effects are exerted by PGE_2 acting on EP_1 and

EP_3 receptor expressing neuronal populations in the pre-optic area. These neuronal populations were described in detail earlier in this thesis (section 2.4.4) Activation of the EP_3 receptor (by the binding of PGE_2) in the pre-optic blunts the activity of GABAergic projection neurons descending from the pre-optic area to the DMH or to the rMR (Yoshida *et al.* 2003). This PGE_2/EP_3 receptor interaction inhibits GABAergic neuron drive by reducing intracellular cAMP levels (Steiner *et al.* 2002), an effect mediated by the protein kinase C pathway (Akundi *et al.* 2005). The resulting disinhibition of GABAergic neurons in the DMH and sympathetic premotor neurons in the rMR activates spinal motor output mechanisms which switch on shivering, brown adipose tissue, and cutaneous vasoconstriction (Nakamura 2011). Taken together, these mechanisms are capable of raising human in-vivo T_c to > 40°C without increases in environmental temperature, an immune response that may be crucial in the defence of infection [due to activation of the heat shock response described previously (Roth and Blatteis 2014)].

2.4.3 Role of COX in non-febrile thermoregulation

Despite the accepted roles of the COX enzyme in febrile thermoregulation (described above), its role in non-febrile thermoregulation is a relatively new concept. Given that many over-the-counter and prescription drugs work by blocking the function of COX, it is important to learn if this has a downstream effect on normal thermogenesis. Consequently, ingestion of a COX inhibitor during cold exposure could disrupt thermogenesis and contribute to hypothermia. The evidence concerning COX in non-febrile thermoregulation is discussed below.

Cold exposure or injection of PGE_2 into the pre-optic area stimulates neurons in the rMR and DMH (Nakamura *et al.* 2002; Nakamura 2011; Nakamura and Morrison 2011; Yoshida *et al.* 2003). This neuronal activation drives the physiological responses necessary during fever (to raise T_c) or cold stress (to maintain T_c). Given that EP_3 receptor expressing neurons and cold

sensitive neurons innervate the same premotor neurons (i.e. DMH and rMR), it is possible that EP_3 receptor activation (through binding of PGE_2) within these areas might aid in, or even drive the thermoregulatory response to cold stress in non-febrile humans. The efficiency in which PGE_2 initiates thermogenesis during fever would make this a useful mechanism for humans to defend their T_c in cold environments. Theoretically, hypothalamic PGE_2 production may increase in response to depolarisation of cold sensitive neurons, although this has not been directly tested. This theory is supported by evidence in animal models (Ayoub *et al.* 2011; Ayoub *et al.* 2004; Bizzi *et al.* 1965; Satinoff 1972), and humans (den Hertog *et al.* 2009; Kasner *et al.* 2002; Mauger *et al.* 2014; Rollstin and Seifert 2013), whereby COX inhibitors (and thus reduced PGE_2 synthesis) have been shown to cause dose dependent T_c reductions in the absence of fever or immune response (non-febrile). In the following sections, non-febrile animal and human studies that have used COX/PGE_2 inhibitors during cold stress, and its effect on T_c regulation, will be discussed. In Table 2.4, several cases of hypothermia induced by COX inhibitors are displayed.

Evidence in animal models

The COX inhibitor salicylate reduced the capability of experimental rats to defend their T_c in cold (2 to 5°C) ambient conditions (Bizzi *et al.* 1965; Satinoff 1972). As COX is an enzyme required for the conversion of arachidonic acid to PGH_2 (Dubois *et al.* 1998), these data (Bizzi *et al.* 1965; Satinoff 1972) suggests COX may participate in cold defence, as blockade of PGE_2 via salicylate significantly decreased T_c compared with control. However, it should be acknowledged that the experimental effect observed (Bizzi *et al.* 1965; Satinoff 1972) may be

Table 2.4. Acetaminophen, ibuprofen, and hypothermia

Reference	Species	Subject(s)	Drug	Study type	Basal core Temperature	Environment	Outcomes
Kasner *et al.* (2002)	Human	Non-febrile stroke patients (n = 20)	APAP (650 mg every 4 hours)	Original research	36.96 ± 0.64°C	N/A	0.22°C mean reduction over 24 hours compared with the placebo (95% CI = -0.08 to 0.51°C, p = 0.14)
Visnjevac *et al.* (2014)	Human	Total Hip Arthroplasty patients (n = 74).	APAP (1 g intravenous) + general anaesthesia	Original research	N/A	N/A	APAP + general anaesthesia did not reduce T_c more than general anaesthesia alone. Reduction ~0.3 in both groups.
Rollstin *et al.* (2013)	Human	37-year-old female	APAP (50 g oral) + 2.5 g diphenhydramine	Case report	N/A	Patient found unconscious outdoors. Ambient temperature unknown.	Patient had a T_c of 17°C upon admission. Drug ingestion occurred ~17 h prior to being found. Diphenhydramine effects on thermoregulation unknown.
Ritter *et al*. (1998)	Human	16-year-old male.	IBU (570 mg·kg^{-1})	Case report	N/A	N/A	T_c on admission was 33.8°C. No other temperature data reported. The skin was cool and dry during initial physical examination.
Desai *et al.* (2003)	Human	7-year-old female.	IBU (6 mg·kg^{-1})	Case report	39.7°C	N/A	T_c fell to 32°C 4 h after IBU administration. Despite active warming T_c did not return to 37°C for 4 days.
Donati *et al.* (2016)	Human	Children aged 0-17 years old. (analysis of events from 1985-2015, n = 12)	IBU (60-270 mg)	Database analysis	N/A but IBU used to treat infection in all cases.	N/A	T_c fell to 34.3 ± 0.6°C in 12 reported cases. Hypothermia developed in < 24 h in all cases.

Ayoub *et al.* (2004)	C57/BL6 Mouse	Male (20 ± 2 g, n = 3 in each group)	APAP (100, 200, or 300 mg·kg^{-1})	Original research	37.6 ± 0.7°C	22 ± 1°C	Dose dependent hypothermia. 0.4, 0.8, and 2°C reduction in basal T_c following a 100, 200, and 300 mg·kg^{-1} dose, respectively.
Ayoub *et al.* (2011)	C57/BL6 Mouse	Male (20 ± 2 g, n = 5)	APAP (200 mg·kg^{-1})	Original research	~ 37.5°C (determined from figure 7. Not stated in results)	22 ± 1°C	~2.3°C T_c reduction at 1 h (figure 7). T_c returned to baseline at 2 h.
Li *et al.* (2008)	C57/BL6 Mouse	Male (25-30 g, n = 6)	APAP (160 mg·kg-1)	Original research	37 ± 0.2°C	23 ± 1°C	2-3°C reduction in T_c at 1 h. T_c maintained hypothermic for the following 3 h.

due to salicylate induced decreases in plasma free fatty acid; reduced free fatty acids can limit substrates available for metabolic heat production (Bizzi *et al.* 1965), especially in brown adipose tissue (section 2.4.8). Therefore, studies using salicylate may not reliably support the hypothesis that PGE_2 contributes to heat production during cold exposure. In contrast, studies using the COX inhibitor acetaminophen do not carry this confounding mechanism [i.e. decreases in plasma free fatty acid (Bizzi *et al.* 1965)]. When acetaminophen was administered intravenously (160 to 300 $mg \cdot kg^{-1}$) in non-febrile mice housed at 22°C, which is beneath their thermal neutral zone, T_c was reduced by ~3°C (Ayoub *et al.* 2011; Ayoub *et al.* 2004; Li *et al.* 2008). Interestingly, it was shown via ELISA that when the maximum T_c reduction was reached (following a 300 $mg \cdot kg^{-1}$ dose), this coincided with a 96% decrease in whole brain PGE_2 concentrations (Ayoub *et al.* 2004). These findings support the notion that PGE_2 located in the brain may play a crucial role in the regulation of non-febrile T_c, at least when mice are housed beneath their thermal neutral zone of 30°C (Ayoub *et al.* 2004; Karp 2012). However, it is important to note that in mice, the hypothermic action of acetaminophen may be independent of COX-2 inhibition, as COX-2 knockout mice showed an identical hypothermic response compared to their wild-type counterparts (Ayoub *et al.* 2004). Rather, the hypothermic effects of acetaminophen in mice may be due to inhibitory actions on the constitutive PGE_2 synthesising enzymes (COX-1). This experiment was later replicated (Li *et al.* 2008) using a lower dose (160 $mg \cdot kg^{-1}$), but with the same mouse strain (C57BL/6) and an identical PGE_2 extraction (Powell 1980) and quantification protocol (identical ELISA method). In that work (Li *et al.* 2008), the basal brain PGE_2 concentrations were ~400% greater (1500 pg·100 µl vs 300 pg·100 µl) than shown previously by Ayoub *et al.* (2004). This suggests that measurement error may have occurred in one of these experiments, however it is not possible to identify in which experiment

this error occurred, as PGE_2 concentrations have not otherwise been measured using whole brain samples within rodents. Interestingly, plasma PGE_2 was reduced by intravenous acetaminophen [160 mg·kg (Li *et al.* 2008)], although this did not reach statistical significance (inferential statistics not reported). This suggests that a reduced systemic PGE_2 might play a role in the hypothermic action of acetaminophen, and thus, systemic PGE_2 might play a role in non-febrile thermoregulation.

Evidence investigating a role for PGE_2 in cats failed to show a statistically significant role for PGE_2 as a cold-defensive molecule (Cranston *et al.* 1975). However, although the results from that study do not show a significant effect of acetaminophen on T_c during cold exposure (5 to 9°C), there is clearly a trend toward a reduced T_c in response to intravenous acetaminophen administration (50 mg·kg). More specifically, the mean T_c in cats treated with the acetaminophen was 0.3°C lower after 60 minutes of cold exposure (Cranston *et al.* 1975). To our knowledge, only one study (subsequently described) has previously investigated this notion (Murray *et al.* 2011b) within humans. Humans are unique among other species as we have primarily adapted in favour of efficiently losing body heat, which resulted in a substantial loss of body hair (Marino 2008b). Consequently, humans lack well insulating fur coats, which is a feature used in cold defence by the mammals [cats and mice (Terrien *et al.* 2011)] previously studied to investigate the relationship between PGE_2 and cold exposure (Aronoff and Romanovsky 2007). Thus, any data found in animal models (particularly mammals with fur coats) should be interpreted with care in the context of human translation (see section 2.3). The consensus opinion that PGE_2 does not contribute to heat production in humans during cold exposure (Aronoff and Romanovsky 2007) should not be ruled out as the reviewed data were primarily obtained from hosts that are devoid of the same cold defence mechanisms as humans.

Observational evidence in non-febrile humans

The possibility that PGE_2 might be involved in human afebrile thermoregulation is supported by several lines of research. In the Vigibase adverse drug reaction reporting database (Lindquist 2008), there have been 185 reports of hypothermia associated with ibuprofen (a non-selective COX inhibitor) and 247 with acetaminophen use (Lindquist 2008). Specific case examinations detail T_c reductions of over 10°C following acetaminophen overdose (Rollstin and Seifert 2013; Van Tittelboom and Govaerts-Lepicard 1989). Here, the doses ingested were so large (30 to 50 g) that they would cause complete blockade of PGE_2 production, and thus any heat production associated with its activity. In stroke patients, it has been shown that intravenous administration of acetaminophen (6 $g \cdot d^{-1}$ i.e. 2 g every 4 hours) lowered T_c in stroke patients that were non-febrile by ~0.4°C (Dippel *et al.* 2001; Dippel *et al.* 2003a). Although these findings support the routine use of acetaminophen in the management of T_c during acute ischemic stroke, it is difficult to speculate if blocked PGE_2 production caused the T_c reductions because the ambient temperature was not reported. Based on the evidence presented in the current work, it is possible that acetaminophen mediated PGE_2 blockade rendered the patient incapable of normal thermoregulation, an effect which may have been exacerbated if the patient collapsed in an environment beneath the thermal neutral zone. Thus, it may be speculated that blocking the production of PGE_2 (mediated by acetaminophen) inhibits the activation of spinal motor outputs, which would normally mediate heat generation (through shivering, brown adipose tissue activation, cutaneous vasoconstriction or tachycardia) in conditions set beneath the thermal neutral zone (Nakamura 2011). The evidence for an involvement of COX or PGE_2 in non-febrile thermogenesis is not limited to observational evidence in mammals. Table 2.3 details cases where COX inhibitors acetaminophen and ibuprofen have had a hypothermic side-effect. The

data presented in the next section details direct links between COX and specific thermogenic effectors.

UCP-1 induction and recruitment of beige adipocytes

There is no evidence for an involvement of COX in classical brown adipocyte formation or function. However, COX does appear to be required for beige cell proliferation and *Ucp-1* induction induced by cold stress or norepinephrine. Vegiopoulos *et al.* (2010) treated mice with the β3-adrenergic receptor agonist CL316243 (CL), and quantified the fold-change in *COX-1 and COX-2* genes in intra-abdominal white adipose tissue, along with plasma PGE_2. Three hours after treatment with CL (which simulates acute cold stress), there was a significant increase in plasma PGE_2 released from cultured intra-abdominal white adipose tissue (50 vs 120 $pg \cdot mg^{-1}$ white adipose tissue) and a 3-fold increase in COX-2 mRNA. When released by white adipose tissue, PGE_2 acts as a signalling molecule for proliferation of progenitor cells into beige adipocytes. Because PGE_2 is a potent thermogenic activator, it is possible that its release from white adipocytes (and thus, presence in plasma) causes an unintended thermogenic side effect. Indeed, injection of low dose PGE_2 (~0.08 µg) caused T_c to increase by 0.3°C (Ootsuka *et al.* 2008). Assuming a PGE_2 release rate from white adipose tissue of 120 $pg \cdot mg^{-1}$ in response to cold stress, the absolute plasma PGE_2 concentrations could exceed 0.2 µg. This represents a 150% increase in plasma PGE_2 compared to a concentration that already activates thermogenesis. Future studies should determine the relationship between fat % and total PGE_2 release during acute cold exposure.

Supporting work from Madsen *et al.* (2010) demonstrated that COX-2 deficient mice had a significantly lower T_c after 1 day cold exposure [4°C (36.5 vs 35.5°C)]. These data supports a role for COX-2 in the adaptive response to cold, but there were no early effects of COX-2 gene

deficiency on T_c maintenance. If COX-2 was directly involved in thermogenesis, then one may expect a more immediate difference in T_c between wild type and COX-2$^{-/-}$ mice, but there are caveats to this argument. Firstly, if a gene (in this case *COX-2*) is not expressed throughout the mouse's lifespan, it is possible that compensatory mechanisms develop to ensure mice maintain a constant T_c, especially if housed beneath their thermal neutral zone. Secondly, the acute hypothermic effect of acetaminophen is not inhibited in *COX-2*$^{-/-}$ mice, but is reduced by ~50% in *COX-1*$^{-/-}$ mice (Ayoub *et al.* 2004). This demonstrates that COX-1 is primarily involved in acute responses to cold, whereas COX-2 is involved in adaptive thermogenic responses.

Shivering

The role of COX in non-febrile shivering has not been extensively explored in humans. Recently, the effect of intravenous parecoxib (a selective COX-2 inhibitor) has been explored for its inhibitory effect on post-operative shivering (Li *et al.* 2014; Shen *et al.* 2015). Shivering is a common side-effect of general anaesthetics because they lower the T_c threshold required for metabolic heat production (Campbell *et al.* 2015). In a double-blind clinical trial, Li *et al.* (2014) separated 120 patients into three groups, in which they were administered parecoxib, tramadol, or a placebo. The total number of patients who shivered in the placebo group was 28, while only 7 and 6 patients shivered in the parecoxib and tramadol treated groups, respectively. These effects were replicated in a later study using similar methods, finding that shivering was observed in 55% of those treated with the placebo, but in only 22% of those of were administered parecoxib (Shen *et al.* 2015). Because parecoxib is a selective COX-2 inhibitor, this implies that the enzyme is required for non-febrile shivering. The criticism of these studies is their use of a subjective scale to assess shivering. While the pitfalls are obvious in quantifying shivering intensity, this method is likely acceptable for reliably stating whether shivering was

present in any form. Future studies should elucidate whether COX inhibitors reduce shivering in response to cold stress in humans, while incorporating sEMG for an accurate estimate of the relative intensity.

Vasoconstriction

Prostaglandins are well known for their conflicting roles in vasomotion, but whether they are involved in acute responses to thermal stress is not well understood. TXA_2 is COX-1 derived and primarily secreted from platelets. It acts as an arterial smooth muscle constrictor through opening L-type Ca^{2+} and TRP channels (Grann *et al.* 2016). Because contraction of this tissue increases mean arterial pressure, hypertensive patients are often prescribed aspirin (a robust COX-1 inhibitor) to reduce TXA_2 synthesis, which may reduce the risk of cardiovascular events (Calonge *et al.* 2009; Hermida *et al.* 2005a; Hermida *et al.* 2005b; Smith *et al.* 1980). If TXA_2 was involved in the autonomic pathways regulating vasoconstrictor responses to skin cooling, inhibition of COX-1 may attenuate sympathetic cutaneous vasoconstriction, leading to greater potential heat transfer from the skin to the environment. Using a daily regimen of either low (81 $mg \cdot day^{-1}$) or high dose (650 $mg \cdot day^{-1}$) aspirin for 7 days, Murray *et al.* (2011a) demonstrated that there was no effect on T_c control or heat production during a 120-minute passive exposure to cold (12°C) ambient air. However, it is unlikely that aspirin was present in the circulation during the cold exposure itself, since it was ingested many hours before each trial. Although aspirin acylates pre-existing COX-1, it would have no effect on COX-1 that is upregulated by cold exposure if it was not ingested immediately prior to the trial. Indeed, COX-1 is upregulated in response epinephrine *in vivo* (Suleyman *et al.* 2009), and epinephrine increased ~20 to 84% during acute cold exposure in humans (Radomski and Boutelier 1982; Wagner *et al.* 1987). Thus, it is possible that the cold induced increase in epinephrine elicits a rise in *COX-1* gene

expression, and the subsequent synthesis of TXA_2 could have a role in cold induced vasoconstriction.

The RhoA/Rho-kinase (ROCK) pathway contributes to reflex vasoconstriction within vascular smooth muscle cells during cooling (Lang *et al.* 2009). Compared with young adults, aged skin does not show a diminished ability to constrict in the cold, but is more reliant on ROCK for this to take place (Thompson-Torgerson *et al.* 2007). Activated ROCK elicits vasoconstriction through mobilisation of alpha2C adrenoceptors from the golgi apparatus to the cell membrane (Bailey *et al.* 2004). There is evidence that COX-1 induced TXA_2 receptors are involved in this pathway, upstream of ROCK activation. For example, human umbilical vein endothelial cells stimulated with a synthetic TXA_2 shows increased activation of ROCK, and Y27632, a ROCK pathway inhibitor, diminishes the physiological effect of TXA_2 in these cells (Song *et al.* 2009). Moreover, Grann *et al.* (2016) showed in isolated rat penile arteries that TXA_2 induced vasoconstriction is critically dependent on the ROCK pathway. This suggests that COX-1 is involved in ROCK mediated smooth muscle contraction, such that pharmacological inhibition of COX-1 may reduce cutaneous vasoconstriction evoked by skin cooling. Using microdialysis infusion of ketorolac, Medow *et al.* (2008) studied the effect of non-selective COX inhibition on cutaneous vascular conductance (CVC) in a thermoneutral environment. An interesting finding from that study was that ketorolac infusion (10 mM) increased resting CVC to ~32% maximal vasodilation, whereas CVC was ~16% max at the untreated site, providing strong evidence that COX vasoconstrictor products (likely TXA_2) are required for vasomotor tone even in thermoneutral conditions.

Vasodilation

During heat stress, acetylcholine is released from cholinergic nerve terminals to induce vasodilation (Holowatz *et al.* 2005). However, the function of acetylcholine is dependent on the activity of co-transmitters, namely nitric oxide and prostanoids. Kellogg *et al.* (2005) used microdialysis at different human forearm skin sites to infuse acetylcholine in conjunction with the non-selective COX inhibitor ketorolac. Acetylcholine infusion increased CVC to ~79% of maximum vasodilation. However, the addition of ketorolac with acetylcholine caused CVC to reach only 55% of max, and this decreased to only ~31% with ketorolac + L-NAME (a nitric oxide synthase inhibitor). This study demonstrates that COX activity is indispensable for acetylcholine induced vasodilation, but it was still unclear if ketorolac influenced skin blood flow in response to heat stress. Holowatz *et al.* (2009) examined the contribution of COX to vasodilation during normothermia and hyperthermia. In that study, the cutaneous vasculature of 13 middle aged males (53 ± 2 years) was treated with ketorolac with and without a T_c increase of 1°C. During normothermia (baseline T_c), ketorolac increased CVC by ~10% compared with the saline control (~18 vs 10% CVC max), but compared with saline, it had no effect on CVC in response to heat stress. In the L-NAME treated site, the rise in CVC evoked by heat stress was reduced by 30%, suggesting that nitric oxide is the primary contributor to vasodilation in response to a rise in T_c. On the other hand, it does appear that COX vasoconstrictor products (probably TXA_2) are involved in vasomotor tone in thermal neutral conditions.

Fujii *et al.* (2014b) examined the contribution of human nitric oxide and COX products in the forearm sweating and CVC response to exercise at a fixed moderate (400 Watts) and high (700 Watts) heat load, using continuous microdialysis infusion of L-NAME and ketorolac, respectively. At a moderate heat load, compared with control solution, ketorolac reduced forearm

sweat output ($p < 0.05$) to a level comparable with L-NAME infusion (~64, 54, and 55 $mg \cdot min^{-1} \cdot cm^{-1}$ with saline, ketorolac, and L-NAME, respectively). At a high heat load, there was no contribution of COX or nitric oxide to forearm sweating at a high heat load ($p > 0.05$). At both moderate and high heat loads, there was no influence of ketorolac on the CVC response at any time-point, whereas L-NAME reduced CVC by ~20% of max throughout the exercise trial. Given that ketorolac reduced sweat output without affecting skin blood flow (CVC), this suggests that COX may play a role within the sweat gland itself. Fujii *et al.* (2014a) examined the distinct role of COX and nitric oxide in the vasodilatory response to methacholine. Methacholine, an acetylcholine receptor agonist, was administered to 10 young adults (23 ± 4 years) via intradermal microdialysis at 1, 10, 100, 1,000 and 2,000 mM doses, and in conjunction with ketorolac, L-NAME, or a combination of the two. Methacholine caused CVC to reach ~100% max at doses of ≥ 100 Mm, but co-administration of ketorolac, L-NAME, or a combination had no influence on this response. As far as the sweating response, methacholine increased sweat output in line with the dose administered, yet ketorolac or L-NAME had no influence on sweat output compared with a saline control ($p > 0.05$).

The contribution of COX to reflex vasodilation may be age dependent. For instance, Fujii *et al.* (2016) studied the sweating and CVC response to 9 young (25 ± 4 years) and 9 older (60 ± 6 years) males in response to PGI_2 at incremental doses of 0.04, 0.4, 4, 40 and 400 μm, each at 25 minutes. PGI_2 increased %CVC max to 20, 37, 53, and 70% at the 0.04, 0.4, 4, 40 and 400 μm dose, which was similar in both young and older subjects. Despite the increased skin blood flow, PGI_2 administration did no induce sweating at any dose ($p > 0.05$). Interestingly, co-administration of the nitric oxide inhibitor NG-nitro-L-Arginine (L-NAA) decreased 4, 40, and 100 μm prostacyclin induced vasodilation by ~20% in the young adults, while it had no

inhibitory effect in the older subjects. This is suggestive of an age dependent crosstalk mechanism between PGI_2 and nitric oxide.

In summary, there are numerous pathways where COX may be involved in normal vasomotor tone, heat gain, and heat loss responses. Evidence for COX mediated vasodilation and sweating is equivocal, and is dependent on both age and the level of heat load placed upon subjects. PGI_2 is the primary vasodilatory COX product, and its effectiveness in this regard is highly dose dependent. Moreover, there is evidence that PGI_2 does not act independently, but instead, there may be a crosstalk mechanism involving nitric oxide. In contrast, there are numerous pathways by which COX products (particularly PGE_2) may be involved in non-febrile thermogenesis, but the tissue releasing PGE_2 is more difficult to predict. While norepinephrine induced PGE_2 release from white adipose tissue intriguing, studies have not yet reported plasma PGE_2 responses to cold exposure in healthy humans. Finally, evidence that acetaminophen induced hypothermia is blunted in COX-$1^{-/-}$ mouse strains suggest that COX-1 is involved in baseline T_c control, especially since brain PGE_2 concentrations were highly correlated with T_c reduction evoked by acetaminophen. In the next section, the pharmacology of acetaminophen and ibuprofen will be introduced.

2.5 Pharmacology of acetaminophen and ibuprofen

2.5.1 Pharmacokinetics

Acetaminophen

Acetaminophen is an over-the-counter drug used for the treatment of mild to moderate pain and fever. It is sold as a single entity formulation or in combination with opioid analgesics such as codeine. Owing to a different molecular mechanism of action, it has very weak anti-

inflammatory properties when compared with traditional NSAIDs such as ibuprofen and aspirin. This is discussed further in section 2.1.2. In adults, acetaminophen is normally prescribed in 1 g doses every 4 hours for the treatment of pain and fever (Bertolini *et al.* 2006). In laboratory settings, it is normally administered in doses corrected for body mass and lean mass to account for changes in the volume of distribution and volume of the liver (ethical constraint). Following oral ingestion, acetaminophen undergoes first-pass metabolism where ~15 to 25% is immediately metabolised in the liver. The majority (~90%) of acetaminophen is conjugated (bound) to glucuronide and sulphate to form non-toxic metabolites APAP-G and -S, respectively. A small amount is converted to N-acetyl-*p*-benzoquinoneimine (NAPQI) by the P450 family of enzymes in the liver (Zurlinden and Reisfeld 2016). This highly reactive metabolite is immediately conjugated with glutathione after therapeutic dose ingestion, but causes severe liver necrosis after toxic doses due to glutathione depletion (Hinson *et al.* 2010). Thus, it has been shown that glutathione administration following high dose acetaminophen is hepatoprotective, and P450 enzyme induction causes further damage. Following glutathione conjugation, the NAPQI-G molecule is rapidly converted to cysteine which is then eliminated in the urine (Mitchell *et al.* 1973). Oral acetaminophen reaches maximum plasma concentration (T_{max}) ~60-120 minutes after ingestion at therapeutic doses. Using gas chromatography, Rawlins *et al.* (1977) demonstrated that acetaminophen C_{max} was attained after ~60 minutes in healthy adult humans. When Singla *et al.* (2012) examined this at higher doses, the mean T_{max} increased to 100 ± x minutes, although no nutritional controls are outlined in that work which likely increase variability. When acetaminophen is ingested in a fed state, concentration max (C_{max}) was reduced by nearly 50%, and the AUC was reduced from 43.5 to 34.6 $\mu g \cdot h^{-1} \cdot ml^{-1}$ (Moore *et al.* 2015). At therapeutic doses, the binding of acetaminophen to plasma proteins is extremely low (~1-5%) compared with

selective COX-2 inhibitors such as celecoxib. The half-life of acetaminophen is ~4 h after therapeutic doses, but is dependent on frailty, nutritional status, and dose. In a group of 55 older people (77 to 84 years old, 29 healthy vs 26 frail), frailty significantly increased the half-life of acetaminophen following a 1000 mg oral dose [2.7 ± 0.5 h in the fit older subjects vs. 3.4 ± 1.2 h in the frail older subjects (Ellmers *et al.* 1991)]. Acetaminophen has a volume of distribution of ~1-2 litres·kg^{-1}, passes through most tissue types via passive diffusion, and readily crosses the blood brain barrier, reaching peak cerebral spinal fluid concentrations ~4 hours after a 1.5 g dose (Singla *et al.* 2012).

Ibuprofen

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) available over-the-counter and on prescription. For over-the-counter use (800 to 1200 mg·day^{-1}), it is recommended for the treatment of mild to moderate pain and inflammation, such as in headache, back pain, toothache, fever, and musculoskeletal ache (Bushra and Aslam 2010). On prescription, where doses are normally 1800 to 2400 mg·day^{-1}, it is used for chronic conditions such as osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and other chronic conditions (Bushra and Aslam 2010). It is also administered to paediatric patients for acute pain and fever at doses of 5 to 10 mg·kg^{-1}, where it is recommended above aspirin due to an increased safety profile, and is also preferred over acetaminophen due to enhanced analgesic and antipyretic effects (Rainsford 2009). This may be due to alternative mechanisms of COX inhibition. Ibuprofen is administered as an equal (racemic) mixture of *R* and *S* enantiomers, where (*S*)-ibuprofen is considered responsible for much of its therapeutic effects, and (*R*)-ibuprofen is considered inactive (Rudy *et al.* 1991). When (*R*)-ibuprofen enters the liver via the portal circulation, 50-65% is converted to the *S* enantiomer by the α-methylacyl-coenzyme A racemase enzyme (Rudy *et al.* 1991).

Regarding prostanoid synthesis, (*S*)-ibuprofen inhibition of this pathway *in vitro* was 150 times more potent than (*R*)-ibuprofen (Evans 1992). Moreover, (*S*)-ibuprofen inhibited TXA_2 formation in the platelet with 10 times greater potency than a racemic mixture of ibuprofen (*S* + *R*), while (*R*)-ibuprofen was ~10-fold less potent than *S* and *R* combined (Villanueva *et al.* 1993). The pharmacodynamics of ibuprofen are described in detail in the next section. Ibuprofen is most commonly prepared in an oral formulation, although it is available via intravenous administration in the USA. Topical and rectal formulations are also available in most Western regions. After oral administration, ibuprofen reaches C_{max} at 1 to 2 hours, but this depends on the coating of the capsules administered and whether it is ingested with or without food. For instance, Legg *et al.* (2014) compared the plasma concentration response to five oral formulations (Advil film-coated, Advil FastGel, Nurofen, Advil, and Nurofen Express) containing 400 mg ibuprofen in 71 participants in a fasted state. Advil film-coated, FastGel, and Nurofen express all reached C_{max} within 40 minutes of oral administration, whereas standard Advil and standard Nurofen took between 80 and 120 minutes to reach C_{max}. Ibuprofen has a low apparent volume of distribution (~0.1 to 0.2 $l \cdot kg^{-1}$) since it is highly bound to plasma proteins [~98% (Mazaleuskaya *et al.* 2015)], resulting in less transport into extravascular compartments compared with drugs with low plasma protein binding i.e. acetaminophen. Unbound ibuprofen readily crosses the blood-brain-barrier (BBB) with considerable efficacy. Parepally *et al.* (2006) showed that rat brain uptake of ibuprofen was not inhibited by the addition of pyruvate, probenecid, digoxin, or valproate, showing that BBB transporters MCT_1, OAT_3 and MRP are not involed in the uptake of ibuprofen into the brain. Ibuprofen is extensivley bound to albumin *in vivo,* and replication of this binding (through the addition of albumin into the ibuprofen preparation) inhibited ibuprofen brain uptake in a dose dependent manner. In live humans,

measuring brain concentrations of any drug is not possible, so the cerebral spinal fluid (CSF) concentration is used as a surrogate for brain concentrations. Work from Kokki *et al.* (2007) showed that ibuprofen readily crosses the BBB in children during inguinal surgery (ages 3 months to 12 years). In that study, the CSF t_{max} was 30 to 40 minutes post infusion of 10 $mg \cdot kg^{-1}$ ibuprofen, and concentrations exceed the unbound plasma concentration in all subjects after 30 minutes. In 46 adult patients undergoing lumbar puncture for nerve compression pain, oral administration of ibuprofen (800 mg) resulted in a plasma and CSF t_{max} of 1.5 and 3 hours, respectively (Bannwarth *et al.* 1995). In contrast to paediatric patients, it took ~90 minutes for the CSF concentration to exceed the unbound plasma concentration of ibuprofen. The half life of ibuprofen in the plasma and CSF was 2.5 and 7.5 hours, respectively.

2.5.2 Pharmacodynamics

Acetaminophen and ibuprofen reduces the synthesis of prostanoids through actions on different active sites of the COX homodimer. Acetaminophen acts on the *peroxidase* active site, whereas ibuprofen (and most NSAIDs) binds to COX on the *cyclooxygenase* active site. As a phenol, acetaminophen reduces the oxidative state of the enzyme, allowing the heme to return to its neutral resting state (Boutaud *et al.* 2002). Since acetaminophen acts as a reducing co-substrate, there is production of acetaminophen in a radical form, and this serves as evidence in favour of acetaminophen acting in this manner (Boyd and Eling 1981). In normal COX metabolism, hydroperoxides oxidise the heme within the *peroxidase* site, yielding a ferryl protoporphyrin IX radical cation. The radical from this form of the heme is donated to a neighbouring Tyr 385 amino acid, generating the Tyr radical required for subsequent generation of PGG_2 (Dubois *et al.* 1998). Because of this mechanism, the potency by which acetaminophen inhibits prostanoid synthesis is dependent on the intracellular concentrations of hydroperoxides, also known as the

peroxide tone. This was confirmed by studies showing that the addition of glutathione peroxidase (a hydroperoxide scavenger) enhances the efficacy of acetaminophen in both COX-1 and COX-2 (Hanel and Lands 1982; Ouellet and Percival 2001). Later experiments confirmed this hypothesis by treating cells with *tert*-butyl hydroperoxide and showing the inhibitory effect of acetaminophen on prostanoid synthesis was inversely proportional to the concentration of *tert*-butyl hydroperoxide (Boutaud *et al.* 2002). Acetaminophen is a potent inhibitor of COX in the central nervous system, endothelial cells (Boutaud *et al.* 2002), neurons (von Bruchlausen and Baumann 1982), and circulating leukocytes (Hinz *et al.* 2008). In contrast, it has very little capacity to inhibit COX bioactivity in platelets or in any locally inflamed tissue (Lages and Weiss 1989). Again, this cell and tissue selectivity is due to differences in the peroxide tone of these cells.

Ibuprofen's primary mechanism of action is inhibition of the COX pathway. It is a non-selective COX inhibitor, since it is equipotent in its inhibition of COX-1 and COX-2 activity. Neupert *et al.* (1997) drew whole blood from adult humans which was i) allowed to clot for 1-hour (to induce COX-1 activity), or ii) stimulated with lipopolysaccharide (to induce COX-2 activity). Blood samples were treated with (*R*)-ibuprofen and (*S*)-ibuprofen to determine their effects on plasma TXB_2 and PGE_2, reflecting COX-1 and COX-2 metabolism, respectively. (*S*)-ibuprofen was equipotent for both COX isoforms, shown by IC_{50} values of 2.1 ± 1.3 μM for COX-1 and 1.6 ± 0.8 μM for COX-2. In contrast, (*R*)-ibuprofen was a weak COX-1 inhibitor (IC_{50} = 34.9 ± 13.2 μM) and showed negligible inhibition of COX-2 (IC_{50} = > 250 μM). To determine the mechanism of ibuprofen at the atomic level, crystallization of COX isoforms in complex ibuprofen were required, and this has now been achieved for both COX isoforms (Orlando *et al.* 2015; Selinsky *et al.* 2001). These works demonstrated that ibuprofen forms a complex with

Arg-120 in the *cyclooxygenase* active site of the enzyme, competing with the arachidonic acid (AA) substrate which binds to the same residue. When this position on the *cyclooxygenase* active site is taken up by ibuprofen, the conversion of AA to PGH_2 is ultimately decreased, resulting in a blunted prostanoid synthesis.

2.5.3 Effect of acetaminophen and ibuprofen on normal body temperature

The notion that acetaminophen and ibuprofen interact with non-febrile thermoregulation is a relatively new concept, especially in humans, but there is evidence from clinical studies dating back to the 1980's. There are several case reports linking acetaminophen and ibuprofen administration and accidental hypothermia, at both therapeutic and high doses. Firstly Denes *et al.* (2002) report on four cases of acetaminophen induced hypothermia where patients with human immunodeficiency virus (HIV) presented to the emergency department with a T_c of 27 - 33°C. It is unknown if the virus had any impact thermoregulatory responses to therapeutic acetaminophen ingestion, but given that metabolism of acetaminophen can change in HIV infected people, this could explain the increased susceptibility (O'Neil et al., 1999). That being said, there are many other reports of acetaminophen hypothermia in otherwise healthy individuals, suggesting that HIV is not a prerequisite for hypothermia to develop after administration. Van Tittelboom and Govaerts-Lepicard (1989) report on four cases where hypothermia developed in febrile children after therapeutic doses of acetaminophen. The development of hypothermia progressing after fever has been well documented since then, and the molecular pathways well described (Romanovsky et al., 2005; Song et al., 2016). It seems apparent from case reports, however, that fever is not required for acetaminophen induced hypothermia. Rollstin and Seifert (2013) document a case of a 37 year old female who reported to the emergency department with a T_{re} of 17°C. It was later determined that the patient ingested

50 grams of acetaminophen 18 hours prior to presentation, and remarkable survived with treatment of N-acetylcysteine therapy.

There are several case reports concerned with ibuprofen and accidental hypothermia. Therapeutic doses of ibuprofen and acetaminophen were used in combination to treat fever in a 15 month old child who presented to the hospital with a T_c of 39.2°C (Richardson and Sills, 2004). However, the child developed hypothermia after drug administration, marked by a T_c of 33.6°C which was sustained for 11 hours before returning to 37°C. Because hypothermia can develop as a by-product of fever, it is difficult to determine if i) administration of acetaminophen and ibuprofen were just coincidental, or ii) the drugs invoked a hypothermic. reaction. A case report from 2003 describes a similar scenario as above but with ibuprofen exclusively. A 7-year old girl reported to hospital with a high fever (and a T_c of 39.3°C), but after a single dose of ibuprofen her T_c decreased to 34.9°C in a 4 hour period (Desai and Sriskandan, 2003). Despite persistent efforts to raise the T_c back to normal with warming blankets, the patient's T_c remained hypothermic for a further four days. Finally, Ritter and Eskin (1998) report on a 16 year old male who consumed approximately 40 grams ibuprofen orally, and presented to the emergency department with a T_c of 33.8°C. The pathophysiology of hypothermia in this case is probably linked to the very large dose causing central nervous system depression and a reduction in normal metabolism (Auriel et al., 2014).

Reports of hypothermia following administration of acetaminophen and ibuprofen are extremely rare. At therapeutic doses, the cause of hypothermia after administration of either drug is largely unknown, and appear to be linked with a hypothermic response to fever. Overdose of acetaminophen and ibuprofen can cause profound hypothermia in the absence of prior fever. The probable cause is drug-induced toxicity within the central nervous system which disrupts normal

metabolism (a constant source of heat production) or thermoregulatory mechanisms in the hypothalamus (Auriel et al., 2014; Smolinske et al.). *Clinical trials* It is general accepted that neurological outcome after acute ischemic stroke is partly determined by the T_c in the first 24 hours after stroke onset (den Hertog et al., 2011). Thus, the "paracetamol (acetaminophen) in stroke (PAIS)" trial used information from case reports and animal work to determine if acetaminophen could be used as a strategy to reduce T_c in acute ischemic stroke victims (Dippel et al., 2003). Kasner *et al.* (2002) demonstrated that acetaminophen reduced average T_c across a 24 hour period by ~0.3°C in non-febrile acute ischemic stroke patients. Because neurological outcome after stroke is inversely related to T_c on admission, this warranted a larger scale trial directly assessing if acetaminophen induced hypothermia improved neurological outcome after stroke. The work eventually progressed to a phase III trial which involved 1400 patients receiving acetaminophen (6 grams daily) or a placebo (den Hertog et al., 2009). There was a small benefit of acetaminophen administration on neurological outcome, but no adjustments were made between febrile and non-febrile patients. Thus, acetaminophen may have improved outcome in febrile stroke patients through its normal, well established antipyretic mechanism (Anderson, 2008).

2.6 Aims and hypotheses

Following completion of this literature review, the general methodologies employed in this thesis will be outlined in the proceeding Chapter. Subsequently, four experimental studies will be presented. Prior to reading the following sections, the aims and hypotheses of this thesis are presented below:

Aims

1. To validate whether acetaminophen administered at therapeutic doses has a hypothermic action in healthy humans. This will be achieved through the completion of Study 1 (Chapter 4).
2. To investigate if acetaminophen administration influences thermoregulatory responses to dry and humid passive heat stress. This will be achieved through the completion of Study 2 (Chapter 5).
3. To use the information gained from Study 1 and 2 and elucidate the therapeutic or pathological potential of acetaminophen during extreme heat or cold stress. This will be achieved through Study 3 (Chapter 6).
4. To investigate the pharmacodynamics behind the responses shown in Study 1, 2 and 3 using a non-selective cyclooxygenase inhibitor. This will be achieved through the completion of Study 4 (Chapter 7).

Null (H_0) and Alternative (H_A) Hypotheses

$H_{01:}$ There will be no difference in the T_{re} response between orally administered acetaminophen and a placebo during a 2-hour exposure to 20°C.

$H_{A1:}$ Acetaminophen will independently reduce T_{re} during a 2-hour exposure to 20°C compared with a placebo.

H_{02}: There will be no difference in the T_{re} rate of rise between acetaminophen and a placebo during compensable and uncompensable passive heat stress.

H_{A2}: Acetaminophen will independently reduce the T_{re} rate of rise during compensable heat stress compared with a placebo.

H_{03}: Acetaminophen induced hypothermia in humans will not be exacerbated by skin cooling.

H_{A3}: Skin cooling will exacerbate the hypothermic action of acetaminophen.

H_{04}: Administration of a non-selective COX inhibitor (Ibuprofen) will not impact thermoregulatory responses to a 2-hour passive exposure to 10°C.

H_{A4}: Administration of a non-selective COX inhibitor will reduce T_{re}, shivering, and metabolic rate during a 2-hour passive exposure to 10°C.

CHAPTER 3: GENERAL METHODOLOGY

This section describes the general procedures that were performed in each experimental chapter. Details of study specific methodologies are present in their respective experimental chapters.

3.1.1 Ethical approval and location of experiments

Ethical approval for Study 1 to 3 was obtained from the Institute of Sport and Physical Activity Research (ISPAR). Study 4 was approved by ISPAR and the University of Bedfordshire Research Ethics Committee (UREC). Ethical approval codes are stated in the "ethical approval" sections of each experimental chapter.

3.1.2 Participants

Each study required male participants aged between 18 and 35 years. Females were not included as participants for logistical reasons associated with i) the menstrual cycle and its potential impact on thermoregulatory responses to thermal stress, and ii) the potential interaction with experimental drugs and those used for contraception. Also, a maximum age of 35 was chosen based on prior work showing no differences in the cardiovascular responses to cold exposure in those aged between 18 – 35 (Hess et al., 2009). Participants were deemed healthy prior to participation. For this conclusion to be made, participants completed a physical activity readiness questionnaire (PAR-Q), blood screening questionnaire, alcohol use identification test, and an acetaminophen (Study 1, 2, and 3) or ibuprofen (Study 4) risk assessment questionnaire. Smokers were not permitted to take part in the study due to the impact smoking has on cardiovascular function (Michalak *et al.* 2012). Participants carrying a musculoskeletal injury, an acute or chronic illness, or those regularly taking any other medications were not permitted to take part in the study. Participants were informed of all the potential risks of taking part in the

experiments, and subsequently all provided verbal and written consent. Each volunteer was medically screened prior to participation (see "general experimental controls" in each experimental chapter).

3.1.3 Anthropometry data

Participant's height (cm), body mass (kg), and body composition (body fat %) were measured using a stadiometer (Harpenden, HAR-98.602, Pembrokeshire, United Kingdom), digital scales (Tanita, BWB0800, Hoogoorddreef 56E 1101 BE Amsterdam, Netherlands), and air displacement plethysmography (Cosmed, Bod Pod, 2000A, Middlesex, United Kingdom), respectively. The reliability of the bod-pod was not measured by the researcher but an independent study showed that the equipment shows acceptable reliability, but may over-estimate body fat in comparison to under water weighing (Tseh et al., 2010).

3.1.4 Control measures and standardisations

The following control measures were used in each experimental protocol to reduce the likelihood of confounding variables impacting experimental dependent variables. The following confounding variables were controlled in all experimental trials:

Prior exercise

Participants were required not to exercise at least 48 hours prior to participation due to the impact this may have had on thermoregulation and cardiovascular responses to cold stress. There was a minimum 30-minutes rest period prior to entry into the environmental chamber to allow T_c to stabilise.

Diurnal variations and circadian rhythms

Experimental trials were completed at the same time of day to account for diurnal variations and circadian rhythms in T_c (Waterhouse *et al.* 2005). There was between subject variation Study 2 due to time constraints for study completion, but no within subject variation for time of arrival. There was no between or within subject variation for time of arrival in any other experiment conducted within this thesis.

Clothing

During Study 1a, 1b, and 2, subjects donned underwear and shorts, equating to a Clo insulation value of ~0.2. In Study 3 and 4, subjects were also permitted to wear socks to minimise the risk of non-freezing cold injury. Additional clothing will likely affect participants T_{sk} and perceptual responses to cold stress. All participants adhered to this control measure.

Acute and chronic alcohol ingestion

Participants were asked not to ingest any alcohol 48 hours prior to participation. On test days, participant's alcohol intake was monitored via breathalyser (AlcoSense, AlcoSense ONE, Maidenhead, United Kingdom). As chronic alcoholism can increase the potential for acetaminophen and ibuprofen hepatoxicity (Prescott 2001), participants were also required to complete a self-assessment alcohol use identification test (AUDIT) questionnaire. If scores on the AUDIT exceeded 8, participants were excluded from the study.

Supplementation and nutritional intake

Participants were restricted from ingesting caffeine 24 hours prior to testing due to the impact this may have had on thermogenesis (Gosselin and Haman 2013). Participants were not permitted to take part in the experiment if they were currently using any non-prescription or prescription medications. This was because it may have impacted thermoregulation, and to avoid

drug interactions with acetaminophen or ibuprofen. Participants were asked to arrive at the laboratory having fasted overnight or for at least 12 hours.

In Study 1, 2, and 3, participants consumed a standardised meal 30 minutes after arrival, having fasted overnight. The meal consisted of 50 g cornflakes, 250 ml semi skimmed milk, and 500 ml water. In total, the meal 303 kcal. In Study 4, participants arrived fasted overnight and did not consume any food prior to cold exposure.

Prior acclimatisation and acclimation

All participants confirmed that they had not visited a hot or cold climate for one week, for at least 3 weeks prior to participation in experimental trials because this may have affected heart rate, T_c and T_{sk}, and thermal sensation during cold exposure (Poirier *et al.* 2015; Saat *et al.* 2005).

Contraindications to acetaminophen or ibuprofen administration

Nutritional restrictions are outlined in sections 3.4.4 and 3.4.5. In addition to these controls, participants were required to complete an acetaminophen risk assessment questionnaire for Study 1 to 3, and an ibuprofen risk assessment questionnaire for Study 4. If participants answered "yes" to any questions they were not permitted to take part in the experiments.

3.1.5 Physiological measurements and apparatus

Measurement of T_c

Rectal temperature was used as an index of T_c throughout this thesis. Rectal temperature measurement has been shown to be the gold standard method of measuring T_c (Huggins *et al.* 2012) due to its accuracy (± 0.1°C) and practicality. T_c was measured using a rectal probe (YSI, 400H/4491H, Hertfordshire, United Kingdom) inserted by participants 10 cm beyond the anal sphincter. Measurement of T_c was continuously displayed on a temperature monitor (Libra

Medical, ET402, Birmingham, United Kingdom). Participant's inserted the rectal thermistor upon arrival to the laboratory to ensure resting T_c levels could be measured and were within the normal resting values (i.e. non-febrile if < 38°C).

Measurement of T_{sk}

In Study 1, 2 and 3, copper based thermocouples (Grant, EUS-U-VS5-0, Dorset, UK) connected to a wireless data logger (Grant, Squirrel Series, Dorset, UK) recorded T_{sk} at four sites; calf, thigh, chest, and triceps. Thermocouples were securely attached to the belly of each muscle by hypafix surgical adhesive tape (BSN medical, D-22771, Hamburg, Germany) on the participant's right side of the body. The weighted T_{sk} of four sites was subsequently calculated using the Ramanathan (1964) equation below:

$$\textbf{Mean } T_{sk} = 0.3*(T_{arm} + T_{chest})+0.2*(T_{calf} + T_{thigh})$$

The 4-point equation was used because it shows strong agreement with more complex equations requiring more contact points (Ramanathan, 1964). However, it was determined that more temperature sites were required to investigate if any effects on skin blood flow and thus, heat loss. To improve the mean T_{sk} prediction in Study 4, a twelve-point system was used. In this study, thermocouples were placed on the belly of the gastrocnemius, rectus femoris, triceps brachii, pectoralis major, trapezius, erector spinae, bicep femoris, and soleus. Because electromyography probes were placed on the participant's dominant side, thermocouples were placed on the participant's right side of the body. The Hardy and DuBois (1938) 12-point equation was used to estimate mean T_{sk}:

$$\text{Mean } T_{sk} = (T_{forehead}*0.07)+(T_{chest}*0.0875)+(T_{abdomen}*0.0875)+(T_{outer\ lower\ arm}*0.14)+(T_{hand}*0.05)+(T_{quad}*0.095)+(T_{shin}*0.065)+(T_{instep}*0.07)+(T_{trapezius}*0.0875)+(T_{lower\ back}*0.0875)+(T_{hamstring}*0.095)+(T_{calf}*0.065)$$

Measurement of heart rate, blood pressure and thermal sensation

Heart rate was continuously measured via a telemetric heart rate monitor (Polar, FS1, Birmingham, United Kingdom) attached to the chest. Blood pressure (Study 3 and 4) was measured using an automated, portable monitoring device (Omron healthcare, M5-1, Milton Keynes, UK). Data was expressed as beat per minute (i.e. $b \cdot min^{-1}$). Participants provided thermal sensation scores verbally at rest and every 10 minutes during experimental protocols.

Measurement of hydration via urine osmolality

Prior to each experimental protocol, participants provided a small urine sample to ensure they were in a euhydrated state. A small sample of urine (~1 ml) was pipetted into a handheld digital refractometer (Atago Vitech, Pocket PAL-OSMO, Warwickshire, United Kingdom) to measure urine osmolality. If scores fell under 600, subjects were considered adequately hydrated. If scores exceeded 600, participants were required to drink a safe volume of water until euhydrated (< 600 $mOsm \cdot kg^{-1}$).

Calibration of equipment

The T_{sk} probes (Grant, EUS-U-VS5-0, Dorset, UK) were checked for accuracy against ambient air temperature measurements. The T_{sk} probes were required to be within 0.1°C of i) each other and ii) the air calibration thermometer. Alternative probes were used if the error was over 0.1°C. The Servomex (Servomex, Servomex mini HF 5200, Sussex, UK) was calibrated on each morning of the trial by laboratory technicians as per manufacturer recommendations.

3.1.6 Experimental design

All experiments (excluding Study 1 a) were double blinded, randomised, and had a crossover design. Randomised treatment allocation was conducted with IBM Statistics Package for Social

Sciences (SPSS) version 21.0 (SPSS Inc, Chicago, IL, USA) by using the 'random sample of cases' function. Each trial was separated by at least 7 days to ensure full clearance of acetaminophen or ibuprofen.

Preparation and administration of acetaminophen, ibuprofen and placebo

All drugs were administered orally in the present study with ~ 150 ml of tap water. Each drug was housed in an opaque silicone coated capsule. Dextrose was used as a placebo in experimental trials 2, 3 and 4 and the mass was matched with experimental drugs administered. Acetaminophen [Paracetamol, Aspar Pharmaceuticals, London, UK (Study 1, 2 and 3)] was administered at 20 mg·kg of lean body mass^{-1} and weighed using an electronic analytical balancer. Acetaminophen was administered at a dose relative to lean body mass to reduce any toxic effects on the liver, as advised by a consultant anaesthetist (Dr David Conn, Exeter Hospital). Because lean body mass is better correlated to liver volume than total body mass (Kwo *et al.* 1998), the dose provided using this system ensures high doses are not given due to a large body fat %. Ibuprofen [Banner Pharmacaps Europe, Tillburg, Netherlands (Study 4)] was administered at a fixed dose of 400 mg, in line with the doses recommended by the food and drug administration (FDA) for acute pain.

3.1.7 Blood collection

Venous blood collection and storage

Venous blood was obtained from the superficial vein in the antecubital fossa of the forearm via venepuncture or cannulation (study dependent). Time points of blood collection are displayed in relevant experimental chapters. Blood was drawn into a 6 ml tubes (Grenier Bio-One, Kremsmunster, Austria) containing either ethylenediaminetetraacetic acid (EDTA) or heparin

before being centrifuged (Heraus Multifuge X3R, Thermo Scientific, Waltham, USA) at 1500 *g* for 5 minutes, at 4°C. Once the plasma component was separated, 1 ml was pipetted (Finnpipette F2, Thermo Scientific, Waltham, USA) and stored in 1.5 ml microcentrifuge tubes (Eppendorf, Grenier Bio-One, Kremsmunster, Austria) in a -80°C freezer until further analysis.

Plasma volume

Blood samples were pipetted and drawn in duplicate into microhaematocrit capillary tubes (Hawksley, Lancing, Sussex, UK) before being centrifuged in a Hawksley HaematoSpin 1400 (Hawksley, Lancing, Sussex, UK) for 3 minutes at 14,000 g. Subsequently, capillary tubes were placed on a Hawksley microhaematocrit tube reader (Hawksley, Lancing, Sussex, UK) and the haematocrit (%) read accordingly. For haemoglobin determination, 10 µl of blood was collected onto a HemoCue® Hb 201 microcuvette (HemoCue® Ltd., Dronfield, Derbyshire, UK) and placed into a HemoCue® Hb 201+ (HemoCue® Ltd., Dronfield, Derbyshire, UK) in accordance with manufacturer's instructions. Haemoglobin and haematocrit concentrations were used to measure changes in plasma volume per the Dill and Costill method (Dill and Costill, 1974) using the following equation:

$$((100*Hbpre/Hb\ post))*((1-(Hct\ post - 100))/(1-(Hct\ pre - 100)))-100$$

Plasma biomarkers were adjusted accordingly to account for any change in plasma volume. The percentage change in plasma volume was either added or subtracted from the absolute concentration of the biomarker in question.

3.1.8 Enzyme linked immunosorbent assays

In Study 1, acetaminophen plasma concentrations were measured using a Neogen ELISA development kit (Neogen, Auchincruive, Aryshire, UK). In Study 4, PGE_2 plasma concentrations

were measured using an Enzo Life Sciences high sensitivity ELISA development kit (Enzo Life Sciences, Exeter, Devon, UK). All assays were conducted in accordance with the manufacturers recommendations and analysed in duplicate.

3.1.9 Statistical analysis

All statistical analyses were completed using *R* software version 3.3.2 (*R* Core Development Team), a language and environment for statistical computing. Normality assumptions were checked using quantile-quantile (QQ) plots. Where data was normally distributed, central tendency and dispersion are reported as mean ± standard deviation (SD). Data was reported as median (range) if it did not follow a normal distribution. Linear mixed models were used to analyse the change in dependent variables over time of exposure, and the fixed and random components of each model are displayed in each experimental chapter. The Akaike information criterion (AIC) was used to determine the fit of the full model compared with the null model, and subsequently to compare models with different correlation structures. The model with the lowest AIC value was chosen based on this procedure (Akaike 1973). A cumulative link mixed model (also known as an ordinal regression model) was used to analyse thermal sensation data, which is presented as an odds ratio (OR) relative to a placebo trial. The alpha level of significance testing was set as $p \leq 0.05$, and 95% confidence intervals (CI) are used to denote the imprecision in the point estimate.

CHAPTER 4. EXPERIMENT 1: EVIDENCE OF ACETAMINOPHEN INDUCED HYPOTHERMIA IN NORMOTHERIC, NON-FEBRILE HUMANS.

4.1 Introduction

Acetaminophen is a widely used analgesic antipyretic drug that is branded as Tylenol in the USA and as paracetamol in the UK. It is available over-the-counter in various single-entity formulations and is commonly prescribed in combination with various opioids to manage moderate pain symptoms. Despite its well-established validity as an analgesic and antipyretic via inhibition of COX (Aronoff *et al.* 2006; Li *et al.* 2008), a growing body of evidence also demonstrates a third, hypothermic action of acetaminophen i.e. T_c reduction in the absence of fever. This side effect may be beneficial for inducing therapeutic hypothermia, but may also increase rates of accidental hypothermia, via exacerbated T_c reductions in environments beneath the thermal neutrality. The hypothermic effects of intravenous acetaminophen have been reported in rodents, with up to a 4°C reduction in T_{re} (Ayoub *et al.* 2011; Gentry *et al.* 2015; Li *et al.* 2008). The hypothermic effects of intravenous acetaminophen have not been confirmed in non-febrile human beings in a controlled laboratory setting.

Confirming if acetaminophen exhibits a hypothermic action in humans is pertinent for several reasons. Firstly, due to the inverse relationship between T_c and neurological outcome after stroke or traumatic brain injury (Reith *et al.* 1996), acetaminophen could be used as a cheap, safe, and easily administered pharmacological method to reduce T_c when more invasive and logistically challenging methods are not available or appropriate (den Hertog *et al.* 2009). Indeed, acetaminophen has been shown to reduce mean T_c in non-febrile stroke patients (Kasner *et al.* 2002), however the acetaminophen dose used was small (650 mg), T_c was only averaged across a

24-hour period, and there were no obvious environmental or nutritional controls in place. Secondly, if acetaminophen reduces the resting T_c of healthy humans, it may be involved in the pathology of accidental hypothermia. For example, if acetaminophen reduces T_c via increasing heat loss or decreasing heat production, this places individuals who consume acetaminophen at greater risk of developing hypothermia, especially in winter months. This is of particular concern to thermoregulatory vulnerable individuals i.e. the very young (aged 0-4 years) or the elderly (age ≥65 years), who together contributed ≥85% of UK hospital admissions in 2014 where hypothermia was the primary or secondary diagnosis (HSCIC 2015). As acetaminophen is the most frequently administered over-the-counter medication worldwide (Blieden *et al.* 2014), any hypothermic action could have deleterious implications for a significant number of people as outlined above. In this preliminary trial, young adults were used as participants to first establish any cause and effect due to ethical considerations.

4.1.1 Experimental aims and hypothesis

The first aim was to determine the peak plasma concentration of acetaminophen following an oral dose of 20 mg·kg of lean body mass^{-1} (part a). The second aim of the present study was to determine whether acute, oral acetaminophen ingestion alters thermoregulatory control during passive exposure to a sub neutral environment of 20°C (part b). The final aim was to assess the reliability of rectal temperature, T_{sk}, heart rate, and thermal sensation responses *within* subjects. Based on prior experiments in mice, it was hypothesised that acetaminophen would significantly decrease T_{re} across the 120-minutes study period, compared with a placebo.

4.2 Methods

For clarity, this methodology is separated into two parts (part a and part b). In part a, the methods involved in determining the plasma concentrations of acetaminophen after an oral dose of 20 mg·kg lean body mass^{-1} is described. In part b, the methods used to determine the effect of acetaminophen on thermoregulation is described. Eight participants from part a went on to participate in part b (see Figure 4.1 for study design).

4.2.1 Ethical approval

All experimental procedures were approved by the University of Bedfordshire's Institute for Sport and Physical Activity Research Ethics committee (approval code 2012ASEP021), and they conformed to the standards set by the World Association Declaration of Helsinki 'Ethical Principles for Research Involving Human Subjects'.

4.2.2 Power analysis

Power analyses were conducted with GPower software version 3.1 (Heinrich University, Düsseldorf, Germany). Using T_{re} data from a previous experiment where acetaminophen was tested as a hypothermic agent in non-febrile stroke patients (Dippel et al., 2003a), it was determined that a total of nine participants were required to achieve a statistical power of 80%.

4.2.3 Participants

Ten Caucasian males [Age (23 ± 2 years), Height (181 ± 8 cm), Mass (80 ± 8 kg), body fat (16 ± 4%)] participated in part a. Thirteen Caucasian males [Age (23 ± 1 years), Height (174 ± 3 cm), Mass (73.6 ± 8 kg), body fat (15.5 ± 4.8%)] participated in part b. Eight participants from part a went on to participate in part b, with two discontinuing their participation for part b (see Figure 4.1 for trial profile). Participants were provided with written information regarding all experimental procedures, with supporting oral explanations from the principal investigator.

Participants then subsequently provided written informed consent. The participants were non-smokers, non-febrile (resting T_{re} 36.5 – 37.5°C), and were free from musculoskeletal injury.

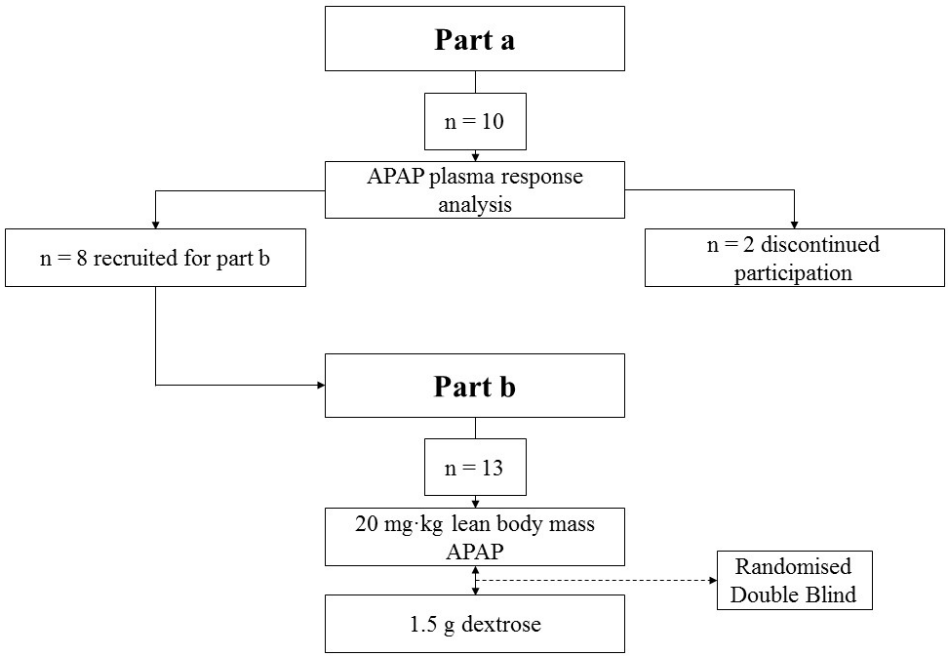

Figure 4.1. Study Design.

4.2.4 Inclusion/exclusion criteria

Prior to each laboratory visit, participants completed an informed consent sheet, alcohol use disorder identification test [AUDIT; (Saunders *et al.* 1993)] a breathalyser test (AlcoSense, One, Berkshire, UK), and an acetaminophen risk assessment questionnaire. To avoid the risk of liver damage inflicted by acetaminophen, participants were not able to participate in the research if they scored above ten on the AUDIT questionnaire or alcohol was present in their bloodstream. No participants presented with any pre-existing medical conditions that may have put them at an increased risk of acetaminophen toxicity. Further details are provided in section 2.14.4 and 2.14.7, respectively. Due to potential thermoregulatory adaptions (Poirier *et al.* 2015), individuals were not permitted to take part in any experimental procedures if they were heat

acclimated or acclimatized. Thus, those who had travelled to a hot climate or participated in a laboratory based heat acclimation protocol less than three weeks prior to the experiment were not permitted to take part (Saat *et al*. 2005). All participants presented with a stable T_{re} (36.5–37.5°C).

4.3 Part a methodology

4.3.1 Part a design

To identify when the peak plasma concentration of acetaminophen arises after oral ingestion (20 mg·kg lean body mass^{-1}), ten Caucasian males visited the laboratory on one occasion. Participants adhered to all experimental controls listed in the "general experimental controls" section.

4.3.2 Part a controls

On the trial day, acetaminophen (Paracetamol, Aspar Pharmaceuticals, London, UK) was administered at a dose equal to 20 mg·kg of lean body mass^{-1}. Each capsule contained a maximum of 500 mg of acetaminophen. The dosing range of acetaminophen in the present work was 1019 to 1420 mg (mean ± SD = 1293 ± 163 mg). This dosing was advised by a leading clinician and consultant anaesthetist, and has been used previously (Mauger *et al.* 2014). All participants arrived at the laboratory having fasted overnight for 12 hours. To control the gastric emptying rate of acetaminophen, participants ingested a standardized meal [cornflakes (50 g), milk (250 ml) and 1 litre of water] 1 hour prior to acetaminophen or placebo ingestion. Experimental trials took place within a custom built environmental chamber (Custom build, T.I.S.S, Hampshire, UK) which simulated the desired environmental condition of 20°C and 40%

relative humidity. The participants' clothing was standardized across all trials, in which they were barefoot, topless, and wore knee length shorts.

4.3.3 Part a protocol

Participants arrived at the laboratory at 08:30. Upon arrival, participant's lean body mass was calculated via air displacement plethysmography (Bod Pod, 2000A, Birmingham, UK). At 08:45 participants consumed a standardised meal (see "part a Controls"). At 09:20, participants remained seated and a 20G cannula (Introcan® Safety Winged, B Braun Medical, Sheffield, UK) was placed in a prominent vein within the antecubital fossa. At 09:30, participants entered the environmental chamber (20°C, 40% r.h.). Fifteen minutes after entry, participants orally ingested acetaminophen (Paracetamol, Aspar Pharmaceuticals, London, UK). Blood samples were drawn into a heparin coated EDTA tube (Vacuette®, Greiner Bio-One, Stroudwater, UK) immediately prior to acetaminophen ingestion, and subsequently at 20, 40, 60, 80, 100, and 120-minutes post ingestion. Peak plasma levels of acetaminophen were quantified using a commercially available ELISA kit (Microplate EIA kit, Alere toxicology, Abingdon, UK) read in duplicate at a dual wavelength of 450 and 650 nm (Sunrise, Tecan, Seestrasse, Männedorf). The average inter-plate CV was 5% (intra-plate CV not applicable due to assay layout). Venous hemoglobin (Hb 201+, Hemocue, Staines, UK) and haematocrit (Haematospin 1300, Hawksley, Sussex, UK) concentrations were taken in duplicate and averaged at each point of blood sampling. This data was used to calculate changes in plasma volume according to the Dill and Costill method (1974), and acetaminophen concentrations were subsequently adjusted in accordance with any changes in plasma volume.

4.4 Part b methodology

4.4.1 Power calculation

Power analyses were conducted with GPower software version 3.1 (Heinrich University, Düsseldorf, Germany). Utilising T_{re} data from a previous experiment where acetaminophen was tested as a hypothermic agent (Dippel *et al.* 2003a), it was determined that a total of 13 participants were required to achieve a statistical power of 90%.

4.4.2 Part b design

To determine the hypothermic effect of acetaminophen, 13 participants visited the laboratory at the same time of day on three occasions (acetaminophen, placebo, and control), each separated by at least seven days. The data from the acetaminophen and placebo trials were used for the determination of acetaminophen's hypothermic action. The data from the placebo and control trials were used to generate reliability statistics. All visits were randomized, and the acetaminophen and placebo trials were double blinded. The trials took place within an environmental chamber (20°C, 40% r.h.) set beneath the thermoneutral zone [i.e., subneutral; (Kingma *et al.* 2012; Kingma *et al.* 2014)] for 120 minutes. A sub-neutral environment was chosen for two reasons, (i) to ensure that heat loss mechanisms would not become activated during the protocol and (ii) to help replicate the sub-neutral conditions in previous animal work concerning acetaminophen induced hypothermia (Ayoub *et al.* 2011; Ayoub *et al.* 2004)]. On each trial day, acetaminophen (Paracetamol, Aspar Pharmaceuticals, London, UK) or a placebo (dextrose, MYPROTEIN, Cheshire, UK) was administered at a dose equal to 20 mg·kg of lean body mass^{-1}. Each capsule contained a maximum of 500 mg of acetaminophen. The dosing range of acetaminophen in the present work was 1019–1420 mg (mean ± SD = 1226 ± 135 mg). Controls for gastric content, nutritional intake, environmental conditions, and clothing were identical to that of part a (see "part a controls").

4.4.3 Part b protocol

Participants arrived at the laboratory at 7am or at 10am, where each participant's time of arrival was consistent through all experimental trials to account for any circadian rhythm or diurnal variations in T_{re} (Waterhouse *et al.* 2005). Upon arrival, participants were instrumented for the measurement of T_{re}, T_{sk}, and heart rate (see "Instrumentation and Equations" for details). Thirty minutes after arrival, participants consumed the standardised breakfast. Sixty minutes after the meal was consumed, participants ingested acetaminophen, a placebo (dextrose), or nothing (control). Participants remained rested in an upright-seated position between meal consumption and acetaminophen or placebo ingestion, and for the duration of data collection to ensure resting physiological status was attained and maintained throughout the data collection. Data collection began immediately after ingestion of acetaminophen or placebo, i.e., 60 minutes after meal consumption. Resting measurements of T_{re}, T_{sk}, heart rate, and thermal sensation were taken 5 minutes prior to chamber entry, and subsequently every 10 minutes for 120-minutes post entry.

4.4.4 Part b instrumentation and equations

Equipment details and equations for T_{re}, T_{sk}, heart rate, and thermal sensation are described within the General Methodology (section 3.1.5).

4.4.5 Statistical analysis

All statistical analyses for were performed using the *'psych'*, *'nlme'*, *'ordinal'*, *'ez'*, and *'stats'* packages in *R* version 3.3.2 (R Core Development Team 2014). Normality assumptions were checked using quantile-quantile plots (Grafen and Hails 2002) and were plausible in all instances. Central tendency and dispersion are reported as means ± standard deviation (SD). Reliability of all variables from consecutive pairs of trials were assessed using data from the

control and placebo trial. The following reliability measures [change in mean (CIM), coefficient of variation (CV), intraclass correlation coefficient (ICC), and typical error (TE) were all reported. The Akaike information criterion (AIC) was used to determine model fit against the null model (Akaike 1973). The correlation structure with the lowest AIC was chosen based on this procedure. A linear mixed model with fixed ('drug', 'time') and random ('subject i.d') effects was fitted with an autoregressive correlation structure (to account for autocorrelation) to examine the effect of acetaminophen on T_{re}, T_{sk}, and heart rate [Time (13 levels): pre, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 minutes × Drug (2 levels): placebo, acetaminophen]. A cumulative link mixed model with fixed ('drug', 'time') and random effects ('subject i.d.') was used to compare thermal sensation scores between placebo and acetaminophen. The thermal sensation analysis is presented as an odds ratio, and the output example is explained in the text. The two-tailed alpha level of significance testing was set as $p \leq 0.05$. 95% confidence intervals (CI) are presented to denote the imprecision of the point estimate.

4.5 Results: Part a

The peak plasma concentration of acetaminophen during the 120-minutes experiment was 14 ± 4 μg/ml (range, 8-19 μg/ml). The time for acetaminophen to reach maximal concentrations was 96 ± 13 minutes (range, 80-100 minutes). Figure 4.2 displays the plasma concentration response of acetaminophen, every 20 min for 2 hours.

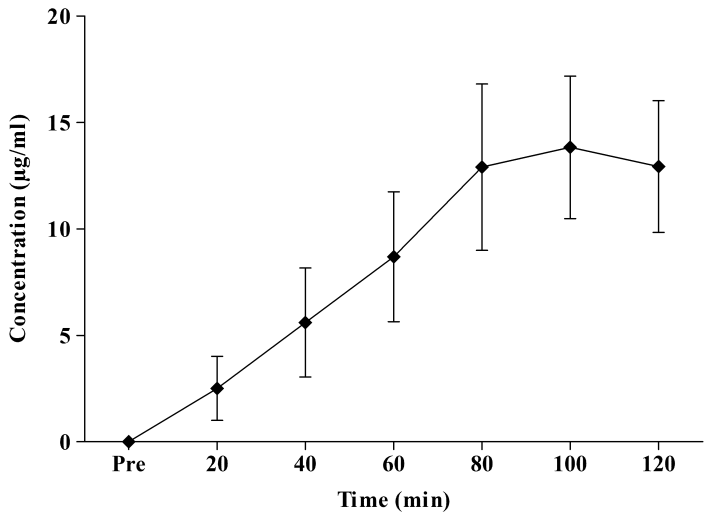

Figure 4.2. Mean ± standard deviation values for plasma acetaminophen concentration response to oral intake of 20 mg·kg lean body mass^{-1} acetaminophen during a resting 120-minutes period. n=10.

4.6 Results: Part b

4.6.1 Reliability

Reproducibility statistics (i.e. control vs placebo) demonstrated that there was no systemic bias for any of the variables tested. Results are presented in Table 4.1.

4.6.2 T_{re}

Main effects were found for condition ($F_{1,1}$ = 28.68, p < 0.001), and time ($F_{1,12}$ = 17.23, p < 0.001) between acetaminophen (36.73 ± 0.1°C; 95% CI = 36.67 to 36.8°C) and placebo (36.83 ± 0.1°C; 95% CI = 36.77 to 36.89°C). A significant interaction effect was also found ($F_{1,12}$ = 51.68, p < 0.001), revealing that mean T_{re} was significantly lower in the acetaminophen group from 30 minutes to the cessation of the trial. The peak T_{re} reduction from baseline arose 110 minutes (6 subjects) or 120 minutes (7 subjects) after ingestion, and the drop ranged from 0.10 to 0.39°C (mean = 0.19 ± 0.09°C). The mean T_{re} response to acetaminophen ingestion is displayed

in Figure 4.3. The individual responses to dextrose and acetaminophen are displayed in Figure 4.4.

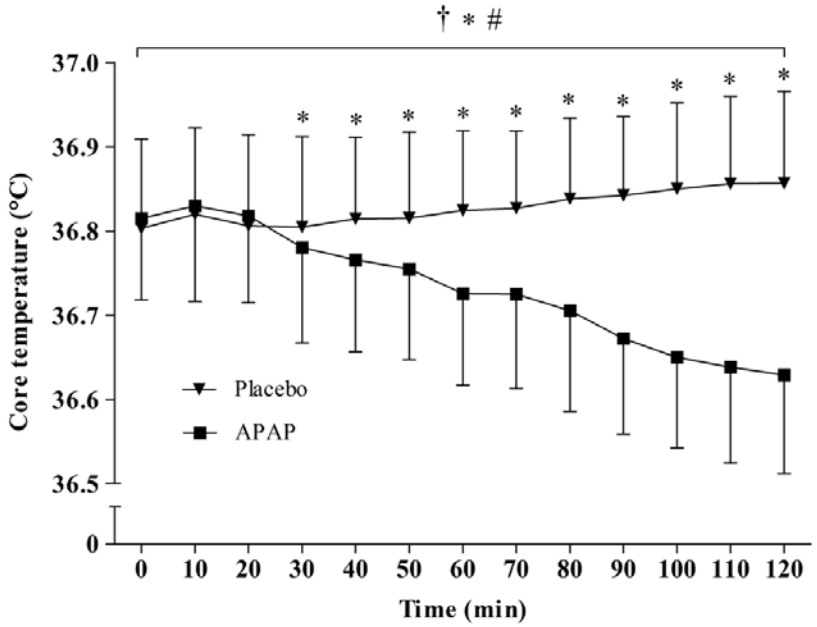

Figure 4.3. Mean ± standard deviation values for T_{re} in both the acetaminophen and placebo conditions. ∗Significant main effect for condition. #Significant main effect for time. †Significant interaction effect. n=13.

4.6.3 T_{sk}

There were no significant main effects for condition ($F_{1,1}$ = 0.01, p > 0.05) between acetaminophen (26.8 ± 1.3°C; 95% CI = 26 to 27.6°C) and placebo (26.9 ± 0.8°C; 95% CI = 26.41 to 27.3°C), but there was a main effect for time ($F_{1,12}$ = 30.46, p < 0.001). No interaction effects were found ($F_{1,12}$ = 0.39, p > 0.05)

4.6.4 Thermal sensation

TS did not change with acetaminophen administration (p > 0.05). Compared with time-point 0, TS was reduced from time-point 20 to 120 (p < 0.05). The odds ratios are displayed in Table 4.2. The average thermal sensation in both trials was 3.5 ± 2 (4 = “comfortable”).

4.6.5 Heart rate

There were no significant main effects for condition ($F_{1,1}$ = 0.76, p > 0.05) between acetaminophen (62 ± 8 b·min^{-1}; 95% CI = 58 to 66 b·min^{-1}) and placebo (63 ± 7 b·min^{-1}; 95% CI = 60 to 66 b·min^{-1}), but there was a main effect for time ($F_{1,12}$ = 5.57, p < 0.001). No interaction effects were found ($F_{1,12}$ = 0.24, p > 0.05).

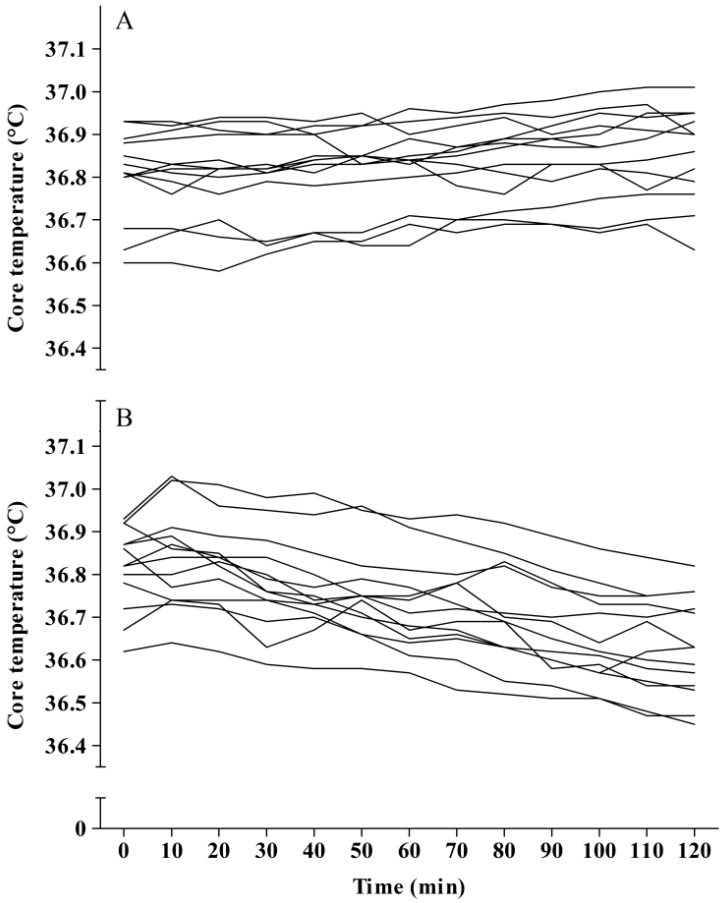

Figure 4.4. Individual T_{re} responses in the placebo (A) and acetaminophen condition (B). n=13.

Table 4.1. Reproducibility of dependent variables commonly used throughout this thesis.

Variable	Placebo	Control	CIM	*p* value	ES	ICC	CV (%)	TE (%)
T_{re}	36.82 ± 0.10	36.82 ± 0.12	0.03 (-0.03 to 0.03)	0.89	0.05	0.91 (0.75 to 0.97)	0.2 (1.8 to 7)	0.03 (0.02 to 0.05)
T_{sk}	31.45 ± 0.54	31.37 ± 0.79	0.1 (0.31 to 0.5)	0.6	0.09	0.54 (0.02 to 0.83)	0.29 (0.18 to 0.72)	0.47 (0.34 to 0.78)
Heart rate	63 ± 4	63 ± 6	0 (-3.43 to 2.63)	0.77	0.06	0.54 (0.05 to 0.83)	6 (4.4 to 10.1)	3.71 (2.69 to 5.98)
TS	3.99 ± 0.04	3.96 ± 0.15	0.03 (-0.06 to 0.12)	0.53	0.2	0.49 (0.42 to 0.9)	3 (2.2 to 4.9)	0.11 (0.08 to 0.18)

Change in mean (CIM), Effect size (ES), Intraclass correlation coefficient (ICC), Coefficient of variation (CV), Typical error (TE). 95% confidence intervals are presented in parentheses where appropriate.

Table 4.2. Changes in TS as a product of time (duration of exposure) and time + drug (interaction effect). Significance codes: < 0.001***, < 0.01 **

	OR	**SE**	***p***
Placebo vs APAP (Intercept)	0.989	2.435	
Time Only			
TIME10	0.288	2.536	
TIME20	0.052	2.994	**
TIME30	0.051	2.948	**
TIME40	0.051	2.948	**
TIME50	0.025	3.047	***
TIME60	0.025	3.024	***
TIME70	0.013	2.975	***
TIME80	0.005	2.925	***
TIME90	0.002	2.851	***
TIME100	0.001	2.854	***
TIME110	0.001	2.879	***
TIME120	0.000	3.211	***
Drug×Time Interaction			
TIME10:DRUGAPAP	1.024	3.712	
TIME20:DRUGAPAP	3.485	4.261	
TIME30:DRUGAPAP	1.991	4.338	
TIME40:DRUGAPAP	1.991	4.338	
TIME50:DRUGAPAP	2.054	4.498	
TIME60:DRUGAPAP	1.009	4.445	
TIME70:DRUGAPAP	0.573	4.039	
TIME80:DRUGAPAP	0.942	3.765	
TIME90:DRUGAPAP	0.984	3.542	
TIME100:DRUGAPAP	0.994	3.514	
TIME110:DRUGAPAP	0.667	3.557	
TIME120:DRUGAPAP	1.512	3.849	

The OR is a score of likelihood relative to 1. For example, for the "time only" model at time point 20, the likelihood of a score being higher than time point 0 is 0.052 out of 1 i.e. ~ 5%. In the same model, the likelihood of scoring higher at 100 minutes than 0 minutes is 0.001 i.e. ~ 0.001%. The "drug×time" model displays the likelihood of a score in the acetaminophen group (APAP) being higher than the placebo at the same time point. For example, at time point 120, the likelihood of a score being higher in the acetaminophen group was 1.512 i.e. ~50%. However, this is not statistically significant due to the high standard error.

4.7 Discussion

4.7.1 Overview of results

The aim of this study was to investigate if oral acetaminophen ingestion exhibited a hypothermic action in non-febrile humans. On average, T_{re} was 0.14°C lower during the 120-minutes exposure to 20°C in the acetaminophen condition compared with a placebo. The maximum (peak) reduction in T_{re} in the acetaminophen condition was 0.19 ± 0.09°C (range = 0.10 to 0.39°C), as shown in Figure 4.2. Having ingested acetaminophen, all participants displayed a gradual decrease in $T_{re,}$ and in seven participants, this did not plateau in the 120-minutes study period (Figure 4.3 and 4.4). However, due to the relatively short experiment time, the notion that T_{re} began to recover before the end of the 120-minutes period in the remaining six participants is speculative. To our knowledge, this is the first study that accurately demonstrates that acetaminophen reduces non-febrile human T_{re} in sub neutral conditions (i.e. beneath thermal neutrality) within apparently healthy human participants (figure 4.3).

4.7.2 Agreement with prior work in this area

The effect of acetaminophen on non-febrile temperature regulation has been investigated previously in mice (Ayoub *et al.* 2011; Ayoub *et al.* 2004; Gentry *et al.* 2015; Li *et al.* 2008) and humans (den Hertog *et al.* 2009; Dippel *et al.* 2003b; Kasner *et al.* 2002). There are also reports of severe hypothermia (T_{re} = 28°C on hospital admission) following acute acetaminophen overdose (Rollstin and Seifert 2012). Prior to the present work, this potentially hazardous side-effect had not been confirmed in passive, nutritionally controlled participants or conducted in a temperature controlled environmental chamber. Despite a significant interaction effect (condition*time) for T_{re}, the 120 minute trial period did not consistently allow for a plateau in T_{re} to be demonstrated i.e. the maximum T_{re} reduction in seven of the thirteen participants was not

seen prior to the final time point of 120 minutes (Figure 4.4). Although T_{re} seemed to plateau in six participants, a trial of longer duration is needed to reliably determine when T_{re} will begin to recover to normal values after administration of acetaminophen. However, given that the cellular target of acetaminophen for inducing hypothermia is not known in humans, it is difficult to predict if the peak reduction in T_{re} is in line with the peak plasma or cerebral spinal fluid (CSF) concentrations.

4.7.3 Pharmacokinetic comparison with prior work

Due to its worldwide use, the pharmacokinetics of acetaminophen has been investigated extensively. However, it was important to determine the short-term concentration response in the present experiment (Study 1) as this has not been analysed (i) following doses of 20 mg·kg of lean body mass^{-1} and (ii) following the implemented nutritional controls. The data presented here is; however, in line with previous work. Early work using gas chromatography demonstrated that in adult humans (fasted and apparently healthy), oral doses of 1000 and 2000 mg acetaminophen reach peak plasma concentrations in ~60 and 120 minutes, respectively (Rawlins *et al.* 1977). An oral dose relative to lean body mass was used in the present experiment because the volume of distribution of hydrophilic drugs correlates more strongly with lean body mass compared with total body mass (Morgan and Bray 1994). Consequently, the doses administered here ranged from 1019 to 1415 mg (mean = 1226 ± 135 mg). Thus, modelling previous pharmacokinetic data (Rawlins *et al.* 1977) and that from part a, it can be predicted that during part b, peak plasma concentrations were reached within the study period of 120 minutes. However, given that T_{re} did not consistently recover in the 120 minutes study period (Figure 4.4), this raises the notion that the peak reduction in T_{re} is more likely to be in line with peak CSF concentrations of acetaminophen, which may take 4 hours to arise after an acute 1000 mg dose (Singla *et al.* 2012).

Future work should elucidate when T_{re} begins to recover after acute acetaminophen exposure, as this may have important implications if acetaminophen is to be used as a hypothermic agent following brain injury (Saxena *et al.* 2015). It would also be beneficial to ascertain if the maximal T_{re} reductions are in line with peak CSF concentrations, as this would help identify if acetaminophen induced hypothermia is mediated within the central nervous system.

4.7.4 Implications for accidental hypothermia

In the United Kingdom, cold-related mortality presently accounts for at least one order of magnitude more deaths than heat-related mortality i.e. around 61 and 3 deaths per 100,000 population per year, respectively (Vardoulakis *et al.* 2014). Consequently, in 2014 there were over 16,000 hospital admissions in the UK whereby hypothermia was the primary or secondary cause (HSCIC 2015). Exposure to environments beneath thermo-neutrality clearly present a major health risk, particularly in thermoregulatory vulnerable populations such as the very young and the elderly, who account for more than 85% of these admissions. The primary deleterious effects of cold on the human body arise when T_c falls below 35°C, although the autonomic physiological responses required to maintain T_c (tachycardia and shivering) can increase cardiovascular strain and can lead to secondary events particularly in elderly individuals (Parsons 2014). The data obtained in this experiment demonstrates that T_{re} is not as efficiently defended when participants ingest acetaminophen in a subneutral environment. This is particularly concerning as the average thermal sensation in both groups (acetaminophen and placebo) was 3.4 ± 2 (4 = "comfortable"), and no participants reported feeling "cold" on the thermal sensation scale. If acetaminophen inhibited normal thermogenic mechanisms in these conditions, it is likely that these T_{re} reductions will be exacerbated in colder environments. More work is needed in this area to confirm when the peak reduction in T_{re} arises, and the variability in this response.

Moreover, it is unclear if acetaminophen induced hypothermia is exacerbated in cold conditions (where there is a greater reliance on thermogenesis). These findings could have implications for public health recommendations.

4.7.5 Possible physiological underpinnings

The observed hypothermic effect of acetaminophen may be due to a decrease in heat production or an increased heat loss. During exposure to cold environments, veno and vasoconstriction occur rapidly and the magnitude depends on the gradient between the T_{sk} and the air temperature (Castellani and Tipton, 2015). Thereafter, metabolic heat production through shivering and BAT activation increases in an effort to maintain T_c close to 37°C. If we assume that the T_{sk} reduction (i.e. 27°C) reflects the level of cold stress in this study, it is likely that a change in vasomotor tone was the dominant factor for preserving T_{re} (Blondin et al., 2014). If this were true, T_{sk} would increase in vasodilatory sites (i.e. glabrous skin), reflecting an increase in skin blood flow. An elevated T_{sk} in these sites would be detrimental for T_c preservation due to the increased gradient between T_{sk} and air temperature, resulting in increased convective heat loss from the skin (Parsons, 2003).

That being said, the reductions in T_{sk} have been shown to initiate a shivering response (Gosselin and Haman, 2013). The possible inhibition of this response with acetaminophen is described in more detail below.

4.7.6 Possible molecular underpinnings

The molecular target for acetaminophen induced hypothermia in humans is not well established, but is likely due to inhibition of the COX enzyme. When administered orally (1000 mg), acetaminophen is a potent inhibitor of COX-2 in intact cells, but may also inhibit COX-1 (Hinz

et al. 2008). Given that participants in this study were exposed to sub-neutral environmental temperatures, it is possible that COX may have been prevented from activating thermogenic responses to this environment. Although the role of COX in non-febrile thermogenesis remains a topic of debate (Aronoff and Romanovsky 2007), recent evidence supports a role for COX in this capacity. For example, it has been demonstrated that COX-2 is essential for UCP-1 induction in beige/brite adipocytes during cold exposure, such that T_{re} was not efficiently defended during acute cold exposure in COX-2 gene deficient mice compared with their wild-type counterparts (Madsen *et al.* 2010). More recently, intravenous parecoxib (COX-2 selective inhibitor) administration significantly reduced post-operative shivering in non-febrile patients. Thus, it is possible that during sub neutral conditions, a reduction in autonomic shivering responses (mediated by acetaminophen induced COX-2 inhibition) could contribute to the decline in T_{re} witnessed in the present work. Indeed, the magnitude of reductions in T_{sk} seen in this study have been shown to initiate shivering in prior research (Gosselin *et al.* 2013).

4.7.7 Delimitations

This experiment contains several delimitations which should be considered in future experiments. In part a, the concentrations of acetaminophen in the plasma were quantified through an ELISA assay. The use of an ELISA in this context is not common in pharmacokinetic research due to the increased specificity and performance of liquid chromatography/mass spectrometry (LC/MS). Despite this, the results in the present study (part a) were in line with prior research in this area (Rawlins *et al.* 1977; Singla *et al.* 2012). The second limitation of this experiment was the anatomical position of the participants (i.e. seated upright). Positioning participants in this way promotes activation of the trunk (anti-gravitational) muscles (abdominal muscles, erector spinae, obliques), which may have interfered with resting metabolic rates and

therefore heat production (Rubini *et al.* 2012). The seated upright position was used in this study to best replicate the behaviours of thermoregulatory vulnerable individuals in their home during winter months. The third limitation of this study is the requirement that participants ingest food (~ 300 kcal) one hour prior to the 20°C exposure. In thermogenesis research, it is advisable that participants enter a cold environment in a fasted state to avoid any potential confounding effects of diet induced thermogenesis (Westerterp 2004). A 300 calorie meal (as used in this study) would be expected to cause a Δ increase in energy expenditure of ~0.2 kcal·minutes^{-1} (Ishii *et al.* 2016). This nutritional intake was used in the present study to reduce heterogeneity of acetaminophen absorption rates (Divoll *et al.* 1982b). A final limitation of this study was the use of doses adjusted for lean body mass. The use of a fixed dose (i.e. 500, 1000, and1500 mg) would have allowed a potential dose response to be identified, an important factor for future public health recommendations. For example, a 500-mg dose of acetaminophen may have no effect on T_{re} stability, and thus be a suitable dose for those at risk of hypothermia in winter months. The absolute doses administered in the present study ranged from 1019 to 1420 mg, with no relationship between dose quantity and T_{re} reduction being apparent.

4.7.8 Conclusions

In conclusion, it has been demonstrated that acute acetaminophen ingestion at a dose of 20 mg·kg lean body mass reduces non-febrile T_{re} during a 120-minute passive exposure to 20°C, 40% r.h (figures 4.3 and 4.4). Future research should seek to determine if acetaminophen reduces the capacity of the thermoregulatory system to maintain T_{re} during cold exposure. If acetaminophen reduces metabolic heat production during exposure to sub neutral environments, an amplified effect on T_{re} may occur when the ambient temperature is markedly reduced. Moreover, it should be determined if the peak reductions in T_{re} are in line with peak CSF

concentrations. Such findings would determine if acetaminophen induced hypothermia may be involved in the pathology of accidental hypothermia, especially during overdose.

CHAPTER 5. EXPERIMENT 2: NO EFFECT OF ACETAMINOPHEN ON CORE TEMPERATURE DURING PASSIVE HEAT EXPOSURE

5.1 Introduction

Heat stress is a topic that receives considerable attention in the context of human health and performance. An individual is under heat stress when active heat loss mechanisms are required to keep T_c stable, marked by an elevation of skin blood flow and secretion of sweat upon the skin surface. As humans, we live most of our lives in a state of heat balance (i.e. S is ~0), and this is primarily mediated through behavioural adaptations. Here, afferent neurons synapse with neurons in the thalamus which project to the primary somatosensory cortex, allowing conscious derivation of the state of the external environment (Craig 2002). Changing clothing, adjusting the thermostat, and seeking the shade on a hot sunny day are all examples of behavioural thermoregulation. If the T_{sk} continues to rise despite these behavioural responses, autonomic heat loss effector pathways are activated. Here, heat sensitive cutaneous nerves project to the anterior hypothalamus, activating cholinergic nerves which serve to increase skin blood flow and sweating (Nakamura and Morrison 2010). However, the T_c will rise if active heat loss mechanisms are insufficient, inducing cardiovascular strain, dehydration, and a reduced cognitive and physical function. This may progress into heat stroke if T_c reaches levels required for protein denaturation, or is raised for a very prolonged period which may activate innate immune responses (Lim and Mackinnon 2006).

In an occupational setting, a reduced ability to maintain cognitive or physical function can have adverse effects on health and safety. Studies demonstrate an increased risk of occupational injury while working in a hot environment (Tawatsupa *et al.* 2013), and there is also a strong negative

correlation between the environmental temperature and labour productivity (Hübler *et al.* 2008; Zander *et al.* 2015), each caused by feelings of discomfort and distraction (Taylor *et al.* 2015). These productivity losses are estimated to incur an annual loss of ~$6.2 billion in Australia (Zander *et al.* 2015) and ~$771 million in Germany (Hübler *et al.* 2008). Although these projections are based on exceptionally hot periods, the climate is consistently warming, and even if Europe achieves their goal of reducing CO_2 emissions to 20% below the level shown in 1990 by 2020, the effects of climate change will persist for at least a further 50 years (Nybo 2016). To offset these health perturbations and productivity losses, knowledge on contributing factors, or methods that may off-set internal heat strain are of interest since they may be used therapeutically to curtail T_c elevations.

In Study 1, it was demonstrated that acetaminophen holds a hypothermic action in healthy males, decreasing T_{re} by ~0.2°C at 120-minutes exposure to mild cold (20°C). That preliminary study warrants future work which will elucidate if the risk of acetaminophen induced hypothermia increases as the ambient temperature declines. However, given that acetaminophen is one of the most commonly administered drugs in the world, any beneficial or adverse effects on heat stress responses should be reported. It is currently unclear how acetaminophen might alter the T_c responses to passive heat stress as there is evidence for conflicting effects on cutaneous vasodilation. For example, acetaminophen is a potent COX inhibitor, an enzyme which is required for vasodilatory responses when young adult humans are subjected to a moderate (400 Watts) heat load (Fujii *et al.* 2014b). The evidence pertaining to this is discussed in section 2.5.8. Thus, it is possible that acetaminophen mediated inhibition of COX could curtail heat transfer from the skin to the environment by inhibiting full reflex vasodilation. A conflicting argument is that acetaminophen administration potentiates heat loss through an action of one of its active

metabolites, N-acetylbenzoquinoneimine (NAPQI). NAPQI is a potent agonist of the TRPA1 ion channel which is present on heat sensitive thermosensory neurons (Nassini *et al.* 2010). When these neurons are activated by NAPQI *in vivo*, there is a profound hypothermic effect in mice, which is highly dose dependent (Gentry *et al.* 2015). Therefore, acetaminophen (or NAPQI) could decrease the rate of heat storage by driving heat loss effector responses, providing a protective effect on T_c during heat stress. Research supporting an effect of acetaminophen during heat stress is varied. During fixed intensity cycling exercise in the heat (70% VO_{2max}), acetaminophen reduced T_{re} prior to and throughout the exercise trial by ~0.15°C (Mauger *et al.* 2014). Additionally, a recent case report showed that acetaminophen was effective in the management of mild heat illness, although its effectiveness was anecdotal and not supported by consistent T_c monitoring before and after its administration (Valente *et al.* 2017). Conflicting evidence shows that acetaminophen had no influence on T_{re} or sweating during exercise at a fixed rate of heat production (8 $W \cdot kg^{-1}$ body mass), but this effect may be dose dependent since there was a trend towards an increased T_{sk} and decreased T_{re} towards the end of the 60-minute cycling trial (Coombs *et al.* 2015). None-the-less, no study has investigated the effects of acetaminophen on T_{re} during passive heat stress i.e. without the confounding effect of exercise.

5.1.1 Experimental aims and hypothesis

Thus, the aim of the present study was to document the thermoregulatory responses to passive heat stress with and without acetaminophen administration. As the hypothermic effect of acetaminophen may be mediated through increasing heat loss (Gentry *et al.* 2015), it was hypothesised that the T_{re} rise would be reduced in the acetaminophen condition compared with a placebo.

5.2 Methods

5.2.1 Ethical approval

All experimental procedures were approved by the University of Bedfordshire's Institute for Sport and Physical Activity Research Ethics committee (approval code 2012ASEP021), and they conformed to the standards set by the World Association Declaration of Helsinki 'Ethical Principles for Research Involving Human Subjects'.

5.2.2 Participants

The 13 Caucasian males who participated in Study 1 part a also volunteered for this experiment. Participants were randomly divided into two groups which involved dry heat exposure [dry, 120 minutes at 45°C, 30% r.h. and humid heat exposure (humid, 45 minutes at 45°C, 70% r.h.). Subject characteristics are displayed in table 5.1.

Table 5.1. Participant anthropometrical characteristics in the hot dry and hot humid conditions. Data is presented as mean ± standard deviation with the range in square brackets.

Exposure	Age (years)	Height (cm)	Mass (kg)	Body Fat (%)	Lean Mass (kg)
DRY (n = 6)	23 ± 2 [21 to 26]	175 ± 7 [167 to 187]	71 ± 8 [61 to 81]	15 ± 5 [12 to 21]	60 ± 6 [51 to 68]
HUMID (n = 7)	23 ± 1 [21 to 24]	174 ± 8 [169 to 188]	74 ± 8 [67 to 86]	17 ± 4 [15 to 24]	61 ± 8 [51 to 71]

5.2.3 General design

Participants were exposed to heat stress (45°C) having ingested acetaminophen (20 $mg \cdot kg^{-1}$ lean body mass) or a sugar placebo (dextrose) 100 minutes prior to environmental chamber entry.

Based on the data from Study 1 part a (Chapter 4), this allowed time for the peak plasma concentration of acetaminophen to arise, ensuring that a therapeutic concentration was present in the circulation during heat stress. The two exposures to DRY (30% r.h.) or HUMID (70% r.h.) heat stress was separated by at least 7 days. Based on pilot work, the total exposure times in each trial was sufficient to induce a significant change in T_{re} over time. Drugs (acetaminophen or placebo) were administered in a double blind, randomised manner.

5.2.4 Inclusion/exclusion criteria

The general inclusion and exclusion criteria are outlined in Chapter 4 (section 4.2.3).

5.2.5 Heat stress protocol

Participants arrived at the laboratory at 07:00 or 10:00 in a fasted state, where each participant's time of arrival was consistent through all experimental trials to account for any circadian rhythm or diurnal variations in T_{re} (Waterhouse *et al.* 2005). Upon arrival, participants were instrumented for the measurement of T_{re}, T_{sk}, and heart rate (see section 3.1.5 for details). Thirty minutes after arrival, participants consumed the standardised meal (see section 3.1.4). One hour after the meal was consumed, participants were administered acetaminophen or a placebo by a laboratory technician. Participants entered the environmental chamber 100 minutes after drug ingestion, for peak plasma concentrations to be reached, as informed by Study 1 part a. Between drug administration and chamber entry, participants were rested in temperate environment. Five minutes prior to entering the chamber, resting measures of T_{re} and T_{sk}, heart rate, and thermal sensation were collected. Participants entered the environmental chamber 3 hours after initial arrival at the lab, in which they remained in a seated upright position for the duration of the heat

exposure. Due to differences in trial length (i.e. 45 and 120 minutes)T_{re}, T_{sk}, heart rate, and thermal sensation were measured every 5 and 10 minutes in the dry and humid trial, respectively.

5.2.6 Instrumentation and equations

Equipment details and equations for T_{re}, T_{sk}, heart rate, and thermal sensation are described within the General Methodology (section 3.1.5). The rate of T_{re} increase was calculated as follows:

$$\text{Rate of change} = (T1-T2)/\text{exposure time} \quad (3)$$

Where T1 is the final T_{re} reached, T2 is the pre/baseline T_{re} attained 5 minutes prior to heat exposure, and exposure time is the duration of heat exposure expressed in minutes. This value was 120 and 45 minutes in the dry and humid trials, respectively. This equation yields a value for the change in T_{re} per minute ($T_{re}\cdot min^{-1}$).

5.2.7 Statistical analysis

All statistical analyses were performed using the *'nlme', 'ordinal', 'ez', 'sjPlot'* and *'stats'* packages in *R* version 3.3.2 (R Core Development Team 2014). Normality assumptions were checked using quantile-quantile plots (Grafen and Hails 2002) and were plausible in all instances. Central tendency and dispersion are reported as means ± standard deviation (SD). The Akaike information criteria (AIC) was used to determine model fit (Akaike 1973). The correlation structure with the lowest AIC was chosen based on this procedure. The rate of T_{re} change ($T_{re}\cdot min^{-1}$) was analysed with a paired t-test. A linear mixed model with fixed ('drug', 'time') and random ('subject i.d') effects was fitted with an autoregressive correlation structure (to account for autocorrelation) to examine the effect of acetaminophen on T_{re}, T_{sk}, and heart rate

in dry [Time (13 levels): pre, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 minutes × Drug (2 levels): placebo, acetaminophen] and humid [Time (10 levels): pre, 5, 10, 15, 20, 25, 30, 35, 40, 45 minutes × Drug (2 levels): placebo, acetaminophen]. A cumulative link model was used to compare thermal sensation scores between placebo and acetaminophen in the thermoneutral and cold conditions. The two-tailed alpha level of significance testing was set as $p \leq 0.05$, and 95% confidence intervals (CI) are presented to denote the imprecision of the point estimate.

5.3 Results

Figure 5.1 displays the T_{re}, T_{sk}, and TS responses to passive heat stress in the dry and humid condition.

5.3.1 T_{re}

Dry

The rate of T_{re} change (°C·min^{-1}) was not different between the acetaminophen and placebo trials (t = 0.43, p = 0.65). Throughout the trial, T_{re} increased at a rate of 0.005 and 0.006°C·min^{-1} in the acetaminophen and placebo trials, respectively.

There was a main effect for drug ($F_{1,2}$ = 181.24, $p < 0.001$) and time ($F_{1,9}$ = 34.19, $p < 0.001$), but there was no interaction effect (drug × time) for the T_{re} response ($F_{1,9}$ = 0.16, p = 0.99) in the placebo (37.15 ± 0.11°C, 95% CI = 37.06 to 37.32) and acetaminophen (37.14 ± 0.13°C, 95% CI = 37.04 to 37.24) trial. Compared with the baseline (0) value, T_{re} was increased from 15 minutes to the end of the trial ($p < 0.05$).

Humid

The rate of T_{re} change (°C·min^{-1}) was not different between the acetaminophen and placebo trials (t = 0.59, p = 0.57). Throughout the trial, T_{re} increased at a rate of 0.023 and 0.021 °C·min^{-1} in the acetaminophen and placebo trials, respectively.

There was a main effect for drug ($F_{1,2}$ = 181.24, p < 0.001) and time ($F_{1,12}$ = 34.19, p < 0.001), but there was no interaction effect ($F_{1,12}$ = 0.16, p = 0.997) for the T_{re} response between the placebo (37.21 ± 0.32°C, 95% CI = 36.95 to 37.47) and acetaminophen (37.19 ± 0.29°C, 95% CI = 36.96 to 37.42) group.

5.3.2 T_{sk}

Dry

There was a main effect for drug ($F_{1,2}$ = 215.45, p < 0.001) and time ($F_{1,12}$ = 77.32, p < 0.001), but there was no interaction effect ($F_{1,12}$ = 0.19, p = 0.991) for the T_{sk} response between the placebo (35.9 ± 1.1°C, 95% CI = 35.02 to 36.8) and acetaminophen (35.9 ± 0.9°C, 95% CI = 35.2 to 36.6) group.

Humid

There was a main effect for drug ($F_{1,2}$ = 351.41, p < 0.001) and time ($F_{1,12}$ = 50.39, p < 0.001), but there was no interaction effect ($F_{1,12}$ = 0.32, p = 0.96) for the T_{sk} response between the placebo (37.5 ± 1.5°C, 95% CI = 36.3 to 38.7) and acetaminophen (37.1 ± 2.2°C, 95% CI = 35.3 to 38.9) group.

5.3.3 Heart rate

Dry

There was a main effect for drug ($F_{1,2}$ = 52.31, p < 0.001) and time ($F_{1,12}$ = 14.51, p < 0.001), but there was no interaction effect ($F_{1,12}$ = 0.41, p = 0.965) for the T_{sk} response between the placebo (82 ± 6 b·min^{-1}, 95% CI = 77 to 87) and acetaminophen (83 ± 13 b·min^{-1}, 95% CI = 73 to 93) group.

Humid

There was a main effect for drug ($F_{1,2}$ = 5.34, p = 0.02) and time ($F_{1,12}$ = 73.89, p < 0.001), but there was no interaction effect ($F_{1,12}$ = 0.32, p = 0.97) for the heart rate response between the placebo (94 ± 4 b·min^{-1}, 95% CI = 91 to 97) and acetaminophen (93 ± 9 b·min^{-1}, 95% CI = 86 to 100) group.

5.3.4 Thermal sensation

Dry

TS did not change with acetaminophen administration (p > 0.05). Compared with timepoint 0, TS was increased from timepoint 10 to 120 (p < 0.05). The odds ratios are displayed in Table 4.2.

Humid

TS did not change with acetaminophen administration (p > 0.05). Compared with timepoint 0, TS was increased from timepoint 10 to 120 (p < 0.05). The odds ratios are displayed in Table 4.3.

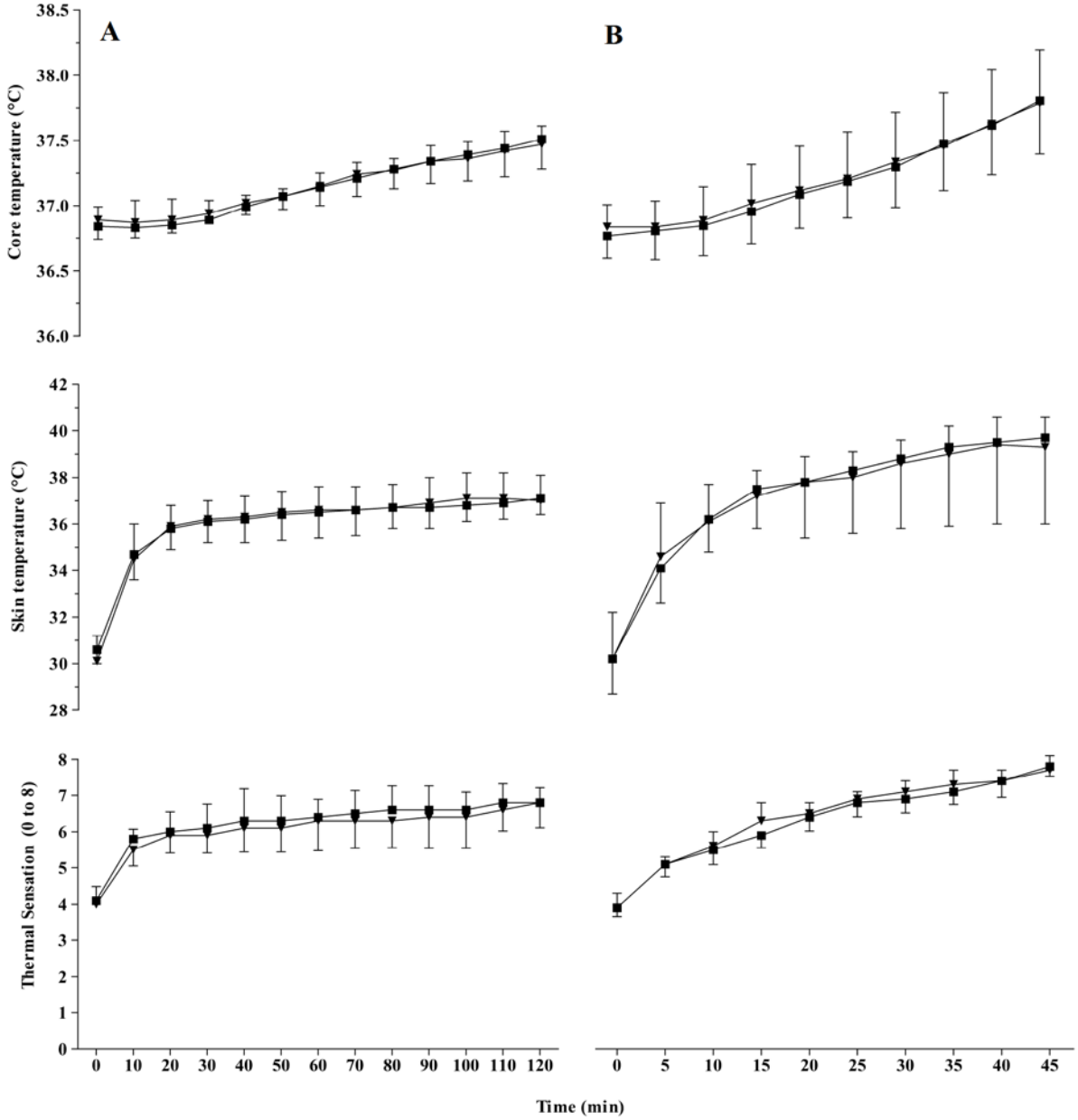

Figure 5.1. T_{re}, T_{sk}, and TS responses to passive dry (A) and humid (B) heat stress. The triangles represent the placebo condition, and the squares represent the acetaminophen condition. DRY n=6, HUMID n = 7.

Table 5.2. Changes in TS as a product of time (duration of exposure) and time + drug (interaction effect) during DRY heat stress. Significance codes: < 0.001***, < 0.01 **

	OR	**SE**	***P***
Placebo vs APAP			
(Intercept)	-0.764	1.427	
Time Only			
TIME20	4.615E+03	4.555	***
TIME40	1.102E+04	4.873	***
TIME60	2.274E+04	5.152	***
TIME80	2.460E+04	5.160	***
TIME100	3.673E+04	5.399	***
TIME120	3.673E+04	5.399	***
Drug×Time Interaction			
TIME20:DRUGAPAP	3.281	5.810	
TIME40:DRUGAPAP	3.786	6.117	
TIME60:DRUGAPAP	5.006	5.932	
TIME80:DRUGAPAP	11.488	6.042	
TIME100:DRUGAPAP	7.232	6.024	
TIME120:DRUGAPAP	24.146	6.268	

Table 5.3. Changes in TS as a product of time (duration of exposure) and time + drug (interaction effect) during HUMID heat stress. Significance codes: < 0.001***

	OR	**SE**	***P***
Placebo vs APAP			
(Intercept)	0.894	3.600	
Time Only			
TIME5	1.387E+05	5.426	***
TIME10	8.274E+06	7.665	***
TIME15	4.233E+09	12.746	***
TIME20	3.561E+10	15.912	***
TIME25	8.616E+11	20.538	***
TIME30	6.748E+12	25.729	***
TIME35	4.299E+13	29.415	***
TIME40	6.459E+14	39.997	***
TIME45	3.914E+15	46.016	***
Drug×Time Interaction			
TIME5:DRUGAPAP	0.624	5.677	
TIME10:DRUGAPAP	0.517	5.856	
TIME15:DRUGAPAP	0.048	6.006	
TIME20:DRUGAPAP	0.277	5.790	
TIME25:DRUGAPAP	0.586	5.714	
TIME30:DRUGAPAP	0.143	5.753	
TIME35:DRUGAPAP	0.0903	5.716	
TIME40:DRUGAPAP	0.040	5.781	
TIME45:DRUGAPAP	0.0522	6.126	

5.4 Discussion

5.4.1 Overview of results

It was hypothesised that acetaminophen would reduce the rate of T_{re} rise during passive heat stress. The experimental hypothesis was not accepted in this experiment, as there was no difference in T_{re} during dry and humid heat stress elicited by acetaminophen. The findings from this study do not support a role for acetaminophen use during heat stress, as there was no improvement in T_{re} maintenance compared with a placebo. Due to its inhibitory effect on COX, it was possible that acetaminophen could reduce heat loss during passive heat stress, but this was not supported in the present study (Figure 5.1). This experiment supports previous work, showing a lack of effect of acetaminophen on the physiological responses to exercise heat stress (Coombs *et al.* 2015).

5.4.2 Comparisons with previous research

Although this is the first study to investigate the effect of acetaminophen on thermoregulatory responses to passive heat stress, its thermoregulatory effects have been reported with exercise in hot ambient conditions (Coombs *et al.* 2015; Mauger *et al.* 2014). When acetaminophen was orally ingested 45 minutes prior to a cycling time to exhaustion trial in the heat (30°C, 50% r.h.), the participant's T_{re} was lower compared with the placebo condition (-0.15°C). Moreover, T_{re} remained ~0.15°C lower throughout the exercise trial, which the authors suggest could contribute to the increased time to volitional exhaustion. Although there was a paradoxical decrease in T_{sk} (~0.7°C) which occurred from 30% of trial through to 100% (i.e. volitional exhaustion), the effect was not large enough to override the acetaminophen mediated reduction in baseline T_{re}. Thus, it appears that acetaminophen inhibits full vasodilation during moderate to high heat load

(increasing heat gain), but induces heat loss in normal ambient conditions, but ambient temperatures outside of the chamber were not reported in that study. It is important to consider that comparing T_{re} responses over a percentage of completion time leads to different absolute time intervals, which is not favourable from a thermoregulatory perspective. To address this issue, Coombs *et al.* (2015) explored if acetaminophen changed the thermoregulatory responses to 60-minute cycle exercise in the heat (34.5°C, 52% r.h.) at a fixed rate of metabolic heat production (8 $W \cdot kg^{-1}$). Using absolute time intervals, they showed that acetaminophen (20 $mg \cdot kg^{-1}$ body mass) had no effect on T_{re}, T_{sk}, or sweating responses (arm and back) compared with the placebo condition. Thus, it appears that the changes in T_{re} evoked by acetaminophen in prior work (Mauger *et al.* 2014) may be due to changes in the baseline T_{re}, an effect which could depend on the ambient conditions outside of the environmental chamber.

5.4.3 Implications

Some pharmaceutical agents increase the risk of heat illness during exposure to hot weather. For example, it was shown that use of ACE inhibitors, diuretics, beta blockers and NSAIDs increased the risk of hospital admissions for heat illness in older people by 2.96 (95% CI = 1.53 to 4.43), 1.95 (95% CI = 1.58 to 2.11), 1.56 (95% CI = 1.30 to 1.84) and 1.51 (95% CI = 1.22 to 1.83) in the period January 2001 and June 2013 (Kalisch Ellett *et al.* 2016). The increase risk of heat illness from NSAID use is likely due to inhibition of COX bioactivity, and thus a reduced synthesis of PGE_2 and PGI_2. These molecules play important roles in kidney function and blood pressure regulation due to their vasodilatory actions. For instance, acetaminophen and ibuprofen depress renal blood flow and glomerular filtration rate due to their inhibitory actions on COX (Colletti *et al.* 1999), an effect which may contribute to excess hospital admission rates under heat stress conditions. Furthermore, there is evidence that COX contributes to vasodilation

(Holowatz and Kenney 2009; Kellogg *et al.* 2005; Medow *et al.* 2008; Noon *et al.* 1998) and sweating (Fujii *et al.* 2014b) responses during heat stress. Although acetaminophen had no effect on thermoregulatory capacity in our participants, this may not be mirrored with other COX inhibitors (NSAIDs) because acetaminophen has additional actions that promote heat loss responses. There is no evidence that NSAIDs induce heat loss responses, so a blunted heat loss response is more likely in drugs such as Ibuprofen, Aspirin, and COX-2 selective inhibitors (compared with acetaminophen). That-being-said, the use of acetaminophen may still be a hazard during heat waves since it can cause renal dysfunction (Satirapoj *et al.* 2007), which is concerning as renal disease/failure increases as a product of heat stress (Hansen *et al.* 2008).

Acetaminophen has been shown to improve repeated sprint and endurance exercise performance (Foster *et al.* 2014; Mauger *et al.* 2010), an effect which the authors attribute to a reduced pain perception. Given the recent finding that cycling time to exhaustion is also improved during heat stress (Mauger *et al.* 2014), and that there is at least no negative impact on thermoregulation, athletes may begin increase self-administration of acetaminophen for ergogenic purposes. It is important to note that such activity is not recommended, since chronic acetaminophen use can lead to liver toxicity and cardiovascular dysfunction, even at therapeutic doses (Hinz and Brune 2012). Moreover, pain relief during exercise is a potential hazard since nociception may serve provide information to the brain of actual or impending tissue damage (Foster *et al.* 2014; Mauger *et al.* 2010). Blocking these signals with acetaminophen could lead to a greater risk of injury, especially in highly motivated athletes.

5.4.4 Delimitations

There are several delimitations to this work which the reader should consider. Firstly, the administration time of acetaminophen was 100 minutes prior to entering the environmental

chamber. This was chosen based on prior pharmacokinetic work (Singla *et al.* 2012), and that of Study One, where the peak plasma concentration of acetaminophen arose at 100-minutes post-ingestion. acetaminophen could not be administered at the onset of heat stress since the humid trial was only 45 minutes in duration, which was not enough time for acetaminophen to reach therapeutic plasma concentrations. The benefit of this design is that acetaminophen would have been present in the circulation at the time of entry into the chamber, however, as systemic acetaminophen was not measured in this study, the actual concentration (and its decay during heat stress) is not known. Because acetaminophen did not differentially alter T_{re} in the dry or humid trial, future research should examine the T_{re} responses to heat stress (120 minutes at 45°C, 30% r.h.) with acetaminophen administered at the onset of the exposure. This would allow for examination of how the T_{re} response changes in line with the acetaminophen concentration, a model that was used effectively in study one. A second limitation is the use of a passive heat exposure and not an exercise model. The benefit of using a passive model is that it closely reflects the conditions where older people would be subjected to heat stress (i.e. in a heat wave), improving the link between acetaminophen use and potential thermoregulatory dysfunction. Because the metabolic heat load seems to determine the requirement of COX for vasodilation, a moderate (~45% VO_{2max}) and high (~80% VO_{2max}) heat load condition may have yielded differences in the T_{re} responses with acetaminophen administration (Fujii *et al.* 2014b). The final limitation to this study is the use of young adults as a model to determine the effect of acetaminophen on thermoregulation. During heat waves, it is the elderly who are considered most at risk, which is in part due to the presence of comorbidities such as diabetes, cardiovascular disease, respiratory disease, and renal impairments (Kenney *et al.* 2014). Also, the thermoregulatory responses are not homogenous between young adults and older people,

largely due to differences in nitric oxide bioavailability (Holowatz *et al.* 2003). Because young adults are generally more efficient at dissipating heat than older people, it is not certain that acetaminophen will have no effect on T_{re} in an aged individual. Indeed, aspirin ingestion resulted in an elevated T_{re} during exercise in the heat in middle aged (50 to 65 years) participants, an effect which occurred through increase in the T_{re} threshold required for cutaneous vasodilation (Bruning *et al.* 2013).

5.4.5 Conclusions

In conclusion, it has been demonstrated that acetaminophen (20 $mg \cdot kg^{-1}$ lean body mass) had no effect on the thermoregulatory response to passive heat stress in young adults. Although acetaminophen did not affect T_{re} during dry or humid heat stress, its potential impact on renal function do not make it a suitable alternative to NSAIDs during a heatwave. Future research should identify if T_{re} is maintained during passive heat stress, particularly in older people, while also assessing its acute effects on renal function.

CHAPTER 6. EXPERIMENT 3: ACETAMINOPHEN INDUCED HYPOTHERMIA IS POTENTIATED DURING ACUTE COLD STRESS.

6.1 Introduction

6.1.1 Background

Accidental hypothermia is characterised by an unintended T_c reduction to 35°C or lower. Such a fall in T_c can induce ventricular fibrillation and ultimately cardiac arrest if T_c declines to < 28ºC (Brown *et al.* 2012; Filippi *et al.* 2014). In the United States, hypothermia was the cause or contributing cause of death in over 5500 cases between 2006 and 2010 (Berko 2016), but this is likely underestimated since T_c needs to be measured at or near the time of death. Nonetheless, data from United Kingdom hospital episode statistics indicate that hypothermia was the primary or secondary diagnosis in over 100,000 hospital admissions from 2005 to 2015 (HSCIC 2015). Although death from hypothermia is rare, it remains a significant health risk in elderly and very young individuals, particularly during winter months and unaccustomed cold spells (Brown *et al.* 2012). Interestingly, there is a growing body of evidence demonstrating that acetaminophen could reduce T_c stability during cold exposure (discussed below), placing users at an increased risk of accidental hypothermia.

Acetaminophen is an over-the-counter drug marketed as paracetamol in Europe and Tylenol in the United States. It is best known for its ability to decrease pain perception and reduce T_c during a fever; each of these actions are in part mediated through an inhibition of COX enzyme activity (Anderson 2008). However, there is evidence of a 'hypothermic' action of acetaminophen, which refers specifically to an acetaminophen-induced reduction in T_c independent of febrile status. In mice, high doses (150 to 300 mg·kg^{-1} body mass) administered intravenously reduced T_{re} by 2 to

4°C (Walker et al. 1981), an effect that was confirmed in subsequent experiments (Ayoub *et al*. 2004; Ayoub *et al*. 2011; Li *et al*. 2008; Massey *et al*. 1982). In humans, there have been 246 reports in Vigibase© (the WHO international database of adverse drug reactions) specific to acetaminophen-induced accidental hypothermia (Lindquist 2008). In addition, several case studies report profound hypothermia following therapeutic doses (Van Tittelboom and Govaerts-Lepicard 1989) and high doses of acetaminophen when ingested orally (Block *et al.* 1992; Rollstin and Seifert 2012). Finally, oral acetaminophen administration (20 mg·kg lean body mass^{-1}) reduced T_{re} in young adults by ~0.2°C (range, 0.10 to 0.39°C) during exposure to mild cold [(20°C) Study 1 b]. Although the T_{re} reductions were small, this hypothermic side-effect of acetaminophen occurred in all thirteen participants. Despite this data, additional criteria, such as the environmental temperature, are needed to accurately predict when acetaminophen poses the greatest risk for hypothermia development. Since the COX pathway could be involved in non-febrile thermogenesis (Ayoub *et al.* 2006), inhibition of this enzyme by acetaminophen might cause T_{re} to fall during cold exposure, while exerting negligible effects on T_{re} while exposed to a warm environment.

6.1.2 Experimental aims and hypothesis

The aim of this pilot trial was to examine the thermoregulatory response to acetaminophen administration (20 mg·kg^{-1} of lean body mass) during a 120-minute exposure to a thermo-neutral and cold environment in healthy adult humans. Due to a potential role of COX in non-febrile thermogenesis (Ayoub *et al.* 2004), it was hypothesised that acetaminophen would reduce T_{re} in cold conditions, but have no effect on T_{re} in thermo-neutral conditions relative to a placebo.

6.2 Methods

6.2.1 Ethical approval

All experimental procedures were approved by the University of Bedfordshire's Institute for Sport and Physical Activity Research Ethics committee (approval code 2014ISPAR011), and they conformed to the standards set by the World Association Declaration of Helsinki 'Ethical Principles for Research Involving Human Subjects'.

6.2.2 Power calculation

Power analyses were conducted with GPower software version 3.1 (Heinrich University, Düsseldorf, Germany). Using T_{re} data from a previous experiment where acetaminophen was tested as a hypothermic agent in non-febrile stroke patients (Dippel et al., 2003a), it was determined that a total of nine participants were required to achieve a statistical power of 80%.

6.2.3 Participants

Nine Caucasian males [Age (22 ± 1 years), height (179 ± 5 cm), mass (80.7 ± 11.9 kg), body fat (20 ± 5 %)] volunteered to take part in the study. Participants were provided with written information regarding all experimental procedures, with supporting oral explanations from the principal investigator. Participants then subsequently provided written informed consent. The participants were non-smokers, non-febrile (resting T_{re} < 38°C), and were free from musculoskeletal injury.

6.2.4 Inclusion/exclusion criteria

Prior to each laboratory visit, participants completed an informed consent sheet, alcohol use disorder identification test [AUDIT; (Saunders et al., 1993)] a breathalyser test (AlcoSense, One, Berkshire, UK), and an acetaminophen risk assessment questionnaire. To avoid the risk of liver

damage inflicted by acetaminophen, participants were not able to participate in the research if they scored above ten on the AUDIT questionnaire or alcohol was present in their bloodstream (i.e. > 0% BAC). In addition, the acetaminophen dose was relative to lean body mass because it is a better indicator of liver volume than total body mass (Kwo *et al.* 1998). No participants presented with any pre-existing medical conditions that may have put them at an increased risk of acetaminophen toxicity. Due to potential thermoregulatory adaptions (Blondin et al., 2014a; Poirier et al., 2015), individuals were not permitted to take part in any experimental procedures if they were heat or cold acclimated or acclimatised. Thus, those who had travelled to a hot/cold climate or participated in a laboratory based heat/cold acclimation protocol less than three weeks prior to the experiment were not permitted to take part. All participants presented with a stable resting T_{re} (36.5-37.5°C).

6.2.5 Experimental design

To determine if acetaminophen reduces T_{re} stability during cold stress, nine participants visited the laboratory on 5 occasions, each separated by at least seven days. On visit 1, participants arrived fasted (overnight) and had their body fat assessed via air displacement plethysmography (Bod Pod, 2000A, Birmingham, UK). Visits 2-5 (experimental trials) were randomised (SPSS Inc., Chicago, USA), double blinded [drug ingestion only (i.e. “A” or “B”)], and followed a repeated measures design. On these visits, participants were exposed to either cold (10°C, 40% r.h.) or thermoneutral (25°C, 40% r.h.) environmental temperatures for 120 minutes, having been administered acetaminophen (20 mg·kg of lean body mass) or a placebo (dextrose). The dose of acetaminophen administered in the present work was 1287 ± 173 mg (range, 1082 to 1486 mg).

6.2.6 Experimental protocol

All participants arrived at the laboratory at 1000. Upon arrival, participants were instrumented for the measurement of T_{re}, T_{sk}, and heart rate (see "Instrumentation and Equations" for details). Thirty minutes after arrival participants consumed a standardised breakfast (1030) and ingested acetaminophen or a placebo one hour after the meal was consumed (~1135). Participants remained rested in an upright seated position between meal consumption and acetaminophen or placebo ingestion to ensure resting physiological status was attained. Participants were wheeled into the environmental chamber immediately following drug administration. Resting measurements of T_{re}, T_{sk}, heart rate and thermal sensation were taken five minutes prior to acetaminophen or placebo ingestion (1120) and subsequently every 10 minutes for 120 minutes' post-ingestion. Blood pressure was taken prior to chamber entry and every 30 minutes (pre, 30, 60, 90, 120 minutes) until the end of the trial.

6.2.7 Instrumentation and equations

T_{re} and T_{sk} was measured via portable data loggers. The T_{sk} from 4 sites was used to estimate mean T_{sk} (Ramanathan 1964). See section 3.1.5 for equipment details and the formula for calculating mean T_{sk}. Section 3.1.5 also details the measurement of heart rate and thermal sensation.

Mean arterial pressure (MAP) was measured using a portable blood pressure monitor (Omron M5-1, Omron, Milton Keynes, UK). Measurements were taken at baseline (pre), and every 30 minutes of the 120-minute exposure period (i.e. 30, 60, 90, and 120 minutes). MAP was later calculated as [(2 × DBP) + SBP]/3, in accordance with Yu et al. (2016).

6.2.8 Statistical analysis

All statistical analyses were performed using the *'nlme', 'ordinal', 'ez', 'sjPlot'* and *'stats'* packages in *R* version 3.3.2 (R Core Development Team 2014). Normality assumptions were checked using quantile-quantile plots (Grafen and Hails 2002) and were plausible in all instances. Central tendency and dispersion are reported as means ± standard deviation (SD). The Akaike information criteria (AIC) was used to determine model fit (Akaike 1973). The correlation structure with the lowest AIC was chosen based on this procedure. A linear mixed model with fixed ('drug', 'time') and random ('subject i.d') effects was fitted with an autoregressive correlation structure (to account for autocorrelation) to examine the effect of acetaminophen on T_{re}, T_{sk}, and heart rate in thermo-neutral and cold conditions [Time (13 levels): pre, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 minutes × Drug (2 levels): placebo, acetaminophen]. The same model with different levels of time [Time (5 levels): pre, 30, 60, 90, 120 minutes) × Drug (2 levels): placebo, acetaminophen] was fitted to determine the effect of acetaminophen on MAP in thermo-neutral and cold conditions. A cumulative link model was used to compare thermal sensation scores between placebo and acetaminophen in the thermo-neutral and cold conditions. The two-tailed alpha level of significance testing was set as $p \leq 0.05$. 95% confidence intervals (CI) are presented to denote the imprecision of the point estimate.

6.3 Results

Thermoneutral

There was no main effect for drug or interaction effect (drug × time) for T_{re}, T_{sk}, heart rate, TSS, or MAP. A main effect for time was present in each of these variables apart from MAP, showing that T_{re}, T_{sk}, heart rate and TSS changed ($p < 0.05$) over time with no differences observed

between acetaminophen and placebo. Descriptive (mean ± SD) data for each 30-minute interval is shown in Table 6.1.

Cold

The T_{re} response during cold exposure differed between the acetaminophen and placebo conditions. An interaction effect ($F_{1,12}$ = 2.25, p = 0.01), main effect for drug ($F_{1,2}$ = 2.25, p < 0.01), and main effect for time ($F_{1,12}$ = 8.33, p < 0.01) was found between placebo (37.06 ± 0.20°C; 95% CI = 36.99 to 37.12°C) and acetaminophen (36.90 ± 0.32°C; 95% CI = 36.79 to 37.01°C). Specifically, T_{re} was 0.18, 0.19, 0.22, 0.27, 0.29 and 0.35°C lower in the acetaminophen trial at time points 70 to 120 minutes compared with the placebo. The peak T_{re} reduction in the nine participants (120 minute compared with baseline) was 0.16 to 0.57°C (mean = 0.40 ± 0.15°C). Mean and individual T_{re} responses over the 120-minute exposure period are displayed in Figures 6.1 and 6.2, respectively.

There were no main effects for drug or interaction effects between drug and time for T_{sk}, heart rate, TSS, or MAP. A main effect for time was present in each of these variables excluding MAP. All descriptive data for each 30-minute interval is shown in Table 6.1. For T_{re}, Table 6.2 displays the model's fixed effects coefficients and random effect variances.

6.3.1 T_{sk}

Thermoneutral

There was no significant interaction effect ($F_{1,12}$ = 0.49, p > 0.05) or main effect for drug ($F_{1,2}$ = 0.13, p > 0.05) between placebo (30.7 ± 0.7°C, 95% CI = 30.7 to 30.9°C) and acetaminophen (30.7 ± 0.5°C, 95% CI = 30.6 to 30.8°C), but there was a main effect for time ($F_{1,12}$ = 4.45, p < 0.05).

Cold

There was no significant interaction effect ($F_{1,12}$ = 0.33, p > 0.05) or main effect for drug ($F_{1,2}$ = 0.06, p > 0.05) between placebo (26.1 ± 2.5°C, 95% CI = 25.2 to 26.9°C) and acetaminophen (26.4 ± 2.5°C, 95% CI = 25.5 to 27.2°C), but there was a main effect for time ($F_{1,12}$ = 114.39, p < 0.05). Mean T_{sk} responses are displayed in Figure 6.1.

6.3.2 Thermal sensation

Thermoneutral

TS did not change with acetaminophen administration and did not change as a product of time (p > 0.05). The odds ratios are displayed in Table 6.2.

Cold

TS did not change with acetaminophen administration (p > 0.05). Compared with timepoint 0, TS was increased from time-point 10 to 120 (p < 0.05). The odds ratios are displayed in Table 6.2.

6.3.3 Heart rate

Thermoneutral

There was no significant interaction effect ($F_{1,12}$ = 0.81, p > 0.05) or main effect for drug ($F_{1,2}$ = 2.13, p > 0.05) between placebo (60 ± 9 b·min^{-1}, 95% CI = 58 to 61 b·min^{-1}) and acetaminophen (63 ± 9 b·min^{-1}, 95% CI = 61 to 64 b·min^{-1}), but there was a main effect for time ($F_{1,12}$ = 2.00, p < 0.05).

Cold

There was no significant interaction effect ($F_{1,12}$ = 0.80, p > 0.05) or main effect for drug ($F_{1,2}$ = 1.57, p > 0.05) between placebo (63 ± 8 b·min^{-1}, 95% CI = 60 to 65 b·min^{-1}) and acetaminophen (60 ± 8 b·min^{-1}, 95% CI = 58 to 63 b·min^{-1}), but there was a main effect for time ($F_{1,12}$ = 2.42, p < 0.05).

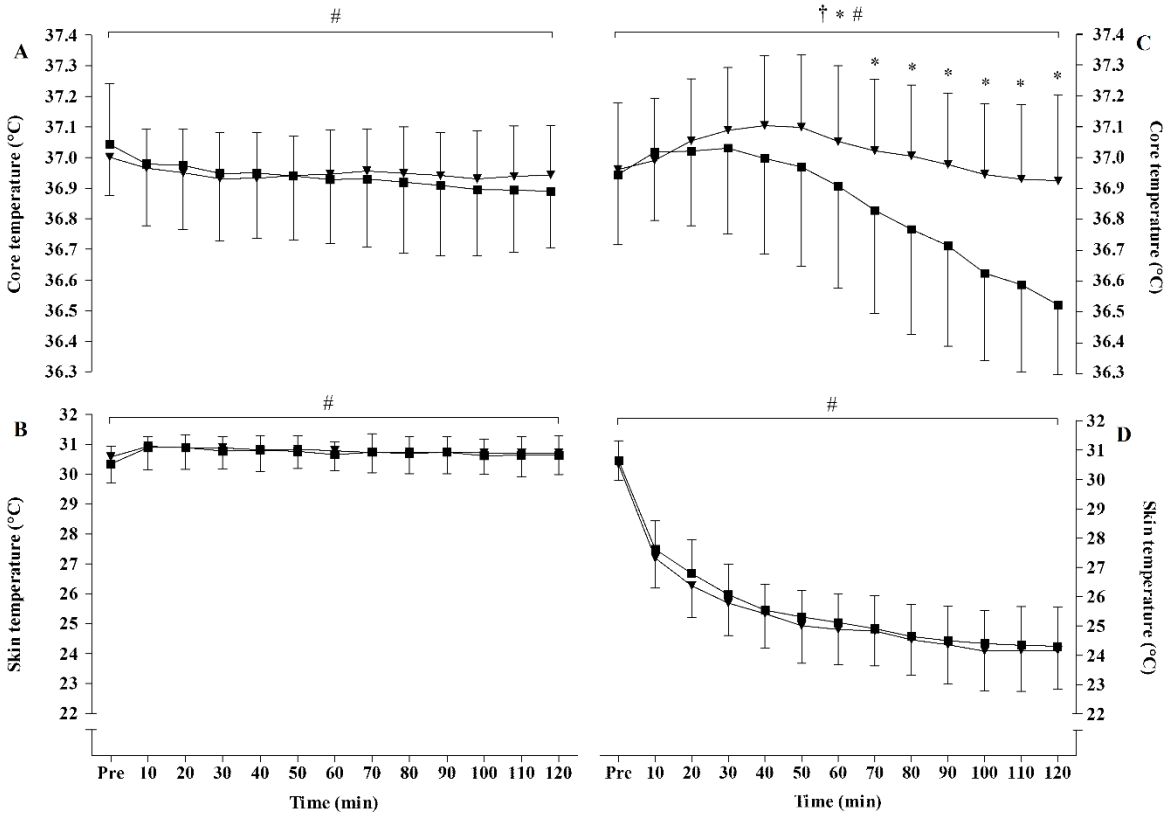

Figure 6.1. (A & C**)** T_{re} in the acetaminophen and placebo conditions during the 25°C and 10°C exposure, respectively. (B & D) T_{sk} in the acetaminophen and placebo conditions during the 25°C and 10°C exposure, respectively. ∗Significant main effect for condition. #Significant main effect for time. †Significant interaction effect. Values are mean ± standard deviation. n=9.

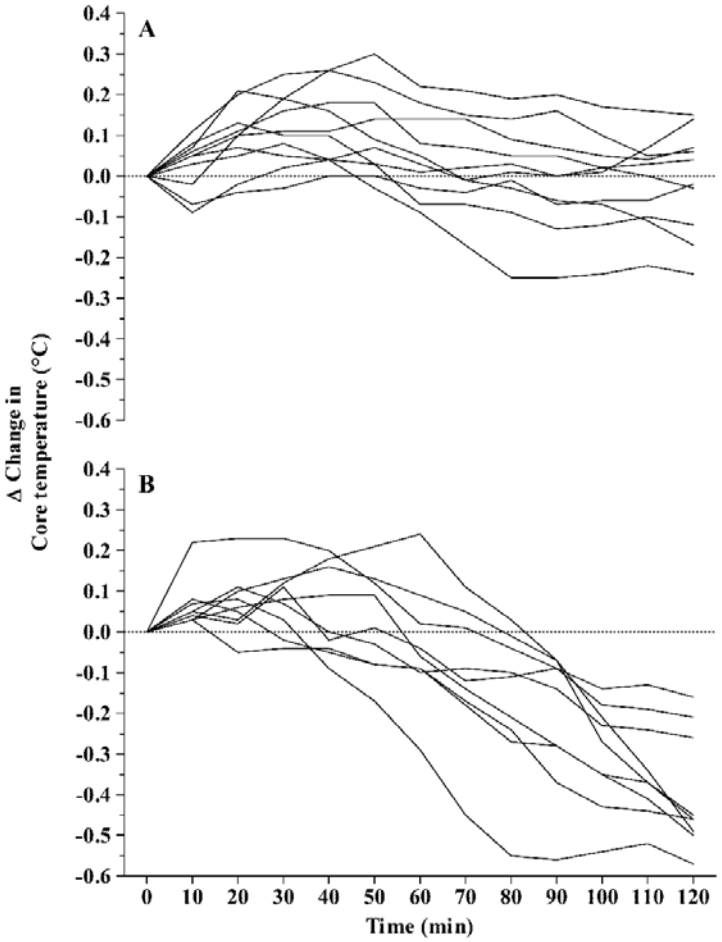

Figure 6.2. Delta (Δ) core temperature responses during cold exposure (10°C) in each participant following administration of a placebo (**A**) or acetaminophen (**B**). n=9.

Table 6.1. Beta coefficients (*B*), 95 % confidence intervals (CI), alpha values (*p)*, and the Phi coefficient are reported for the fixed components (drug & time) during exposure to cold stress (10ºC). The standard deviation of the intercept and residual are reported for the random effect (subject ID).

	Core Temperature (ºC)		
	B	*CI*	*P*
Fixed Parts			
Intercept	36.95	36.71 to 37.13	**<.001**
Drug×Time Interaction			
DRUGAPAP:TIME10	0.03	-0.11 to 0.17	.694
DRUGAPAP:TIME20	-0.03	-0.17 to 0.11	.672
DRUGAPAP:TIME30	-0.06	-0.20 to 0.09	.442
DRUGAPAP:TIME40	-0.10	-0.24 to 0.04	.179
DRUGAPAP:TIME50	-0.12	-0.26 to 0.02	.109
DRUGAPAP:TIME60	-0.13	-0.28 to 0.01	.076
DRUGAPAP:TIME70	-0.18	-0.32 to -0.03	**.021**
DRUGAPAP:TIME80	-0.21	-0.36 to -0.07	**.006**
DRUGAPAP:TIME90	-0.24	-0.38 to -0.10	**.002**
DRUGAPAP:TIME100	-0.29	-0.43 to -0.15	**<.001**
DRUGAPAP:TIME110	-0.31	-0.45 to -0.17	**<.001**
DRUGAPAP:TIME120	-0.36	-0.50 to -0.22	**<.001**
Phi Coefficient			
0.938			
Random Parts (Subject ID)			
		Standard Deviation	
Intercept		0.13	
Residual		0.16	

Table 6.2. Changes in TS as a product of time (duration of exposure) and time + drug (interaction effect). Significance codes: < 0.001***, < 0.01**

	10°C			25°C		
	OR	**SE**	***p***	**OR**	**SE**	***P***
Placebo vs APAP (Intercept)	0.575	2.540		0.989	2.435	
Time Only						
TIME10	2.55E-03	2.918	***	0.773	2.459	
TIME20	2.57E-04	2.981	***	1.302	2.529	
TIME30	2.57E-04	2.981	***	1.352	2.524	
TIME40	7.53E-05	3.130	***	2.013	2.533	
TIME50	3.41E-05	3.202	***	2.992	2.532	
TIME60	1.59E-05	3.340	***	2.013	2.533	
TIME70	1.11E-05	3.351	***	2.992	2.532	
TIME80	3.49E-06	3.530	***	4.031	2.596	
TIME90	1.46E-06	3.622	***	4.031	2.596	
TIME100	1.10E-06	3.728	***	4.031	2.596	
TIME110	1.46E-06	3.619	***	4.219	2.624	
TIME120	6.74E-07	3.792	***	4.219	2.624	
Drug×Time Interaction						
TIME10:DRUGAPAP	0.805	3.883		2.233	3.631	
TIME20:DRUGAPAP	1.736	3.831		1.952	3.725	
TIME30:DRUGAPAP	0.156	3.767		1.880	3.718	
TIME40:DRUGAPAP	0.576	3.724		1.899	3.720	
TIME50:DRUGAPAP	0.436	3.727		1.278	3.711	
TIME60:DRUGAPAP	0.940	3.754		1.899	3.720	
TIME70:DRUGAPAP	0.442	3.730		1.278	3.711	
TIME80:DRUGAPAP	0.815	3.789		1.423	3.781	
TIME90:DRUGAPAP	1.293	3.739		2.046	3.748	
TIME100:DRUGAPAP	1.307	3.733		4.080	3.747	
TIME110:DRUGAPAP	0.644	3.696		5.780	3.886	
TIME120:DRUGAPAP	1.143	3.780		8.252	3.973	

Table 6.3. Descriptive data for each of the five response variables in the thermo-neutral condition (25°C). Descriptive data are the mean values (± standard deviation) during the 120-minute exposure period. The range is provided in parentheses.

		Time-point (minutes)				
		Pre	**30**	**60**	**90**	**120**
T_{re} (°C)	**Placebo**	37.00 ± 0.13 (36.80 - 37.15)	36.93 ± 0.15 (36.72 - 37.13)	36.95 ± 0.15 (36.73 - 37.15)	36.94 ± 0.14 (36.75 - 37.15)	36.94 ± 0.16 (36.74 - 37.21)
	APAP	37.04 ± 0.20 (36.78 - 37.25)	36.95 ± 0.22 (36.78 - 37.14)	36.93 ± 0.21 (36.77 - 37.05)	36.91 ± 0.23 (36.68 - 37.10)	36.89 ± 0.19 (36.62 - 37.10)
T_{sk} (°C)	**Placebo**	30.6 ± 0.9 (28.7 - 31.8)	30.9 ± 0.7 (29.9 - 31.9)	30.8 ± 0.7 (29.8 - 31.7)	30.7 ± 0.7 (29.5 - 31.8)	30.7 ± 0.7 (29.3 - 31.7)
	APAP	30.3 ± 0.6 (29.0 - 31.1)	30.8 ± 0.5 (29.9 - 31.4)	30.7 ± 0.4 (29.9 - 31.2)	30.7 ± 0.5 (29.9 - 31.5)	30.6 ± 0.6 (29.6 - 31.6)
HR (b.min^{-1})	**Placebo**	65 ± 8 (53 - 79)	59 ± 8 (50 - 76)	58 ± 10 (46 - 79)	58 ± 9 (48 - 74)	60 ± 10 (49 - 86)
	APAP	68 ± 8 (53 - 81)	62 ± 10 (49 - 80)	65 ± 10 (50 - 84)	59 ± 7 (49 – 68)	59 ± 10 (42 - 71)
TS (0 to 8 scale)	**Placebo**	4.0 ± 0.1 (4.0 - 4.5)	4.1 ± 0.3 (4.0 - 5.0)	4.2 ± 0.4 (4.0 - 5.0)	4.3 ± 0.4 (4.0 - 5.0)	4.4 ± 0.6 (4.0 - 5.5)
	APAP	4.0 ± 0.2 (3.5 - 4.5)	4.2 ± 0.3 (4.0 - 5.0)	4.3 ± 0.4 (4.0 - 5.0)	4.4 ± 0.4 (4.0 - 5.0)	4.6 ± 0.5 (4.0 - 5.0)
MAP	**Placebo**	91 ± 7 (83 - 103)	91 ± 9 (73 - 101)	91 ± 10 (81 - 113)	92 ± 4 (88 - 99)	90 ± 6 (82 - 99)
	APAP	88 ± 6 (80 - 97)	91 ± 6 (82 - 100)	88 ± 9 (78 - 111)	88 ± 5 (83 - 97)	91 ± 6 (85 - 104)

Acetaminophen (APAP), Core temperature (T_{re}), Skin temperature (T_{sk}), Heart rate (HR), Thermal sensation (TS), Mean arterial pressure (MAP). Values are means ± standard deviation

Table 6.4. Descriptive data for each of the five response variables in the cold condition (10°C). Descriptive data are the mean values (± standard deviation) during the 120-minute exposure period. The range is provided in parentheses.

		Time-point (minutes)				
		Pre	**30**	**60**	**90**	**120**
T_{re} (°C)	**Placebo**	36.98 ± 0.20 (36.70 - 37.13)	37.09 ± 0.19 (36.79 - 37.38)	37.03 ± 0.22 (36.72 - 37.34)	36.97 ± 0.23 (36.71 - 37.29)	36.96 ± 0.25 (36.64 - 37.19)
	APAP	36.97 ± 0.21 (36.61 - 37.36)	37.05 ± 0.26 (36.59 - 37.49)	36.94 ± 0.31 (36.52 - 37.45)	36.76 ± 0.30* (36.33 - 37.29)	36.58 ± 0.23* (36.11 - 36.87)
T_{sk} (°C)	**Placebo**	30.5 ± 0.5 (29.6 - 31.3)	25.8 ± 1.0 (24.7 - 27.6)	24.9 ± 1.0 (23.8 - 26.9)	24.4 ± 1.0 (23.2 - 26.5)	24.2 ± 1.0 (22.8 - 26.6)
	APAP	30.7 ± 0.7 (29.6 - 31.8)	26.1 ± 1.0 (24.7 - 28.2)	25.1 ± 1.0 (23.7 - 26.6)	24.5 ± 1.2 (23.0 - 26.3)	24.3 ± 1.3 (22.4 - 26.5)
HR ($b.min^{-1}$)	**Placebo**	68 ± 7 (54 - 79)	62 ± 9 (48 - 74)	61 ± 4 (55 - 67)	57 ± 8 (48 - 68)	60 ± 9 (51 - 75)
	APAP	66 ± 11 (50 - 79)	59 ± 9 (41 - 70)	58 ± 10 (39 - 73)	54 ± 7 (42 - 64)	57 ± 9 (41 - 70)
TS (0 to 8 scale)	**Placebo**	4.1 ± 0.2 (4.0 - 4.5)	2.8 ± 0.4 (2.0 - 3.0)	2.3 ± 0.5 (1.5 - 3.0)	1.9 ± 0.2 (1.5 - 2.0)	1.8 ± 0.4 (1.0 - 2.0)
	APAP	3.9 ± 0.2 (3.5 - 4.0)	2.3 ± 0.4 (2.0 - 3.0)	2.2 ± 0.4 (1.5 - 3.0)	1.8 ± 0.6 (1.0 - 3.0)	1.7 ± 0.5 (1.0 - 2.5)
MAP	**Placebo**	92 ± 10 (78 - 104)	97 ± 9 (86 - 112)	99 ± 8 (90 - 110)	97 ± 7 (88 - 111)	105 ± 8 (92 - 117)
	APAP	93 ± 6 (78 - 102)	94 ± 9 (74 - 102)	103 ± 7 (91 - 111)	96 ± 6 (88 - 104)	99 ± 6 (77 - 104)

Acetaminophen (APAP), Core temperature (T_{re}), Skin temperature (T_{sk}), Heart rate (HR), Thermal sensation (TS), Mean arterial pressure (MAP). * denotes significant difference between the acetaminophen and placebo condition.

6.4 Discussion

6.4.1 Overview of results

It was hypothesised that acetaminophen would reduce T_{re} in cold conditions, but have no effect on T_{re} in thermo-neutral conditions relative to a placebo. The experimental hypothesis was accepted. The major finding of the present study was that, compared with a placebo, acetaminophen administration reduced T_{re} (0.16 to 0.57°C decrease after 120-minutes exposure) during an acute cold stress (10°C), while it appeared to have no effect on thermoregulation at a thermo-neutral ambient temperature (25°C). During cold exposure, acetaminophen caused T_{re} to fall by ~0.40°C compared with the baseline value at 120 minutes, while it did not decline in the placebo trial. The variability in the response may be due to between subject differences in the rate of acetaminophen absorption, but unfortunately this was not analysed in this pilot trial. The hypothermic response to acetaminophen ingestion observed in the current study corroborates our prior work in humans, in which acetaminophen reduced T_{re} by ~0.19°C in humans exposed to milder cold [20°C, Study 1b]. Furthermore, this is the first study to demonstrate that the ambient temperature can dictate the degree of hypothermia induced by acetaminophen. During cold exposure, this pilot trial shows that healthy young adults could not defend their T_{re} following acetaminophen administration (Figure 2). Given that elderly individuals already struggle to defend their T_{re} without prior drug ingestion (Collins *et al.* 1995), it is reasonable to suspect that acetaminophen would cause T_{re} to decline at a faster rate, increasing the risk of accidental hypothermia.

6.4.2 Comparison with previous research

The notion that ambient and skin temperature dictates the magnitude of acetaminophen's hypothermic action is in line with previous research. In a recent experiment, Coombs and colleagues (2015) demonstrated that acetaminophen had no effect on sweat output and T_{re} during 1-hour exercise in hot conditions (34°C, 52% r.h.) at a fixed rate of heat production (8 W·kg^{-1}). In that study, the mean skin temperature increased by 1°C during the trial (up to ~35°C), a condition in which no heat producing mechanisms will be active (Nakamura and Morrison 2010). Because the mean skin temperature during cold stress was ~24°C at the end of the trial (Figure 2), cutaneous vasoconstriction and active thermogenesis were required for T_{re} to remain stable (Haman *et al.* 2004a; Haman *et al.* 2004b; Nakamura and Morrison 2011). The presence of thermogenesis and vasoconstriction indicates that acetaminophen may reduce T_{re} through inhibition of at least one of these mechanisms, but the precise mechanism needs to be confirmed in future work. Study 1 demonstrated that acetaminophen reduced T_{re} by 0.10 to 0.39°C (mean ± SD, 0.19 ± 0.09°C) at rest when the mean skin temperature was ~27°C. Similar reductions in skin temperature induce shivering thermogenesis (Gosselin and Haman 2013), which, if inhibited by acetaminophen, may explain the small reduction in T_{re} seen in Study 1.

Studies in mice have shown T_c fell by 0.40, 0.80, and 2°C following 1-hour acetaminophen infusion of 100, 200, and 300 mg·kg^{-1} body mass respectively (Ayoub *et al.* 2006). Thus, acetaminophen-induced hypothermia is not only dependent on ambient temperature, but also on the dose administered. It is important to note here that mice are often housed in environments of 18 to 20°C, which is 8 to 10°C beneath their normal thermo-neutral zone (Speakman and Keijer 2013). These housing conditions are consistent in experiments concerning acetaminophen-induced hypothermia in rodents (Ayoub *et al.* 2004; Li *et al.* 2008; Massey *et al.* 1982; Walker *et al.* 1981), such that these animals constantly produce heat to maintain their T_c. Inhibition of this

heat production through acetaminophen may explain its hypothermic action, a notion that should be confirmed through the administration of high dose acetaminophen in mice housed within and below their thermo-neutral zone (i.e. 30°C and 20°C, respectively).

6.4.3 Possible physiological underpinnings

The results from the present study raise an interesting question; does acetaminophen increase whole body heat loss, or decrease whole body heat production? Because either action would theoretically result in a decreased T_{re} in the present work, the T_{re} data collected does not provide clues as to which answer is in fact true. Coombs *et al.* (2015) documented that acetaminophen ingestion had no effect on regional sweat output (a marker of heat loss) during cycling at a fixed heat production. However, due to the high evaporative requirement for heat balance in that study, any effect of acetaminophen on heat loss would be difficult to detect due to an already high sweat rate. If the evaporative requirement for heat balance is reduced to zero i.e. resting in a cool environment, a small increase in heat loss by acetaminophen would likely result in a reduced T_c, as observed in the present study. An increased T_{sk} at vasodilatory sites (i.e. hands and feet) would provide evidence for heat loss activation, but a limitation of the 4-point T_{sk} equation used in this study is that temperature is measured on largely non-vasodilatory sites (Taylor et al., 2014). Thus, it remains possible that heat loss was elevated in the cold with acetaminophen, despite prior work finding a null effect during exercise in hot humid conditions.

The fact that T_{re} rose in the first 30 minutes of cold stress has been shown in previous studies where humans were exposed to cold stress. For example, Murray *et al.* exposed young adults to 12°C air and found a 0.2°C T_{re} increase during the first 30 minutes of exposure. In that study, and in line with the present work, T_{re} returned to normal in the control conditions after this period i.e. from 30 to 120 minutes. Interestingly, this transient rise in T_{re} was not witnessed when

participants ingested acetaminophen. Although the lower T_{re} was not statistically significant until 80 minutes, the depressed thermogenic response in the first third of the trial may be attributed to an altered "null zone". The null zone is a narrow range of internal temperatures (approximately ± 0.5°C) in which neither heat loss or heat gain responses are activated, and was first described in 1983 using a method that clamped the T_{sk} during exercise and recovery (Mekjavić *et al.* 1991). The fact that in the acetaminophen trial, reductions in T_{sk} were not met with an increased T_{re} suggests that the "null zone" was shifted to the left i.e. thermogenic responses were activated for a lower drop in T_{sk} in the placebo condition. During fever, the null zone is shifted to the right i.e. shivering activated at a higher T_c (Nakamura, 2011). Acetaminophen depresses thermogenesis during fever by shifting the null zone back to normal values i.e. 36.5 – 37.5°C (Li et al., 2008). It is unknown if this explains the reduced thermoregulatory responses to cold with acetaminophen, but this could be directly investigated using similar methods to Mekjavić *et al.* (1991), and comparing the responses between a placebo/control and acetaminophen.

6.4.4 Implications

Given acetaminophen reduced T_c stability in healthy adult males its hypothermic effect is likely to be larger in populations already considered vulnerable in sub-neutral ambient temperatures (i.e. the very young and the elderly). Accidental hypothermia is a rising global health concern. In the USA, the Centre for Disease Control and Prevention report that hypothermia was the cause of nearly 17,000 deaths from 1999 to 2011 (Xu 2013). In the UK, hospital episode statistics show that there were over 108,000 admissions to NHS hospitals from 2005 to 2014, where hypothermia was the primary or secondary cause (HSCIC 2015). This database also shows that the very young (0-4 years; 43,868 admissions) and the elderly (≥ 65 years; 48,477 admissions) make up 85% of the total admissions. This is concerning for two reasons. Firstly, acetaminophen

is the most frequently administered analgesic among frail and pre-frail elderly individuals (Koponen *et al.* 2013), with no age-related delay in drug absorption (Divoll *et al.* 1982a). Secondly, acetaminophen is the analgesic of choice in neonates (Allegaert and van den Anker 2016). In the perioperative setting, T_c monitoring after acetaminophen administration in these vulnerable groups is recommended. A 2011 study showed that intravenous acetaminophen (~20 $mg \cdot kg^{-1}$ body mass) did not cause hypothermia in 93 neonates (Hopchet *et al.* 2011). However, the ambient temperature was not reported (presumably 23-25°C), and only the skin temperature was measured. This is problematic since our work showed a clear reduction in T_c without a change in skin temperature between acetaminophen and placebo (Study 1, Chapter 4). Moreover, neonates are exposed to cold stress when wet with amniotic fluid, during transportation, or during surgery. Based on our data, we propose that acetaminophen may increase the risk of neonatal hypothermia only when coupled with one of these cold stressors, and not in a thermo-neutral environment.

6.4.5 Proposed molecular mechanism

As acetaminophen is a potent COX inhibitor in non-inflamed tissue (Aronoff *et al.* 2006; Boutaud *et al.* 2002; Hinz *et al.* 2008; Hinz and Brune 2012), it is likely that the hypothermic action of acetaminophen is also mediated through inhibition of this enzyme. There are two COX isoforms (COX-1 and -2), and their function is to convert arachidonic acid to PGH_2 (Simmons *et al.* 2004), which cell specific isomerases and synthases then convert to prostanoids (prostaglandin E_2, F_2, D_2, and I_2) or thromboxane. The strongest evidence that acetaminophen induced hypothermia is mediated through COX inhibition was provided by Ayoub and colleagues (2004). They demonstrated that acetaminophen reduced T_{re} by 1.5°C in COX-1 knockout mice, where it fell by nearly 4°C when administered to wild-type mice. In addition,

they showed a strong positive correlation between brain PGE_2 concentrations and T_{re}. This data demonstrates that COX-1 may be required for normal T_{re} maintenance in mice housed in sub-neutral ambient temperatures, and that PGE_2 is the prostaglandin responsible for this phenomenon.

PGE_2 is indispensable for lipopolysaccharide induced fever (rise in T_{re}) because it dis-inhibits pre-optic area neurons that induce shivering and brown adipose tissue activation, such that nanoinjection of PGE_2 into the pre-optic area of anaesthetised mice induces a sharp rise in thermogenesis, and consequently T_{re} (Nakamura and Morrison 2011). Production of PGE_2 is a crucial component of the febrile response because the increased internal temperature creates a sub-optimal environment for bacterial growth and viral replication (Small *et al.* 1986). In section 2.4.3, it was argued that PGE_2 release is required for full thermogenesis during acute cold exposure, and suggested that pharmacological inhibition of COX metabolism (and thus, PGE_2 production) would reduce T_c stability in non-febrile cold exposed humans. Given that acetaminophen is a potent COX inhibitor in non-inflamed tissue, the present study provides compelling evidence that this enzyme is required for full thermogenesis in cold exposed humans. Confirmation of this theory may have major implications for acetaminophen users who are vulnerable to hypothermia (i.e. the elderly and the very young).

6.4.6 Limitations

One limitation of the present study is the use of young, healthy individuals as participants because this age demographic does not represent an at-risk group vulnerable to hypothermia. However, this age group was chosen because the body of evidence supporting the experimental hypothesis is too small to warrant investigation in a vulnerable age group (i.e. the elderly). Given that acetaminophen significantly reduce T_c in a group capable of strong metabolic responses to

cold stress, this warrants future research in elderly individuals. The second limitation to this study was the use of a cold stressor which does not reflect the minimum indoor temperatures witnessed during winter months, which is ~15°C. The reason for using severe cold stress was to clearly establish if a link existed between acetaminophen administration and cold-induced thermogenesis. The use of a higher ambient temperature may have resulted in an under-estimation of the effect of acetaminophen on non-febrile thermoregulation. A final limitation of this study was the absence of plasma acetaminophen concentrations during the environmental exposures. A dose-concentration relationship was clearly defined in Study 1a. However, plasma acetaminophen concentrations in this study would have allowed the reader to establish whether the inter-subject variability was due to differential plasma acetaminophen concentrations.

6.4.7 Conclusions

In conclusion, this study demonstrates that acute acetaminophen ingestion (20 mg·kg lean body mass) reduces T_c maintenance during acute cold exposure. Future work should determine if these hypothermic effects are limited to acetaminophen, or are witnessed in other COX inhibitors such as Ibuprofen, Aspirin, and Coxibs. If all COX inhibitors reduce thermogenesis during cold exposure, the prescription of these medications should be carefully considered in winter months and in the perioperative environment, especially in those already vulnerable to hypothermia.

CHAPTER 7. EXPERIMENT 4: EFFECT OF IBUPROFEN, A NON-SELECTIVE CYCLOOXYGENASE INHIBITOR, ON THERMOGENESIS DURING ACUTE COLD EXPOSURE.

7.1 Introduction

In Study 3 (Chapter 6), it was demonstrated that acetaminophen induced hypothermia is exacerbated in line with reductions in ambient temperature and T_{sk}. In a thermoneutral environment (25°C), there was no hypothermic side-effect of acetaminophen since T_{re} was stable throughout the passive 120-minute exposure (Figure 6.1). However, when the ambient temperature decreased to 10°C (reducing T_{sk} to 24°C), acetaminophen reduced T_{re} by 0.4°C (range = 0.16 to 0.57°C) after 120-minutes ($p < 0.001$). The results from that work raise concerns regarding the safe use of acetaminophen in those vulnerable to accidental hypothermia. For instance, it was shown that older people may not maintain a constant T_{re} during acute cold stress, an effect attributable to a reduction in endogenous heat production and possibly a reduced thermal sensitivity. The added administration of a drug which has a hypothermic side effect (such as acetaminophen) may accelerate reductions in T_{re}, leading to a greater risk of accidental hypothermia.

Information regarding acetaminophen's hypothermic mechanism of action is absent in humans. In mice, this side-effect may be mediated through interaction with TRPA1 (Gentry *et al.* 2015), hypothalamic $GABA_A$ receptors (Ahangar *et al.* 2016), or inhibition of COX metabolism (Ayoub *et al.* 2006). COX deserves specific investigation because it is the drug target of alternative analgesic and antipyretic medications such as ibuprofen, aspirin, and Coxibs. If COX is required for non-febrile thermogenesis, administration of any COX inhibitor should be avoided during

uncompensable cold stress. There is growing evidence that COX is involved in thermogenesis induced by skin cooling. In COX-1$^{-/-}$ mice, the hypothermic effect of intravenous acetaminophen (300 mg·kg^{-1}) was reduced by ~50% compared with wild-type mice. Moreover, the T_c reductions elicited by acetaminophen was strongly related to total brain PGE_2 concentrations, showing that COX-1 derived PGE_2 may play a role in normal T_c regulation. In section 2.5.3, evidence suggestive of a COX involvement in non-febrile vasomotion, shivering, and brown adipose tissue activation is discussed, as well as PGE_2's hyperthermic mechanism of action. Aside from the metabolic pathways that initiate thermogenesis, COX induced TXA_2 may also be involved in the veno-vasoconstrictor response to cold stress.

Although acetaminophen is a potent COX inhibitor *in vivo* (Hinz *et al.* 2008), its actions on TRPA1 and $GABA_A$ mean that is its hypothermic action could be independent of COX. Furthermore, actions on each of these sites combined may be responsible for the physiological change in T_c (during cold stress), which is a powerfully regulated variable in human biology. Thus, using acetaminophen as a model to investigate if COX is involved in non-febrile thermoregulation specifically is problematic. A preferred option may be to administer ibuprofen, as it is not known to have any additional mechanistic actions besides non-selective inhibition of COX-1 and COX-2. Additionally, ibuprofen blocks COX metabolism within the *cyclooxygenase* active site of the enzyme, whereas acetaminophen works on the *peroxidase* active site (Boutaud *et al.* 2002). This is an advantage since ibuprofen is effective in all tissue types regardless of the peroxide tone. Finally, ibuprofen crosses the blood brain barrier, resulting in non-selective COX inhibition in and outside of the central nervous system (Parepally *et al.* 2006). Taken together, administration of ibuprofen during acute cold stress is an effective tool to determine if COX is involved in thermogenesis.

7.1.1 Experimental aims and hypothesis

The aim of this trial was to examine the thermoregulatory response to ibuprofen administration (400 mg) during a 120-minute exposure to a cold environment (10°C) in healthy adult humans. It was hypothesised that ibuprofen would have a hypothermic side-effect by reducing metabolic rate and shivering thermogenesis.

7.2 Methods

7.2.1 Ethical approval

All experimental procedures were approved by the University of Bedfordshire's Institute for Sport and Physical Activity Research Ethics committee and University Research Ethics Committee (UREC), and they conformed to the standards set by the World Association Declaration of Helsinki 'Ethical Principles for Research Involving Human Subjects'.

7.2.2 Power calculation

Power analyses were conducted with GPower software version 3.1 (Heinrich University, Düsseldorf, Germany). Using T_{re} data from Study 3 (Chapter 6) it was determined that a total of six participants were required to achieve a statistical power of 90%. Specifically, the maximum reduction in T_{re} induced by acetaminophen (0.42°C) and the variability in the response (0.13°C) were parameters in the model.

7.2.3 Participants

Six Caucasian males [Age (20 ± 1 years), height (182 ± 6 cm), mass (82 ± 20 kg), body fat (16 ± 7 %)] volunteered to take part in the study. Participants were provided with written information regarding all experimental procedures, with supporting oral explanations from the principal

investigator. Participants then subsequently provided written informed consent. The participants were non-smokers, non-febrile (resting T_{re} < 38°C), and were free from musculoskeletal injury.

7.2.4 General experimental controls

The general experimental controls are described within the General Methodology (section 3.1.4). In addition, participants were required to complete the American College of Sports Medicine cardiovascular screening questionnaire prior to participation in the experiment. On the morning of each trial, participants were required to detail any medicinal activity in the prior 48 hours. No participants reported any pharmaceutical use in this time-period.

7.2.5 Experimental design

To determine the hypothermic effect of ibuprofen, 6 participants visited the laboratory at the same time of day on two occasions (ibuprofen vs placebo), each separated by at least seven days. All visits were randomized, and the ibuprofen and placebo trials were double blind. The trials took place within an environmental chamber set at 10°C for 120 minutes. This temperature was chosen as it allowed for thermoregulatory comparisons with Study 3 (Chapter 6) and was sufficient to induce shivering early in the trial (based on pilot data). Ibuprofen (Ibuprofen Modified Release Capsules, Banner Life Sciences, North California, USA) was administered at a 400-mg dose, and the placebo (dextrose, MYPROTEIN, Cheshire, UK) was presented in the same number of capsules to improve blinding.

7.2.6 Experimental protocol

All participants arrived at the laboratory at 0830. Upon arrival, participants were instrumented for the measurement of T_{re}, T_{sk}, and heart rate (see “Instrumentation and Equations” for details). On arrival, participants rested supine for 15 minutes, and subsequently a 5-minute resting sEMG

from the *vastus medialis* and a 2-minute expired air sample was taken (for energy expenditure). At ~0900, participants' maximal voluntary contraction (MVC) was taken on an isokinetic dynamometer (details below). At ~0920, 20G cannula was inserted into the participants' antecubital region and blood samples were taken 5-minutes prior to chamber entry, and subsequently at 60 and 120 minutes (inside the chamber). At ~0930, participants were instrumented for the measurement of T_{re}, T_{sk}, heart rate, and their body mass was taken (see section 3.1.5 for details). At this point, participants rested for 30 minutes in a supine position for resting physiological status to be attained. Ten-minutes prior to chamber entry, measurements for T_{re}, T_{sk}, heart rate, TS and MAP were taken. One-minute prior to entry into the cold chamber (10ºC, 40%), participants were administered 400 mg ibuprofen or a placebo in a double-blind manner (i.e. pills were made and administered by a laboratory technician). Participants laid supine in the chamber for 120-minutes with measurements for T_{re}, T_{sk}, heart rate, TS measured every ten-minutes. MAP was measured at 30, 60, 90, and 120 minutes, and a 2-minute expired air samples was taken at 40, 80, and 120 minutes. A 20-mL venous blood sample was drawn at 60 and 120 minutes. *Vastus medialis* sEMG was recorded at 5-20, 25-40, 45-60, 65-80, 85-100, and 105-120 minutes, in line with Haman *et al.* (2004a).

7.2.7 Instrumentation and equations

Thermometry

The T_{sk} from 12 sites was used to estimate mean T_{sk} (Hardy and DuBois, 1938). See section 3.1.5 for equipment details (T_{re} and T_{sk}) and the formula for calculating mean T_{sk}.

Metabolic rate

Changes in energy expenditure [EE ($J \cdot kg \cdot min^{-1}$)] were quantified using indirect calorimetry (Servomex, Servomex mini HF 5200, Sussex, UK). Two-minute expired air samples were taken at time point 0, 40, 80, and 120 minutes. The expired gas samples were collected in Douglas bags. These samples were analysed for volume of gas expired (litres), O_2 consumption, and CO_2 production.EE was calculated using the Weir equation (Weir 1949).

Maximal voluntary contraction and shivering activity

Shivering EMG was normalised and expressed as a percentage of the maximal voluntary contraction (%MVC). MVC measurements were performed on the morning of each experimental trial using an isokinetic dynamometer (Kin Com 125E Plus, Chattecx, Tennessee, USA). Subjects sat securely in an upright position and the load cell was attached slightly above the ankle. The load cell arm was placed at a 45° angle from its vertical position, and participants performed three maximal knee extensions pushing against the load cell. Each MVC was held for 5 seconds and was separated by a 30 second rest. All six MVCs from the two trials were later analysed with MATLAB, and the MVC which yielded the greatest EMG signal was used to normalise the shivering EMG values from both trials. Shivering intensity was measured using surface EMG at the *vastus medialis*. This muscle was chosen since its activity is induced by skin cooling by as little as 27°C (Haman *et al.* 2004a; Haman *et al.* 2004b), whereas T_{sk} is expected to reach ~25°C in the present work (Based on Study 3). Raw EMG signals were analysed by an external collaborator (Dr Denis Blondin, Université de Sherbrooke) with the use of custom-designed MATLAB algorithms (Mathworks). Dr Blondin was not aware which dataset corresponded to the placebo or ibuprofen trial at the time of analyzing this data. EMG signals were filtered to remove spectral components below 10 Hz and above 500 Hz, as well as 60 Hz contamination (and associated harmonics). Shivering intensity of the VM and its mean (EMG_{shiv})

was determined from root mean square values (RMS) rectified from EMG signals using a 50ms overlapping window (50 %). Baseline RMS values ($RMS_{baseline}$: 5 min RMS average measured before cold exposure) were subtracted from RMS shivering (RMS_{shiv}) as well as RMS_{mvc} values. EMG_{shiv} was then normalized to RMS_{mvc} by using the following equation:

$$EMG_{shiv}\ (\%MVC) = \frac{RMS_{shiv} - RMS_{baseline}}{RMS_{mvc} - RMS_{baseline}} \times 100$$

7.2.8 Statistical analysis

All statistical analyses were performed using the *'nlme', 'ordinal', 'ez'*, and *'stats'* packages in *R* version 3.3.2 (R Core Development Team 2014). Normality assumptions were checked using quantile-quantile plots (Grafen and Hails 2002) and were plausible in all instances. Central tendency and dispersion are reported as means ± standard deviation (SD). The Akaike information criterion (AIC) was used to determine model fit against the null model (Akaike 1973). The correlation structure with the lowest AIC was chosen based on this procedure. A linear mixed model with fixed ('drug', 'time') and random ('subject i.d') effects was fitted with an autoregressive correlation structure (to account for autocorrelation) to examine the effect of ibuprofen on T_{re}, T_{sk}, EE, shivering, and heart rate [Time (13 levels): pre, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 minutes × Drug (2 levels): placebo, ibuprofen]. A cumulative link mixed model with fixed ('drug', 'time') and random effects ('subject i.d.') was used to compare thermal sensation scores between placebo and ibuprofen. The two-tailed alpha level of significance testing was set as $p \leq 0.05$. 95% confidence intervals (CI) are presented to denote the imprecision of the point estimate.

7.3 Results

7.3.1 T_{re}

The T_{re} response to cold exposure was not different between groups. There was no main effect for drug ($F_{1,2} = 0.91$, $p = 0.34$), time ($F_{1,12} = 1.69$, $p = 0.07$), and there was no interaction effect ($F_{1,12} = 0.38$, $p = 0.97$). At 120 minutes, T_{re} was reduced by 0.04 ± 0.10°C and 0.08 ± 0.17°C in the placebo and ibuprofen group, respectively. The T_{re} response to cold stress in each group is displayed in Figure 7.1.

7.3.2 T_{sk}

The T_{sk} response to cold exposure was not different between groups. There was a main effect for drug ($F_{1,2} = 45.83$, $p < 0.01$) and time ($F_{1,12} = 112.77$, $p < 0.001$), but there was no interaction effect ($F_{1,12} = 0.69$, $p = 0.76$). At 120 minutes, mean T_{sk} fell by 4.3 ± 0.8°C and 4.7 ± 0.4°C in the placebo and ibuprofen trials, respectively. The T_{sk} response to cold stress in each group is displayed in Figure 7.1.

7.3.3 Heart rate

The heart rate response to cold exposure was not different between groups. There was no main effect for drug ($F_{1,2} = 1.22$, $p = 0.27$) and time ($F_{1,12} = 0.05$, $p = 0.90$), and there was no interaction effect ($F_{1,12} = 0.50$, $p = 0.90$).

7.3.4 Energy expenditure ($J \cdot kg^{-1} \cdot min^{-1}$)

EE did not change with ibuprofen administration. There was no main effect for drug ($F_{1,2} = 1.22$, $p = 0.56$) and no interaction effect ($F_{1,12} = 1.51$, $p = 0.23$), but EE did increase over time ($F_{1,12} = 59.63$, $p < 0.001$). Compared with baseline, EE rose by 77 ± 22 kJ in the placebo condition and

by 61 ± 18 kJ in the ibuprofen condition, but this was not significantly different ($p > 0.05$). The EE response is displayed in Figure 7.2.

7.3.5 Shivering thermogenesis

The shivering responses of the vastus medialis was not different between groups. There was no main effect for drug ($F_{1,2} = 1.25$, $p = 0.28$) and no interaction effect ($F_{1,12} = 1.10$, $p = 0.38$), but shivering did increase over time ($F_{1,12} = 52.84$, $p < 0.001$). In the final 30 minutes of the trial, shivering activity reached 1.10 ± 0.20 and 1.26 ± 0.30 %MVC in the placebo and ibuprofen group, respectively. The shivering response is displayed in Figure 7.3.

7.3.6 Thermal sensation

The thermosensory responses to cold exposure were no different between the ibuprofen and placebo group. Table 7.1 demonstrates effects for time ($p < 0.001$), whereas there was no main effect for condition and no interactions between groups at any time point ($p > 0.05$).

Table 7.1. Changes in TS as a product of time (duration of exposure) and time + drug (interaction effect). Significance codes: < 0.001***, < 0.01**

	OR	**SE**	***P***
Placebo vs IBU (Intercept)	1.818	3.688	
Time Only			
TIME10	9.77E-04	4.197	***
TIME20	9.24E-04	3.834	***
TIME30	9.24E-04	3.834	***
TIME40	7.80E-04	3.940	***
TIME50	4.19E-04	3.860	***
TIME60	1.85E-04	3.981	***
TIME70	1.91E-04	4.058	***
TIME80	8.40E-05	4.244	***
TIME90	1.32E-04	4.238	***
TIME100	8.40E-05	4.244	***
TIME110	8.40E-05	4.244	***
TIME120	1.27E-04	4.083	***
Drug×Time Interaction			
TIME10:DRUGIBU	0.180	6.143	
TIME20:DRUGIBU	0.134	5.561	
TIME30:DRUGIBU	0.138	5.571	
TIME40:DRUGIBU	0.143	5.547	
TIME50:DRUGIBU	0.266	5.308	
TIME60:DRUGIBU	0.601	5.299	
TIME70:DRUGIBU	0.536	5.401	
TIME80:DRUGIBU	0.787	5.419	
TIME90:DRUGIBU	0.248	5.466	
TIME100:DRUGIBU	0.165	5.380	
TIME110:DRUGIBU	0.108	5.387	
TIME120:DRUGIBU	0.168	5.256	

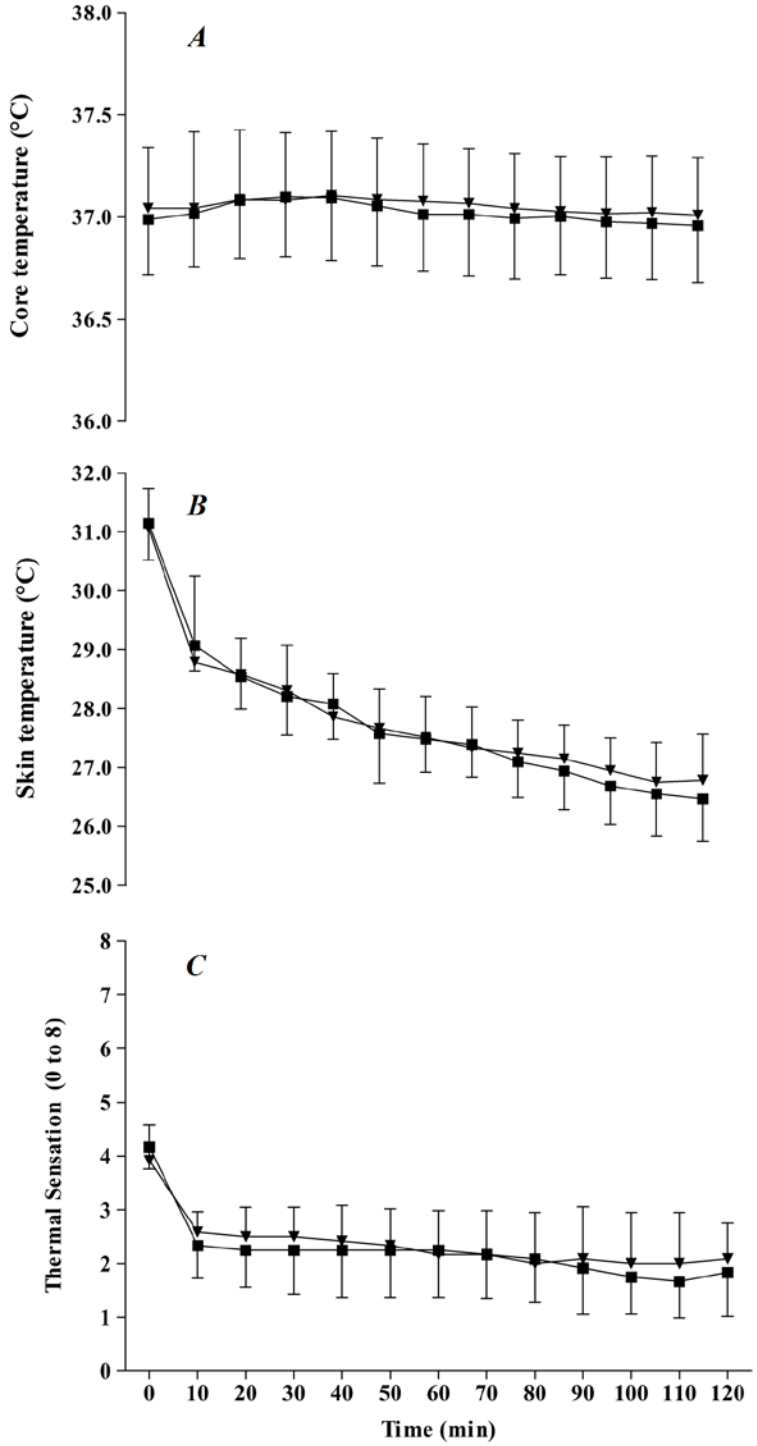

Figure 7.1. Mean ± standard deviation values for T_{re} (A), T_{sk} (B), and TS (C) in the placebo and ibuprofen conditions during a 120-minute exposure to 10°C. n=6.

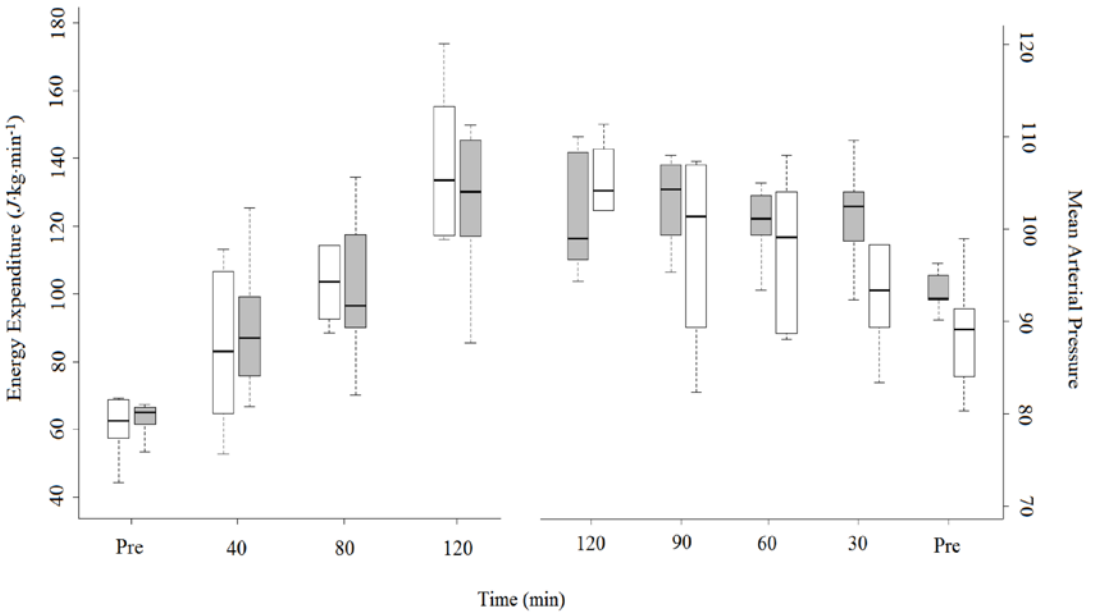

Figure 7.2. Boxplots displaying the change in EE and MAP during the 120-minute exposure to 10°C. The white boxes and shaded boxes denote the placebo and ibuprofen trials, respectively. n=6.

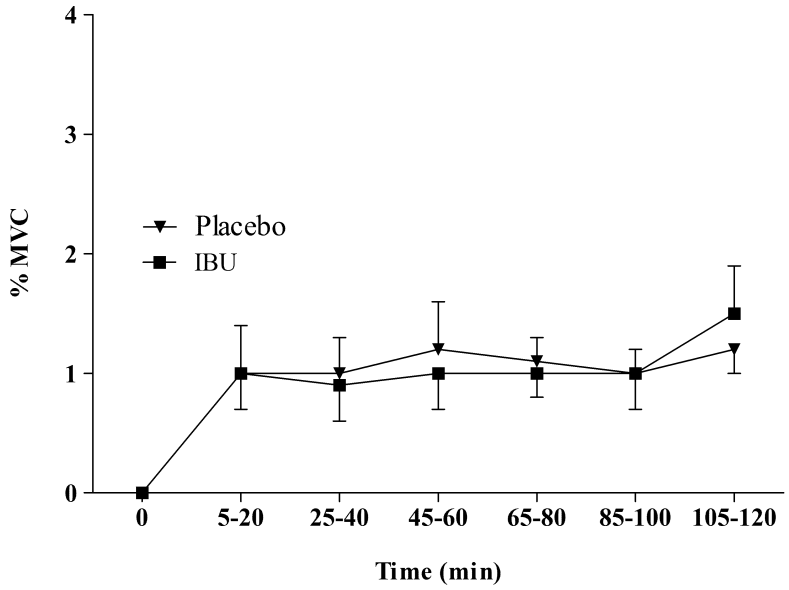

Figure 7.3. Shivering intensity of the *vastus medialis* during the 120-minute cold exposure, expressed relative to an isometric MVC. n=6.

7.4 Discussion

7.4.1 Overview of results

The experimental hypothesis was not accepted in this paper since neither thermogenic nor T_{re} responses to cold differed with ibuprofen administration (400 mg) compared to a placebo ($p > 0.05$). As ibuprofen is a potent non-selective COX inhibitor, this suggests that neither COX isoform has a significant role in the acute thermogenic responses to skin cooling.

This is the first study to compare thermoregulatory responses to cold stress with prior administration of ibuprofen (T minus 5 minutes). However, the effect of chronic aspirin administration on acute thermoregulatory responses to cold has been investigated previously (Murray *et al.* 2011a). Unlike other NSAIDs, aspirin inhibits COX-1 in a non-reversible manner, rendering aspirin an effective antithrombotic drug since it inhibits COX-1 for the life-span of the platelet (platelet COX-1 produces TXA_2 which increases thrombus formation (Schror 1997)]. Although these anti-thrombotic effects had no influence on T_{re}, T_{sk} or heat production during acute cold exposure, it is uncertain whether aspirin was present in the blood stream at the onset of the trial. This limitation was described in more detail in section 2.5.8, but in brief, if COX activity was induced by cold stress it is unlikely that it was inhibited due to the administration timing on the trial day. This was addressed in the present work since prior pharmacokinetic analysis suggested that therapeutic concentrations would be maintained from 15 minutes through to the end of the 120-minutes trial (Legg *et al.* 2014), suggesting that ibuprofen was present in the circulation during the cold exposure trials. Although there was no measurable difference in thermoregulatory responses between the two trials, this does not conclusively demonstrate that COX is not involved in these processes. Although *free* ibuprofen readily crosses the blood-brain barrier, it is extensively bound to plasma proteins (99%) and cannot cross into the brain in this

form (Parepally *et al.* 2006). Thus, the concentration of ibuprofen within the brain may not have been sufficient for COX inhibition in the hypothalamus, so the reduction in T_{re} seen with acetaminophen in identical conditions (Study 3) may be because a greater concentration of the drug reached this brain region. However, this is speculative since mammalian hypothalamic concentrations have not been obtained with either drug following administration.

The thermometric and metabolic responses to this level of cold stress are similar to that seen in previous studies. For example, Gosselin and Haman (2013) demonstrated that EE rose to ~125 $kJ \cdot kg^{-1} \cdot min^{-1}$ after 120-minutes of 10ºC cold exposure, while T_{re} remained stable and T_{sk} fell to ~ 26ºC. Indeed, there are many studies, including Study 3 (placebo trial), which show that T_{re} should remain stable with a 120-minute passive cold exposure to 10ºC (Blondin *et al.* 2010; Haman *et al.* 2004a; Haman *et al.* 2004b). While pilot work determined that a 2-minute sample provided a suitable volume of expired air for metabolic analysis, it is a relatively small sample for a 120 minute trial i.e. it reflects only 6 minutes of VO_2 data. The use of an online gas analysis system in future studies can provide a more detailed analysis of metabolic heat production throughout the course of the exposure and for longer time-periods. The shivering activity of the *vastus medialis* reached a peak of ~1.5% MVC in the current study, while it reached ~3% MVC in prior work where humans were exposed to 10ºC for 120-minutes, but this was achieved with a water perfused suit (Haman *et al.* 2004a). Such a garment would affect nearly 100% of the body surface area, whereas only ~50% of the body surface area was influenced by the cold air in the present study because the posterior side of the body was in contact with a hospital bed. A consequence of these methodological differences is that fewer skin cold thermoreceptors would have been activated during the cold exposure in the present study, perhaps contributing to the lower shivering activity observed. Another contributing factor is that participants in the work of

Haman *et al.* (2004a) had a lower and less variable body fat % (13 ± 1.9% vs 16 ± 7%), and a greater layer of body fat may have reduced the signal from the shivering muscle to the sEMG electrode.

The specific possibility that COX products (prostanoids) are involved in T_c defence against the cold has been investigated in rodents. Solomonovich and Kaplanski (1985) administered indomethacin (a highly potent and non-selective COX inhibitor) intraperitoneally to rats which were subsequently exposed to an ambient temperature of 4 ± 1°C, 23 ± 2°C and 34 ± 2°C. In addition, the authors measured hypothalamic PGE_2 concentrations following each exposure. In line with the present study, acute COX inhibition had no influence on the T_c of rodents, arguing against a COX mediated thermogenesis or vasoconstriction in response to cold. After 2 -hours cold exposure, hypothalamic PGE_2 concentrations were reduced from 8 $pg{\cdot}mg^{-1}$ to 0.7 $pg{\cdot}mg^{-1}$. This shows that PGE_2 is either constitutively expressed in this tissue or that COX is upregulated by cold exposure, but since baseline PGE_2 concentrations were not made in another group of animals, it is not possible to know which is true in either case. Ayoub *et al.* (2004) studied the hypothermic response to high dose acetaminophen infusion (300 $mg{\cdot}kg^{-1}$ body mass) in wild-type, COX-1 and COX-2 genetic knockout mice housed at 20°C. They demonstrated that the hypothermic response to acetaminophen persisted in COX-$2^{-/-}$ mice, but was reduced by ~50% in the COX-$1^{-/-}$ strain. This suggests that COX-1 may participate in normal T_c regulation, at least in mice housed beneath thermo-neutrality. Later work confirmed this hypothermic side-effect in mice, but showed that neither plasma nor brain PGE_2 concentrations were not influenced by acetaminophen in mice housed at 20°C (Li *et al.* 2008). This contradicts prior work in which there was a pronounced reduction in whole brain PGE_2 concentrations following acetaminophen

infusion, but this may be due to the difference in the relative doses administered (160 vs 300 $mg \cdot kg^{-1}$ body mass).

7.4.2 Conclusions

In summary, the administration of ibuprofen, a non-selective COX inhibitor, had no effect on heat production (shivering and metabolic rate) or thermometric (T_{re}, T_{sk}) responses to cold stress. Despite evidence that acetaminophen reduces T_{re} during mild (Study 1) and extreme (Study 3) cold stress, this side-effect was not mirrored with ibuprofen, suggesting that acetaminophen induced hypothermia (Study 1b and 3) is independent of COX inhibition.

CHAPTER 8: SYNTHESIS OF EXPERIMENTAL FINDINGS AND GENERAL DISCUSSION

8.1 General discussion

The aim of this chapter is to provide a general overview of the experimental findings from studies 1 to 4. This chapter will revisit the aims and objectives of the thesis and reviewing if each, in turn, have been achieved. The aim of this chapter is to integrate the findings of all the studies completed in this thesis with brief conclusions from all chapters leading to an overall conclusion from the thesis.

One of the main aims of this thesis was to determine if acetaminophen administration interacted with normal, non-febrile T_c regulation. During fever, T_c is raised through the actions of PGE_2 within the pre-optic anterior hypothalamus, and acetaminophen can reduce this hyperthermia through inhibition of PGE_2 synthesis within the brain (Li *et al.* 2008). Walker *et al.* (1981) were the first to document that high dose acetaminophen infusion causes hypothermia in non-febrile mice, and they also showed that this side-effect occurred prior to any hepatotoxicity. Since then, there have been many other reports of acetaminophen induced hypothermia in mice (Ahangar *et al.* 2016; Ayoub *et al.* 2004; Briyal and Gulati 2010; Gentry *et al.* 2015; Li *et al.* 2008; Massey *et al.* 1982; Walker *et al.* 1981), and several studies have shown the absolute T_c reduction is dose dependent (Ayoub *et al.* 2004; Gentry *et al.* 2015; Walker *et al.* 1981). Evidence of a hypothermic side-effect of acetaminophen prompted clinical research trials to investigate whether acetaminophen administration improved neurological outcome in ischemic stroke patients by reducing T_c and brain temperature (de Ridder *et al.* 2013; den Hertog *et al.* 2009; Dippel *et al.* 2001; Dippel *et al.* 2003a; Kasner *et al.* 2002). Although preliminary trials showed

a slightly reduced T_c with acetaminophen over 24 hours (~0.2°C), this did not have a clinical benefit in larger trials [paracetamol (acetaminophen) in stroke trial (PAIS)] when neurological outcome was assessed. These trials did not support acetaminophen administration in acute ischemic stroke patients, and consequently, the interaction of acetaminophen with non-febrile T_c in humans was not explored further. In this PhD project, the author viewed the animal model data from a different perspective; if acetaminophen reduces human T_c in those without a fever, this may increase the risk of accidental hypothermia in those who already struggle to maintain their T_c during cold exposure. If so, acetaminophen use in these vulnerable people could have serious implications in the healthcare sector, especially since there were already 17,500 hospital admissions from accidental hypothermia in 2014 (HSCIC 2015). To support this view, there are several case reports of acetaminophen induced hypothermia following administration of high (Rollstin and Seifert 2012) and therapeutic doses (Van Tittelboom and Govaerts-Lepicard 1989).

To address this question, a preliminary experiment was developed which investigated if oral acetaminophen administration (20 mg·kg^{-1} lean body mass) interacted with human T_{re} during a passive exposure to mild cold [Chapter 4, (20°C, 40% r.h for 120 minutes)]. This ambient temperature was chosen as it replicated previous experiments in mice which were house beneath their thermal neutral zone (Ayoub *et al.* 2004), and acetaminophen has not been shown to exert a hypothermic action in mammals housed at or above their thermal neutral zone. In this preliminary trial, the confounding effect of clothing was removed to ensure a more uniform T_{sk} distribution across the body. This mechanistic approach removes the opportunity for behavioural thermoregulation and is a useful approach for determining any cause and effect. However, it should be noted that, in real life, young adults are fully capable of behavioural adaptations such as increasing clothing insulation and increasing the room temperature from the thermostat.

However, behavioural thermoregulation is not always possible. Indeed, the mechanistic approach used for the following PhD studies can reflect conditions experienced by frail older people who are the most vulnerable to accidental hypothermia. Due to medical and socioeconomic issues, frail individuals may not be able to readily increase clothing insulation or alter the thermostat of the room (Collins, 1995). Although the hypothermic effect of acetaminophen should be investigated in this specific population, a clear cause and effect trial was warranted in the first instance to build a rationale for future studies investigating this in different population groups.

Although T_{re} was the primary outcome variable in Study 1, its effects on T_{sk} were also of interest. A raised T_{sk} is evident of increased heat transfer from the skin to the environment, since an augmented skin blood flow will increase the local temperature of the skin (Romanovsky 2014). If acetaminophen reduced T_{re} during the 120-minute exposure, a concomitant increase in T_{sk} would suggest that this effect is due to a reduction in the autonomic vasoconstrictor response to cold. Participant's TS was an additional marker of interest throughout this thesis. The ability to perceive changes in T_{re} and T_{sk} is indispensable for adequate behavioural thermoregulation, such that appropriate changes in clothing or the ambient temperature can be made to keep T_{re} at ~37°C. If there was a reduction in T_{re} without an associated decrease in TS, it suggests that participants do not perceive they are colder with acetaminophen, placing that at an even greater risk of accidental hypothermia. Study 1 (Chapter 4) was to be the first experiment in humans which maps these thermoregulatory variables following acetaminophen administration, while controlling the clothing insulation, ambient temperature, and gastric emptying rate of acetaminophen (by controlling food consumption prior to its ingestion). As a dose was administered relative to lean body mass, the plasma response to first documented (Study 1, part a) to ensure that plasma concentrations were in line with that seen previously. In part b (where

the thermoregulatory response was examined), young adults were chosen as participants in place of older people as they generally maintain their T_{re} during cold exposure. Because skin cooling may already decrease the T_{re} of older people without acetaminophen administration, it would be difficult to know acetaminophen's independent influence on T_{re}, even if an effect existed.

In Study 1 part a, it was demonstrated that oral acetaminophen administered at 20 mg·kg of lean body mass reached maximal plasma concentrations [14 ± 4 μg/ml, (range, 8-19 μg/ml)] at 80 to 100-minutes post-ingestion. These concentrations were in line with prior pharmacokinetic work, where acetaminophen was orally administered at a 1 g or 2 g dose (Singla *et al.* 2012). An interesting finding in part b was that acetaminophen reduced T_{re} in all 13 participants relative to the baseline value (0.19 ± 0.09°C decrease at 120 minutes), an effect not seen when participants were administered a sugar placebo. There are several reasons why there was a consistent effect on T_{re}. in this experiment compared with prior work in stroke patients. Firstly, the 20°C environment in Study 1 was sufficient to reduce T_{sk} to 27°C, a temperature which activates metabolic pathways required thermogenesis (Gosselin and Haman 2013). Although the ambient temperature in hospital wards may be ~22°C, the clothing insulation and bedding is sufficient to ensure that the T_{sk} is maintained within a thermoneutral range. Thus, it can be concluded that the hypothermic response to acetaminophen depends on whether participants are exposed to an environment beneath their thermal neutral zone. Secondly, T_{re} was continuously monitored in Study 1b, whereas it was only measured after 24 hours in the PAIS trial (de Ridder *et al.* 2013). Based on our results, the hypothermic action of acetaminophen could occur rapidly after its appearance in the systemic circulation, such that previous likely missed an initial reduction in T_{re}. This is pertinent since T_{re} on hospital admission is a key predictor of neurological outcome following an acute traumatic brain injury (Titus *et al.* 2015). Thirdly, acetaminophen was

administered as a 1 g oral or rectal dose in the PAIS trial. Since the hypothermic action of acetaminophen is highly dose dependent (Gentry *et al.* 2015), a 1 g dose may not have been sufficient to induce a reduction in T_{re}. A final limitation of the PAIS trial was the use of tympanic temperature in an unknown quantity of patients. Although the same method was used at baseline, there can be substantial variability with tympanic temperature and rectal temperature depending on the model of the sensor and the inter-rater reliability.

In experimental Chapter 5 (Study 2), the influence of acetaminophen on thermoregulation during uncompensable dry and humid heat stress was examined. Prior work has demonstrated negative effects of over-the-counter drugs aspirin (oral) and ketorolac (intradermal infusion) on heat loss effector responses in older and young males, respectively (Bruning *et al.* 2013; Fujii *et al.* 2014b). For example, Bruning *et al* (2013) showed that a 7-day regimen of low dose aspirin (81 $mg \cdot day^{-1}$) caused the threshold for heat loss effector responses to increase by ~0.3ºC. In the present acute study, the administration time was 100 minutes prior to entry into the environmental chamber so that C_{max} was attained at this point, in line with Study 1a. The dry and humid heat exposures caused T_{re} to rise at a rate of ~0.02 and 0.005°C·min^{-1}, respectively, but T_{re} and other thermoregulatory responses were not different between the acetaminophen and placebo group, suggesting that acetaminophen does not alter these responses or effect the thresholds for heat loss activation. An important limitation of the study was that T_{re} was the only variable associated with any change in body heat storage. Indeed, many studies demonstrate that the T_{re} likely underestimates absolute heat storage compared with those attained using direct calorimetry (Larose *et al.* 2014). Although direct calorimeters are not commercially available, an improved estimated of heat storage could have been attained with additional T_{re} measurement sites (i.e. oesophageal), and through measurement of whole body sweat losses. The reason that

acetaminophen was examined for both dry and humid heat stress was due to evidence that COX may only be involved in the sweating response at low heat loads (Fujii *et al.* 2014b). The lack of effect for acetaminophen during heat stress supports previous work which drew similar conclusions (Coombs *et al.* 2015).

In experimental Chapter 6 (Study 3), the strength of acetaminophen induced hypothermia was compared between exposure to a thermoneutral environment (25°C) and severe cold stress (10°C). Since acetaminophen had no effect on T_{re} during heat stress (Chapter 4), but reduced T_{re} during mild cold stress (Chapter 5), it is likely that acetaminophen depresses thermogenesis, while exerting little to no effect on active heat loss mechanisms. Because autonomic thermogenesis is governed by initial changes in Tsk, and acetaminophen may reduce efferent drive for this response, it was hypothesised that the strength of acetaminophen induced hypothermia would increase in line with requirement for thermogenesis. Thus, it was predicted that there would be no acetaminophen induced hypothermia in thermoneutral conditions, but a significant effect on T_{re} would occur during cold stress. In that study, the experimental hypothesis was accepted because acetaminophen had a hypothermic side-effect during cold stress, but not in thermoneutral conditions. Indeed, T_{re} fell by ~0.4ºC at 120 minutes compared with baseline, while T_{re} was well maintained in the placebo condition. These findings have implications for accidental hypothermia, especially because older people have the highest hospital admission rates in the UK (HSCIC 2015) and commonly use acetaminophen for the management of chronic pain (Koponen *et al.* 2013).

In Study 4, it was demonstrated that ibuprofen, a non-selective COX inhibitor, had no influence on thermogenesis during an acute exposure to cold (10ºC for 120-minutes). Prior to this work, there was considerable evidence both for (Section 2.4.3) and against (Aronoff and Romanovsky

2007) such a role for COX. In the present work, ibuprofen did not influence metabolic heat production, shivering at the vastus medialis, thermometry (T_{sk}, T_{re}), or thermal perception of cold ($p > 0.05$). The thermometric and metabolic responses were comparable to the placebo condition in Study 3, and prior studies where healthy humans were exposed to 10ºC for over ~120-minutes (Blondin *et al.* 2010; Gosselin and Haman 2013; Haman *et al.* 2004a; Lee and Haymes 1995; Murray *et al.* 2011a). For example, when Gosselin and Haman (2013) exposed young males to 10ºC for 2 hours, T_{re} remained stable at ~37ºC, and EE increased to ~130 $kJ \cdot kg^{-1} \cdot min^{-1}$; these responses were mirrored in the present study. Shivering activity of the *vastus medialis* peaked at ~1.5% MVC, which is relatively low when compared with previous work. For example, when Haman *et al.* (2004a) exposed young adult males to 10ºC for 2 hours, *vastus medialis* shivering activity rose to ~3.5% MVC at the end of the trial. Although the mean T_{sk} response was similar in both studies, methodological differences may explain the lower shivering response in the present study. Since Haman *et al*. (2004a) used a suit perfused with 10ºC water, so ~95% of the body surface area would be exposed to cold, whereas participants laid supine in Study 4 while exposed to 10ºC air. Here, only ~50% of body surface area (the anterior side) would have been exposed to the cold, so less cold thermoreceptors would have been activated to activate thermogenesis. It is also worth noting that local T_{sk} measurements can be confounded if they come into contact with the water perfused garment (communications with experts), perhaps explaining why Tsk was similar in both studies despite changes in body surface area exposed to cold. Based on prior work, it is most likely that COX is involved in chronic adaptations to cold rather than acute responses. For example, COX-2 release of PGE_2 is involved in the browning of white adipose tissue and the induction of UCP-1 in these beige cells (Madsen *et al.* 2010; Vegiopoulos *et al.* 2010). One day after transfer into a cold environment, blockade of COX-2 expression with

indomethacin caused T_{re} to fall by 2ºC, whereas T_{re} only fell by 1ºC in mice treated with saline (Madsen *et al.* 2010). While use of COX inhibitors do not augment heat loss in acutely cold exposed humans (Study 4), they may reduce beige cell following cold acclimation, but this should be determined in future work. Overall, these results suggest that the use of COX inhibitors (NSAIDS & Coxibs) does not represent a risk for accidental hypothermia during winter months. The results also suggest that acetaminophen induced hypothermia is independent of COX inhibition in humans, which contradicts prior work where this side effect was largely reduced in COX-$1^{-/-}$ mice (Ayoub *et al.* 2004).

8.2 Limitations

The use of rectal temperature as a surrogate for T_c is widely used, but it may not reflect whole body heat loss since it measures the temperature of a specific tissue. In addition, there may be a lagged effect regarding the T_{re} response to heat stress compared with oesophageal temperature and intestinal temperature (Lee *et al.* 2000). Although measuring heat balance in real time is only available with direct calorimetry, additional T_c measurement sites would provide a greater estimation of internal temperature. In this project, rectal temperature was chosen since it allows comparisons with previous work in mice (Ayoub *et al.* 2011) and is also efficient from a logistical point of view. Secondly, the lack of circulating plasma acetaminophen concentrations in Study 1b, Study 2, and Study 3 is a major limitation since it is still unknown what concentration is required to mediate the demonstrated physiological responses. However, since the plasma response to 20 $mg \cdot kg^{-1}$ lean body mass was mapped in Study 1a, the concentrations attained in the subsequent experiments can be predicted with reasonable confidence, as there were no changes to the study design which would influence the pharmacokinetics of acetaminophen. Thirdly, Study 2 adopted a design which required acetaminophen to be

administered 100 minutes prior to heat exposure. This approach ensured therapeutic plasma concentrations were attained during the humid heat exposure (45°C, 70% r.h.), since this trial was only 45 minutes in duration. For example, if acetaminophen was administered at rest, concentrations would only be ~5 to 10 $\mu g \cdot ml^{-1}$ at the end of the humid exposure, as informed by Study 1a. In retrospect, administration of acetaminophen immediately prior to chamber entry in the dry heat stress trial (45°C, 30% r.h.) would have improved the comparison with Study 1 and 3. It would also have demonstrated how physiological changes occur as a product of increase acetaminophen concentrations (if an effect existed). Fourthly, the major rationale for investigating whether over-the-counter drugs impact T_c regulation during thermal stress is linked to vulnerable populations, such as the elderly and neonates. These two age groups are the most vulnerable to accidental hypothermia (Collins 1995; Onalo 2013), yet the experimental subjects in this series of studies used healthy young males. However, such an effect needed to be investigated in a healthy model prior to work in vulnerable populations. Indeed, the work demonstrated in Study 1 and 3 serves as a rationale for future investigations into the contribution of acetaminophen to hospital admission rates for accident hypothermia. Fifth, heat production (indirect calorimetry) and shivering (sEMG) responses to cold were only measured in Study 4. The lack of these measures in Study 1 and 3 make it difficult to know if acetaminophen induced hypothermia was due to activated heat loss effectors or increase in basal vasodilation, or through a reduction in heat production. The primary aim of these studies was to determine if acetaminophen reduced T_c, since this was most relevant to clinicians. The data from Study 1 and 3 warrant further work which should measure expired air and shivering responses to acetaminophen during cold exposure. Sixth, the posture of the participants was different in Study 4 (supine), compared with experiments 1-3 (seated). Participants were in a supine position in the

final study because this decreased the potential for subtle behavioural responses i.e. adjusting their position for comfort. The limitation to this is that ~50% of the body surface area is in contact with a non-conductive hospital bed, contributing to an elevated mean T_{sk} (~2ºC) compared with Study 3. The elevated requirement for thermogenesis in Study 3 may have contributed to a greater hypothermic effect of the experimental drug.

8.3 Conclusions

In conclusions, this project has demonstrated that acetaminophen reduces T_c during cold stress, and this effect is unlikely to be mediated by COX inhibition, at least in isolation. Despite its ability to inhibit COX in endothelial cells, and thus inhibition of vasodilatory prostaglandins (Boutaud *et al.* 2002), acetaminophen did not affect the rate of T_c rise during exposure to dry and humid passive heat stress. Future work should determine the factors that influence the hypothermic response to acetaminophen i.e. age, body morphology, dose administered, drug tolerance, ambient temperature, and prior acclimatisation to cold. Moreover, the mechanism of acetaminophens hypothermic should be confirmed in humans because it may lead to the development of drugs used for the induction of therapeutic hypothermia.

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APPENDICES

9.1 Ethical approval confirmation for Study 1 and 2.

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11 February 2013

Ethical scrutiny confirmation

Proposer:	Josh Foster
Proposal short title:	The pre-cooling kinetics of paracetamol: An investigation into paracetamol's effect on core body temperature during thermal homeostasis, compensable heat stress, and uncompensable heat stress

Dear Proposer

Your proposal has now received ethical scrutiny from the Institute for Sport and Physical Activity Research Ethics panel.

I can confirm that this has now been approved, please find below your approval number:

Approval number: **2012ASEP021**

Please note that if it becomes necessary to make any substantive change to the research design, the sampling approach or the data collection methods a further application will be required

You are now clear to proceed with data collection for this project.

Thank you very much for your patience in this matter

Regards

David Kirk

Prof David Kirk (ISPAR Director)

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9.2 Ethical approval confirmation for Study 3.

University of Bedfordshire

10/12/14

Ethical Approval Confirmation

Proposer: Dr Lee Taylor

Proposal title: The influence of paracetamol (acetaminophen) on thermoregulation during acute cold exposure: Are prostaglandins involved?

Dear Proposer

Your proposal has now received ethical approval from the Institute for Sport and Physical Activity Research Ethics Panel.

Approval number: 2014ISPAR011

Please note that if it becomes necessary to make any substantive change to the research design, the sampling approach or the data collection methods a further application will be required.

Please be advised that your research project may be subject to an ethical audit at any given time. If you require any further information please contact the ISPAR Ethics Chair, Dr Laura Charalambous.

You are now clear to proceed with the data collection for this project.

Kind Regards

Dr Laura Charalambous (ISPAR Ethics Chair)

9.3 Ethical approval confirmation for Study 4.

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UNIVERSITY RESEARCH ETHICS COMMITTEE

21 June 2016

Re: UREC77 Involvement of cyclooxygenase in non-febrile temperature regulation.

Dear Mr Foster

The above proposal was submitted to UREC for ethical approval. Your proposal has now been considered by the Committee and I am pleased to inform you that ethical approval has been granted subject to the following conditions:

1. The amendments agreed in discussion with the external reviewer (appendix 1).

2. Re-writing the Participant Information Sheet in lay language. The following amendments are suggested to indicate the ways in which the document needs to be revised:
   a. In the 'purpose of the study', it might not be clear to the potential participant what 'endogenous heat production' is. This should be clearly explained in lay terms.
   b. What is BOD POD?
   c. What is air displacement?
   d. Will the lay person understand what 'will be instrumented for the measurement' means?
   e. What are 'skin thermistors'?
   f. What is the 'antecubital region'?
   g. Use a 'trained person (phlebotomist)' instead of 'train phlebotomist' as not everyone will know what a phlebotomist does.

   The reviewers strongly advise that you invite 3-5 lay people to trial the PIS and use this feedback to edit before resubmitting for approval.

3. Ensuring that the Participant Information Sheet is amended so that the P.I. introduces themself by name and explaining that the study is part of the requirements of a research degree with the University of Bedfordshire.

4. Correction of the following typographical errors:
   a. In the section "what will participants be asked to do" the line: 'on each laboratory visit a breathanalyser…' should read 'on each laboratory visit, a breathanalyser…'

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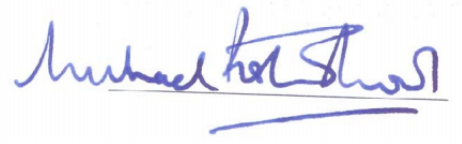
5. Clarity regarding the following:
   a. Where visits 2-3 are described, it is mentioned: "The three conditions include:
      400 mg ibuprofen (capsule form)
      Placebo (dextrose placebo, capsule form)
      Is there a third condition?

Please advise when these conditions have been met. Please note that if it becomes necessary to make any substantive change to the research design, the sampling approach or the data collection methods, a further application will be required.

I wish you every success with your research.

With best wishes



Professor Michael Preston-Shoot
Executive Dean of the Faculty of Health and Social Sciences
Chair of UREC