CALORIE RESTRICTION AND ITS IMPACT UPON THE BEHAVIOURAL, PHYSIOLOGICAL, MOLECULAR, AND METABOLIC INDICATORS OF ILLNESS

Submitted by

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Kent, S., **MacDonald, L.**, Radler, M., & Paolini, A.G. (2009, July). *Calorie restriction attenuates sickness behaviour.* The 3rd International Symposium on Physiology and Pharmacology of Temperature Regulation, Matsue, Japan.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AL	Ad libitum
ANOVA	Analysis of variance
BBB	Blood brain barrier
COX-2	Cyclooxygenase 2
CORT	Corticosterone
CR	Calorie restriction
CRH	Corticotrophin-releasing hormone
DNA	Deoxyribonucleic acid
EE	Energy expenditure
GR	Glucocorticoid receptor
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-1ra	Interleukin 1 receptor antagonist
IκB-α	Inhibitory factor kappa B alpha
LPS	Lipopolysaccharide
LSD	Least Significant Difference
mPGES-1	Microsomal prostaglandin E synthase-1
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPY	Neuropeptide Y
PGE_2	Prostaglandin E_2
POMC	Pro-opiomelanocortin
qPCR	Quantitative real time polymerase chain reaction
RQ	Respiratory quotient
SEM	Standard error mean
SOCS3	Suppressor of cytokine signalling 3
T_b	Body temperature
T_a	Ambient temperature
TNF-α	Tumour necrosis factor alpha

THESIS SUMMARY

The physiological outcomes of a diet reduced in calories have been well established. However, there has been limited research on the impact of calorie restriction (CR) on sickness behaviour. This thesis aims, through a number of studies, to explore the relationship between a CR diet and behavioural, physiological, molecular, and metabolic indicators of illness. The first series of studies examined the effect of CR on body weight, core body temperature (T_b), and locomotor activity. The second series of experiments investigated the impact of CR on sickness behaviour development and the physiological, molecular, and metabolic indicators of illness. The first series of experiments indicated that there was a dose-dependent effect of a 50% CR duration on body weight, T_b, and locomotor activity in rats, with a 28 day CR demonstrating the largest change in the above variables. In addition, there was a dose-dependent effect of CR severity in mice with the 50% CR demonstrating the largest change in variables compared to the 25% CR over 28 days. The second series of experiments indicated that a 50% CR for 28 days in mice and rats attenuated sickness behaviour (fever, anorexia, cachexia) after administration with lipopolysaccharide (LPS). In addition, in the CR mice there was a shift towards a central anti-inflammatory bias as indicated by several hypothalamic immune and diet related markers changing at two and four hours post-LPS. In the CR rats there was a significant increase in peripheral corticosterone at two hours post-LPS and also a significant attenuation of the increase in interleukin 6 at two hours post-LPS. The next experiment in rats indicated that there was a dose-dependent attenuation of sickness behaviour in relation to the duration of the CR regimen. Rats CR for 28 days demonstrated the largest attenuation of sickness behaviour measures, whereas the rats CR for 14 and 21 days demonstrated slight and moderate attenuations respectively in sickness behaviour measures post-LPS. In the next experiment it was demonstrated that the rats CR to 50% for 28 days demonstrated a reduction in metabolic rate after the CR period and no change in metabolism post-LPS. In the final experiment the 50% CR rats selected a higher ambient temperature (T_a) compared to control rats. Further, the CR rats were able to produce a febrile response post-LPS once at this heightened T_a; however, other measures of sickness behaviour remained attenuated in the CR rats. These findings suggest that a 50% CR leads to altered inflammatory pathways (namely a bias towards anti-inflammatory) and that when the CR animals are able to self-select their preferred T_a it possibly becomes less metabolically costly for them to increase their T_b post-LPS.

STATEMENT OF AUTHORSHIP

Except where reference is made in the text of this thesis, this thesis does not contain material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma. No other person's work has been used without due acknowledgement in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

This thesis contains several journal articles. As such, a number of people have made contributions to the studies and material presented in this thesis. Associate Professor Stephen Kent and Associate Professor Antonio Paolini made a number of contributions on a conceptual level and in the research design and analysis of these studies. Morgan Radler assisted with the feeding regimens and collection of some of the behavioural data; Agnes Hazi assisted with the collection of data for the hormonal assays; and Denovan Begg assisted with the data analysis for the metabolic data. For the most part, however, I conducted the empirical research presented here, produced all of the written material, and provided the conceptual and theoretical framework for the thesis.

All research procedures reported in this thesis were approved by the La Trobe University Animal Ethics Committee (AEC approval numbers 08/05(P), 08/28(P), and 10/46(P)).

Leah MacDonald
December 22nd, 2011

FORMAT AND STRUCTURE OF THESIS

The Higher Degrees Committee of La Trobe University permits Doctor of Philosophy candidates to submit a series of manuscripts (published, in-press, in-submission) as an alternative to the traditional thesis style. The guidelines for this format are supplied in Appendix A. This alternative format was adopted for two reasons: to invite ongoing international peer review and critique; and, to facilitate thought and discussion in research communities as a result of any publications. The articles presented here represent a series of studies that follow a logical sequence. The introductory and general discussion chapters are more economical than those of a traditional thesis, and serve to integrate and highlight the main conclusions of each article.

This thesis is comprised of nine chapters. The first chapter provides a general introduction to the immune system and calorie restriction, a review of the literature on physiological, immunological, molecular, hormonal, and behavioural outcomes of a calorie-restricted diet, and a rationale for the proceeding chapters. The empirical work is presented as a series of seven chapters. Each chapter contains a detailed introduction, an outline of the methodology, results and discussion. The ninth chapter provides a general discussion that highlights and integrates the main findings and issues raised by each paper. A framework for future research directions is also provided.

This thesis and its chapters have been formatted in accordance with the guidelines of the American Psychological Association (APA, 2001). However, the format and style for each empirical chapter accords with the requirements of the international peer-reviewed journal to which they were submitted. The only exception is that figures and tables for each study are presented in the body of the manuscript rather than being attached at the end.

Reference

American Psychological Association. (1994). *Publication manual of the American Psychological Association.* (6th ed.). Washington, DC: American Psychological Association.

THESIS OUTLINE

A brief outline of the thesis is provided here to assist the reader, as it comprises a series of published papers, unpublished papers submitted for publication, and unpublished papers.

CHAPTER 1

Chapter 1 provides a general framework and introduction to the thesis topic, highlights areas of research that have been unexplored in this field, and presents the research issues to be addressed in the thesis.

CHAPTER 2

Chapter 2 characterised changes in body weight, body temperature, and locomotor activity after a period of calorie restriction in rats. A further aim was to characterise the dose-dependent changes in these variables in relation to the duration of the calorie restriction regimen.

CHAPTER 3

Chapter 3 had a similar aim as that of Chapter 2 in that the aim was to characterise the changes in body weight, body temperature, and locomotor activity after a period of calorie restriction in a different species, mice. A further aim was to investigate the possible dose-dependent impact of the calorie restriction severity on the abovementioned variables.

CHAPTER 4

Chapter 4 (MacDonald et al., 2011) provides a comprehensive characterisation of the dose-dependent (in relation to severity of calorie restriction regimen) attenuation of sickness behaviour in mice. Further, it characterises a number of hypothalamic immune and diet related mRNA expression levels at 0, 2, and 4 hours after lipopolysaccharide administration.

CHAPTER 5

Chapter 5 further characterises the attenuation of sickness behaviour by investigating the longest period of calorie restriction (28 days) and serum levels of corticosterone, interleukin-6, and interleukin-10 at 0, 2, and 4 hours after lipopolysaccharide administration.

CHAPTER 6

Chapter 6 provides a characterisation of the dose-dependent (in relation to the duration of the calorie restriction regimen) attenuation of sickness behaviour post-exposure to lipopolysaccharide after a period of calorie restriction.

CHAPTER 7

Chapter 7 (submitted for publication) aims to provide a comprehensive account of the metabolic changes after a period of calorie restriction in rats and then also after exposure to lipopolysaccharide.

CHAPTER 8

Chapter 8 (submitted for publication) details the impact of ambient temperature on sickness behaviour production in calorie restricted rats after exposure to lipopolysaccharide.

CHAPTER 9

Chapter 9 provides a general discussion of the complete findings presented in the thesis in light of previous literature. The methodological limitations of the studies and directions for future research are explored.

CHAPTER 1

INTRODUCTION: OVERVIEW OF THE IMMUNE SYSTEM AND CALORIE RESTRICTION

The immune system is highly complex and organised system which aims to fight infection and disease. Although the benefits of a properly functioning immune system are numerous, the immune system can sometimes act in an unregulated manner, such as in chronic illnesses like autoimmune disorders. Chronic inflammation is detrimental to the body's functioning and has been implicated in the aging process, leading to many damaging changes within the body which results in the break-down of cells. The only natural intervention currently known to both reduce inflammation and retard the aging process is calorie restriction (CR). It has been shown in numerous species that CR results in a marked increase in both mean and maximum life-span and a reduction in age-related diseases. One hypothesis of how CR increases the life-span is through reducing inflammation.

This review will firstly discuss the immune system, including the specific and non-specific immune responses; with a focus on the non-specific immune response and how an invading pathogen initiates an acute phase response. CR will be discussed in relation to the inflammation hypothesis of aging, its effect on metabolism, autoimmune diseases, and a number of aspects of immune functioning such as mortality after infection, macrophage activity, and cytokine release. Potential mediators of CR's impact on immune system functioning will be discussed and finally, comparability issues within CR research will be highlighted.

The immune system

The immune system is a complex system that protects the body against infection and disease. It consists of specific (or learned/acquired) and non-specific (or innate) response patterns, with immune cells activating the brain, causing cellular, biochemical,

physiological, cognitive, and behavioural changes that result in sickness (Hart, 1988; Kent, Bluthé, Kelley, & Dantzer, 1992). The most important function of the immune system is to recognise the body's own healthy cells from invading pathogens, and destroy these invading pathogens accordingly (Janeway, 1992). Specific immunity will be discussed briefly; however, non-specific immunity will be the focus of this review, and thus will be discussed in more detail.

Specific or adaptive immunity

Specific immunity is a process whereby the immune system protects the body against specific antigens. These antigens are identified and then engulfed by antigen-presenting cells, such as a macrophage; this results in the unique aspects of the antigen being displayed on the outside of the cell (Maier & Watkins, 1998; Mosser & Edwards, 2008). T cells (a category of white blood cell) are able to recognise the unique aspects of the antigen through receptors and subsequently each T cell has the capacity to recognise one and only one antigen. After the antigen encounters the T cell, the binding of the antigen to the T cell causes a series of cell divisions, and the number of T cells specific to that antigen increase (Davis & Bjorkman, 1988; Maier & Watkins, 1998). The T cells help a second class of white blood cells, B cells, to proliferate and secrete an antibody directed at the specific antigen, which then destroys, or arranges for the destruction of the antigen (Davis & Bjorkman, 1988). Due to the time taken for the body to discover the antigen within the body, and the large number of cell divisions needed for the specific immune response to mount a defence, the specific immune response is not an immediate process. It can take from eight to 12 hours for each cell division to occur and many are required before an effective response is mounted (Maier & Watkins, 1998).

Non-specific or innate immunity and the acute phase response

Non-specific or innate immunity

The innate immune system has been viewed as an evolutionary adaptation whose function was to contain the infection until the specific (or adaptive) immune response can be activated (Medzhitov & Janeway, 1997b). This response functions primarily to restrict tissue damage at the site of infection (Medzhitov & Janeway, 1997a). Macrophages, the immune system's first line of defence, are also involved in non-specific immunity; they engulf non-self-cells, and respond not to a specific antigen, but to general features of non-self-cells (Medzhitov & Janeway, 1997a). Macrophages express a set of receptors known as Toll-like receptors (TLR), which are responsible for recognizing the difference between normal cell tissues, and molecules broadly shared by pathogens, or collectively referred to as pathogen-associated molecular patterns (PAMP) (Dantzer, 2006). Stimulation of TLRs leads to the activation of nuclear transcription factor κB (NF- κB), which enters the nucleus of the cell and stimulates the up-regulation of numerous genes. The response time of the non-specific immune system is almost immediate, one to two hours, due to no need for immune cells to proliferate (Maier & Watkins, 1998). The macrophages can destroy antigens, or they can release nitric oxide, which is a gas that easily moves from inside the cell to the region surrounding it. Nitric oxide acts to interfere with mitochondrial respiration, which is essential for the survival of cells, and can therefore, reduce the growth of bacteria and viruses. In addition to macrophages destroying antigens, stimulation of TLRs leads to macrophages releasing pro-inflammatory cytokines, such as interleukin-1α and β (IL-1), IL-6, tumor necrosis factor α (TNF- α), and interferon-γ (IFN-γ) (Medzhitov & Janeway, 1997a).

Acute phase response

The non-specific response usually leads to an acute phase or innate immune response and is the body's global reaction to an infection and involves communication between the immune system and the central nervous system (Janeway, 1992). During the acute phase response new homeostatic set points are organised so that defence mechanisms can take priority over normal homeostatic states. Experimentally, the acute phase response can be mimicked by injection with an exogenous pyrogen. A common means of mimicking the behavioural and physiological characteristics of a bacterial infection, and therefore an innate immune response, is by administering lipopolysaccharide (LPS), which is the active fragment of the cell wall of Gram-negative bacteria. LPS is recognised within the body when monomers are extracted from the bacterial membrane by a LPS-binding protein, which then transfers the LPS monomer to a lipid binding site on CD14 (a protein on the surface of the TLR4) (Dantzer, 2006). CD14 promotes the binding of the LPS monomer to TLR4, which then alerts the macrophages to the invading antigen. Without CD14, the TLR4 is reduced in its capacity to recognise the LPS monomer, only performing this function when very high concentrations of LPS are present (Dantzer, 2006).

Physiological expression of the acute phase response

The acute phase response can be expressed both physiologically and behaviourally. Physiologically, the body adjusts to the acute phase response by increasing slow wave sleep, increasing levels of white blood cells, producing more immune related proteins (such as cytokines and positive acute phase proteins), and producing a febrile response (Luheshi, 1998). Fever is the temporary resetting of the body's hypothetical thermostatic set point, causing a regulated increase in core body temperature (T_b) (Hasday, Fairchild, & Shanholtz, 2000). Thermoregulatory processes mediate this increase in core

T_b by increasing the firing rate of cold-sensitive neurons (which drive heat production and conservation) and decreasing the rate of firing of heat-sensitive neurons (which drive heat loss) (Dascombe, 1986). As such, it differs from hyperthermia, which is an unregulated increase in T_b (Leon, 2001). Fever does not only occur during an acute phase response, fever can occur after surgery, trauma, chemical or thermal insults, and any stimuli that are capable of eliciting an inflammatory response (Roth, Rummel, Barth, Gerstberger, & Hübschle, 2006). Further, fever can be caused by viral or bacterial infection (Kluger, 1991) or by stress (De Paula, Steiner, & Branco, 2000). The regulated increase in T_b aids the immune response and decreases the reproduction of heat sensitive pathogens, which reproduce best at, or just below core T_b (Kluger, 1991; Maier & Watkins, 1998).

Fever is produced via communication between the immune system and the brain, specifically the hypothalamus (Leon, 2001). Macrophages present as the first line defence against infection, and are primarily involved in removing Gram-positive (for example, staphylococci) and Gram-negative (for example, salmonella) bacteria from the blood via phagocytosis and intracellular killing (Sun, Muthukumar, Lawrence, & Fernandes, 2001). In response to an infection, macrophages release pro-inflammatory cytokines (Leon, 2001). Pro-inflammatory cytokines (IL-1α and β, IL-6, TNF- α, IFN- γ) and anti-inflammatory cytokines [IL-1ra (receptor antagonist), IL-2, IL-4, IL-10, IL-12] are released centrally and peripherally, and play a role in the production and regulation of the febrile response and alert the hypothalamus (how this is achieved is described below) that there is a pathogen in the body (Mouihate, Ellis, Harre, & Pittman, 2005). IL-6 is the principle endogenous pyrogenic cytokine, which activates central nervous system (CNS) mechanisms during inflammation and infection (Cartmell, Poole, Turnbull, Rothwell, & Luheshi, 2000). It has been established that of all the pro-inflammatory cytokines, peripheral levels of IL-6 most closely correlate with the magnitude of fever (Cartmell et

al., 2000; LeMay, Vander, & Kluger, 1990), and it has been demonstrated that IL-6 deficiency or treatment with IL-6 anti-serum can almost completely block fever induced by LPS (Cartmell et al., 2000).

Cytokines alter the activity of the enzyme cyclooxygenase (COX), which has two isoforms, COX -1 (constitutive) and COX-2 (inducible) (Coceani & Akarsu, 1998). The latter is the rate-limiting step in the biosynthesis of prostaglandin E_2 (PGE_2) (Vane, Bakhle, & Blotting, 1998); which has traditionally been seen as the final mediating step in the process of fever generation. PGE_2 is a signalling molecule in the major thermoregulatory site, the medial preoptic area (MPOA) of the hypothalamus (Kluger, 1991). Antipyretic drugs such as aspirin and ibuprofen act to block the COX enzymes and therefore attenuate the febrile response (Plaisance & Mackawiack, 2000).

Peripherally produced cytokines reaching the brain

The means by which peripherally produced cytokines alert the brain to a pathogenic substance within the body remains controversial (Cartmell et al., 2000). Two main theories have been suggested to explain how these hydrophilic peptides, which have a relatively large molecular weight, reach the brain. The first of these is a slower pathway, involving the peripherally produced cytokines gaining direct access to the brain. There is a consensus that cytokines do not enter the brain at just any point along the blood-brain barrier (BBB) due to their large size and hydrophilic nature (Dantzer, 2004). However, it has been postulated that cytokines gain access to the brain via the circumventricular organs which are situated within the third and fourth ventricles and lack the endothelial BBB, one of which is the organum vasculosum of the lamina terminalis (OVLT) (Johnson & Gross, 1993). Furthermore, the OVLT is interconnected with the MPOA of the hypothalamus, where cytokines travel to alert the brain of a pyrogen within the body.

The second of these theories is that the signal reaches the brain via a neural message transported via the vagus nerve. This nerve, which extends from the brain stem to the stomach and intestines, provides afferent input from the gut, lymph nodes, thymus, and spleen to the brain (Maier & Watkins, 1998; Romanovsky, Simons, Szekely, & Kulchitsky, 1997). Cells located between vagal nerve fibres, connective tissue, and in paraganglia also possess receptors for IL-1β, which binds after peripheral stimulation with LPS (Goehler et al., 1999), and stimulates the sensory activity of the vagus nerve (Ek, Kurosawa, Lundeberg, & Ericsson, 1998). Vagotomy studies, whereby the vagus nerve has been severed below the diaphragm, have demonstrated that inactivation of the vagus nerve results in attenuated fever and sickness behaviour in response to intraperitoneal administration of LPS (Hansen & Kruger, 1997; Layé et al., 1995). However, when exposed to large doses of LPS the vagotomised animals are able to demonstrate a fever (Romanovsky et al., 1997). This suggests that the vagus nerve, at least partially, mediates the activation of the hypothalamus in response to cytokines and induces sickness behaviour. Furthermore, vagotomised animals also demonstrate attenuated mRNA levels of IL-1β in the hypothalamus and hippocampus (Layé et al., 1995); however, serum levels of IL-1β and IL-6 do not differ between vagotomised and control animals after an intraperitoneal injection of LPS (Hansen et al., 2000). This indicates that a vagotomy does not alter peripheral immune responses but blocks the central immune response.

Behavioural expression of the acute phase response

Behavioural consequences of the acute phase response are often referred to as sickness behaviour, or behavioural depression, which is characterised by fever, a decrease in food and water intake, a decrease in activity and social interactions, and body weight loss (Kent et al., 1992). Sickness behaviour is adaptive, and is not simply a result of

debilitation and reduced efficiency of bodily functions; instead, it supports the physiological changes that occur after a pathogen enters the body. An example of the adaptive value of sickness behaviour can be seen during the febrile response, which is metabolically very costly to the organism (Hart, 1988). The behavioural depression and anorexia seen during fever are a planned behavioural response in order to initiate the rise in body temperature needed to develop a fever, and also to maintain the febrile response (Hart, 1988). Decreased activity can also lead to decreased heat loss, which is important during the development of fever, as increased heat generation and decreased heat loss are favoured (Hart, 1988). It has been shown that lizards maintained at a low ambient temperature (T_a; and as they are poikilotherms their T_b is closely linked to the T_a) exhibited a higher rate of mortality after being inoculated with a bacteria compared to lizards housed in a higher T_a (Kluger, Ringler, & Anver, 1975). This pivotal research highlights the importance of an increased temperature, and therefore fever, during an infection. The authors concluded that the elevated temperature aided the host's defence mechanisms during infection (Kluger et al., 1975). This finding was supported by investigations in mammals (rabbits) demonstrating that a higher febrile temperature was associated with higher survival rates. However, this increased survival only occurred up until a temperature increase of 2 ¼ °C, after which mortality increased (Kluger & Vaughn, 1978).

Due to the increased set point for T_b, the organism feels cold at T_a that were previously thermoneutral. This leads the organism to attempt to reach the new thermal equilibrium by decreasing heat loss and increasing heat production. For example, curling up and seeking a warm T_a are aimed at decreasing heat loss (Dantzer et al., 1998) and shivering and non-shivering thermogenesis aid heat generation (Hasday et al., 2000). Non-shivering thermogenesis helps to increase T_b by brown-adipose tissue turning fat stores

into heat (Cannon & Nedergaard, 2004). However, heat production is metabolically costly therefore heat conservation is preferred. In addition, the decreased food and water intake seen during sickness is also designed to conserve heat loss through immobility.

An example of the adaptive value of anorexia during illness comes from research in rats that were prevented from expressing the normal anorexic response to infection. Rats force fed their baseline food intake after exposure to Listeria monocytogenes experienced increased mortality compared to animals that are left to experience the full anorexic effect during sickness (Murray & Murray, 1979). If an animal is not hungry, then it does not have to search for food and expose its body surface during movement (Maier & Watkins, 1998). These behaviours are activated in an attempt to increase T_b to match the new hypothetical set point for T_b that has occurred during the febrile response (Leon, 2001). As the behaviour and physiology of the organism combine in the most efficient way to fight infection, sickness behaviour increases the efficiency of the physiological changes during a febrile response (Hart, 1988).

Chronic Inflammation, the Inflammation Hypothesis of Aging, and Calorie Restriction

Although inflammation is a tightly regulated and adaptive process, with age inflammation can become unregulated. This is potentially caused by increased pro-inflammatory cytokine levels, which is postulated to be at the time of menopause and andropause when the regulation of cytokines (in particular IL-6) by sex steroids becomes disrupted (Ershler & Keller, 2000; O'Mahony et al., 1998). Increasingly, it has been postulated that inflammation plays a key role in the aging process, as outlined by the oxidative stress hypothesis of aging (Yu, 1996). Although there are many pharmaceutical interventions aimed at treating and preventing chronic inflammation, one non-

pharmaceutical intervention that has been suggested is CR. In addition to altering the efficiency of the immune system, CR has also been associated with increasing or maintaining healthy cardiovascular fitness and reducing the occurrence of other age-related diseases. These include the reduction of blood pressure, cholesterol, triglycerides, lowering the risk of diabetes and cardiovascular disease (Lane, Ingram, & Roth, 1999; Walford, Mock, Verdery, & MacCallum, 2002), decreased incidence of cancer (Matsuzaki et al., 2000), decreased incidence of autoimmune diseases (Kubo, Day, & Good, 1984), a reduction in post-ischemic inflammation (Chandrasekar, Nelson, Colston, & Freeman, 2001), facilitating the survival of dopamine neurons in a primate model of Parkinson's Disease (Maswood et al., 2004), and protecting hippocampal neurons in a rodent model of Alzheimer's Disease (Halagappa et al., 2007; Wang et al., 2005; Zhu, Guo, & Mattson, 1999). Previous research has demonstrated that CR has provided valuable insight and avenues of exploration for investigating treatments and preventative options for many diseases.

CR will be discussed below in relation to the inflammation hypothesis of aging; CR's impact on metabolism and T_b; the role of CR as used as an intervention for the treatment of autoimmune diseases; and the role of CR in the functionality of the immune system in respect to behaviour, mortality after infection, and macrophages and cytokines.

Inflammation hypothesis of aging

CR is the most widely used intervention in the study of gerontology (Masoro, 2000) and is the only manipulation that has consistently been shown to extend the mean and maximum life span in a wide variety of organisms, such as yeast (Lin, Ford, Haigis, Liszt, & Guarente, 2004), spiders (Austad, 1989), flies (Magwere, Chapman, & Partridge, 2004), mice (Conti et al., 2006; Kubo et al., 1984; Sohal, Ferguson, Sohal, & Forster,

2009), rats (Han et al., 1995; McCay, Crowell, & Maynard, 1935), and primates (Mattison, Lane, Roth, & Ingram, 2003; Messaoudi et al., 2006). CR has been widely studied in rodents and other mammals, and can be achieved by reducing the average unrestricted food intake. Traditionally, the reduction in food intake includes a balanced decrease in calories, protein, vitamins and minerals, while avoiding malnutrition (Weindruch & Walford, 1988). Depending on the severity of the CR, there is a limited period of weight loss, after which body weight is maintained and slightly can increase with time (Weindruch & Walford, 1988).

CR has been established to prolong the lifespan (Weindruch, Walford, Fligiel, & Guthrie, 1986) and it has been postulated that the mechanism by which CR achieves this is by reducing oxidative stress caused by unregulated increases in reactive oxygen species (ROS; Barja, 2004; Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; Shigenaga, Hagen, & Ames, 1994). Unregulated inflammation is suggested to be caused by increased pro-inflammatory cytokine levels (Ershler & Keller, 2000; O'Mahony et al., 1998). Regulation of the inflammatory process depends upon ROS; it is thought that ROS act as signalling molecules, and initiate responses leading to the resolution of the inflammatory process (Bodamyali, Stevens, Blake, & Winyard, 2000). ROS are very important in many physiological functions; however, when an organism is placed under environmental stress, ROS levels increase dramatically, causing damage to cells (Shigenaga et al., 1994), which can lead to chronic inflammation (Chung, Kim, Kim, Choi, & Yu, 2002). Chronic inflammation is seen in a variety of diseases, such as arthritis (Cooke, Hurd, Ziff, & Jasin, 1972), multiple sclerosis (Bitsch, Schuchardt, Bunkowski, Kuhlmann, & Bruck, 2000), and irritable bowel syndrome (Collins, Piche, & Rampal, 2001).

One widely held view is that aging is associated with an increased number of free radicals, resulting in chronic oxidative stress, which is an imbalance between the reactive oxygen in the body and the body's ability to repair the damage caused by the ROS (Shigenaga et al., 1994). The presence of free radicals and the absence of antioxidants (which prevent the oxidization of molecules) can lead to peroxidation (oxidative degradation) of lipids and nucleic acids (especially DNA), resulting in changes in the structure of proteins (Shigenaga et al., 1994).

It has been demonstrated that CR reduces energy expenditure (EE) in rats (Duffy et al., 1989) rhesus monkeys (Lane, et al., 1996), and humans (Heilbronn et al., 2006). Oxidants are produced as a by-product of aerobic metabolism or increased EE (Shigenaga et al., 1994); therefore, it is thought that the lower the aerobic metabolism (or EE), the lower the oxidative damage. In relation to CR, a significant reduction in oxidative damage to mitochondrial DNA has been demonstrated in rats CR to 47% for 12 months (Lopez-Torres, Gredilla, Sanz, & Barja, 2002). This reduction of oxidative damage was not due to a decrease in mitochondrial oxygen consumption, but was due to the mitochondria releasing less ROS (Lopez-Torres et al., 2002). In addition, in rhesus monkeys it was shown that after 10 years on a 30% CR, the level of lipid peroxidation in skeletal muscles was significantly reduced compared to the age-matched controls (Zanial, Oberley, Allison, Szweda, & Weindruch, 2000). Finally, it was demonstrated in humans that a 6-month CR of 25% resulted in a significantly reduced EE and DNA damage as determined in blood cells (Heilbronn et al., 2006). Interestingly, the reduced DNA damage was not associated with reduced oxygen consumption in a metabolic chamber (Heilbronn et al., 2006), which is consistent with the findings of Lopez-Torres et al. (2002); it was not the reduced oxygen consumption that reduced the oxidative damage; it was the reduced release of oxygen species.

Further, as mentioned earlier, it is thought that CR regulates immune function during aging by limiting the age-related increase in pro-inflammatory cytokine levels (Spaulding, Walford, & Effros, 1997). The means by how CR exerts its anti-inflammatory effect is thought to occur due to increased levels of glucocorticoids, which are dose-dependently increased in response to CR (Holmes, French, & Seckl, 1997; Levay, Tammer, Penman, Paolini, & Kent, 2010) and act to increase the transcription of anti-inflammatory cytokines and decrease the transcription of pro-inflammatory cytokines (Barnes, 1998).

However, there is also evidence suggesting that reduced oxidative damage may not be the only mechanism by which CR extends the life span. Huffman et al. (2008) demonstrated that DNA damage was accelerated by obesity in a rodent model, and that this acceleration can be slowed with mild to moderate CR. Furthermore, they investigated why exercise fails to extend the life span in comparison to CR under carefully matched conditions, and found that mice that had been on an exercise regimen did not have greater oxidative damage compared to the CR mice (Huffman et al., 2008). Therefore, the failure of exercise to extend life must not be due to greater oxidative damage. When hormone pathways were compared between the mice that were exposed to exercise and the CR mice, CR resulted in more favourable, and therefore life prolonging changes in hormone pathways, which included insulin and insulin-like growth factor (Huffman et al., 2008). Higher levels of insulin-like growth factor have been linked to an increased risk of some cancers (Chan et al., 1998; Renehan et al., 2004). The hormone levels and stress-related proteins of the mice on CR resembled most closely that of young mice, whereas the mice who exercised exhibited hormones and stress proteins that did not resemble young mice (Huffman et al., 2008). CR reduces the amount of oxidative stress in the mice; however,

so does exercise. Therefore, CR is likely to initiate other life prolonging processes irrespective of reduced oxidative damage.

Calorie restriction, body temperature, and metabolic rate

Mammals use significant amounts of energy to maintain a relatively high and constant core T_b; therefore, it is not surprising that reducing energy consumption (such as via CR) results in a lowered T_b as the organism lowers its metabolic rate to conserve energy (Blanc et al., 2003; Duffy et al., 1989; Gonzales-Pacheco, Buss, Koehler, Woodside, & Alpert, 1993; Heilbronn et al., 2006; Lane et al., 1996). Furthermore, severe under-nutrition and a period of 25% CR in humans have been shown to decrease core T_b (Heilbronn et al., 2006; Keys, Brozek, Henschell, Mickelsen, & Taylor, 1950). As mentioned previously, CR has also been shown to reduce EE in animals and humans (Blanc et al., 2003; Duffy et al., 1989; Forsum, Hillman, & Nesheim, 1981; Gonzales-Pacheco et al., 1993; Lane et al., 1996; Leibel, Rosenbaum, & Hirsch, 1995; Martin et al., 2007b; Ramsey, Roecker, Weindruch, & Kemnitz, 1997).

Animals

The average daily core T_b in rats has been shown to be reduced after a 40% CR for 12 months (Duffy et al., 1989). Rats that were fed during the dark phase demonstrated a similar circadian T_b rhythm compared to the control rats; however, on average T_b was 1 °C lower during both the light and dark phases. Interestingly, another group of CR rats fed during the light phase demonstrated large increases in light phase T_b, in anticipation of food delivery thus altering the circadian rhythm of the rats T_b; however, they still demonstrated reduced dark phase T_b compared to the controls (Duffy et al., 1989). In non-human primates, rectal temperature has been shown to be significantly reduced by 0.5 °C

in rhesus monkeys restricted to a 30% CR; however, this reduction was not evident until two-years after the initiation on CR (Lane et al., 1996). Furthermore, these temperature differences did not emerge until the monkeys were exposed to 30% CR, whereas when the monkeys were CR 10% and 20% of the controls' food intake the differences were non-significant.

In addition to EE, another aspect of metabolic rate, respiratory quotient (RQ) which is often used in the calculation of basal metabolic rate via the calculation of oxygen consumption and carbon dioxide output, has been shown to be reduced in rats that were CR to 30 – 40% of the control animals' food intake for 17 days (Rothwell & Stock, 1982). RQ was reduced during the latter part of the dark phase; however, the control and CR rats demonstrated similar RQ levels during the light phase. Further, rats exposed to a 40% CR for 14 months found that the CR rats RQ was markedly reduced compared to controls (Duffy et al., 1989). In contrast to the above findings, the CR rats demonstrated a reduced RQ during the light phase and a rapid increase in RQ surrounding the time they were fed, which was not seen in the control rats. These discrepancies may stem from the large difference in the period of time the rats were CR. These authors' suggested that this rapid increase of RQ in the CR rats was due to a change in metabolic processes dependent on food availability. This indicated a shift from protein and fat metabolism to carbohydrate metabolism. Once the CR rats finished eating their RQ levels dropped to their pre-feeding levels as their carbohydrate stores depleted (Duffy et al., 1989).

In contrast, some researchers have seen no change in metabolic processes after a period of CR; for instance, six week old rats CR to 40% demonstrated a very similar RQ as control animals after 4½ months of CR (McCarter, Masoro, & Yu, 1985). A number of methodological factors may have accounted for these differences in research findings, such as age of onset of the CR regimen, with CR initiated whilst the animal is not yet

regarded as an adult yielding no change in EE (Ramsey et al., 1997). The grouping together of resting and total EE often also results in a finding of no change in EE (McCarter et al., 1985). The CR animals may only demonstrate a reduction in EE and RQ during the resting phase of the circadian cycle, and not an overall change, thus if total EE or RQ is calculated any differences may be masked (Ramsey et al., 1997).

Humans

More recently, CR in humans was shown to decrease core T_b and metabolic rate (Heilbronn et al., 2006). Three different dietary manipulations were investigated in regards to metabolic rate in overweight, non-obese males and females. These included a 25% reduction in calorie intake, 12.5% reduction in calories and 12.5% increase in EE, and an 890 kcal/day diet (Heilbronn et al., 2006). In addition to finding that core T_b was reduced, EE was significantly reduced in all three dietary manipulation groups, metabolic adaptation was associated with reduced thyroid hormones, and DNA damage was reduced compared to baseline and controls (Heilbronn et al., 2006). Interestingly, the reduction in DNA damage seen in the three dietary manipulation groups was not associated with reduced oxygen intake, which would be expected under the hypothesis that reduced oxygen intake leads to a reduction in DNA damage, as mentioned above. This would then subsequently lead to reduced oxidative stress; which would reduce DNA damage. A similar conclusion can be made in this study as the investigation conducted by Huffman et al. (2008) in mice; reduced DNA damage may not be the sole mechanism by which CR extends life, and there are most likely other processes taking place.

In addition, it was demonstrated in adult humans, that a 25% CR for three months reduced resting metabolic rate beyond what was expected from changes in weight and body composition (Martin et al., 2007b). Similarly, the men and women involved in the

Biosphere 2 experiment demonstrated a significantly reduced 24 hour EE than would be predicted for their age, sex and body composition (Weyer et al., 2000). Biosphere 2 was an enclosed, ecological space in which four men and four women lived for two years with no material entering or exiting the enclosure. Due to the crops failing to produce as much food as expected the participants ate 71 – 79% of the usual adult food intake (Weyer et al., 2000). Furthermore, the participants EE remained at reduced levels even after the men and women resumed an ad libitum (AL) diet for six months, and had recovered to their existing weight levels.

Calorie restriction as an intervention for autoimmune diseases

Research has shown that CR can retard a variety of autoimmune diseases (Fernandes, Yunis, & Good, 1976; Kubo et al., 1984; Kubo, Gajjar, Johnson, & Good, 1992; Ogura, Ogura, Ikehara, & Good, 1989; Piccio, Stark, & Cross, 2008). Kubo et al. (1992) investigated the effect of CR on an autoimmune prone strain of mice, which produce significantly reduced levels of IL-2. IL-2 stimulates the growth and survival of antigen selected cytotoxic T cells, and stimulates the development of T cells' immunologic memory. The deficiency of IL-2 in both the spleen and the lymph nodes was reversed comparable to normal levels after a 50% CR from 6 weeks to three months of age (Kubo et al., 1992). Furthermore, even when the CR was initiated at 12 weeks of age, after disease manifestations in the autoimmune mice had already begun to appear, the reduced calories still increased survival, and increased the levels of IL-2 in the mice (Kubo et al., 1992). CR has also been shown to attenuate the progression of experimental autoimmune encephalomyelitis, a rodent model of multiple sclerosis (Piccio et al., 2008). The mice CR to 60% demonstrated less severe inflammation, less demyelination, less axon injury,

higher plasma concentrations of corticosterone (CORT), and decreased plasma concentrations of leptin and IL-6 (Piccio et al., 2008).

It was also demonstrated that male mice restricted to 115 calories per week (although it was not indicated what level of CR this equated to) after 12 months of age demonstrated significantly decreased levels of serum immune complex (which is instrumental in causing age-related renal and other diseases) (Weindruch & Walford, 1988). In a similar study, a 40% CR from four months of age in mice prone to autoimmune disease reduced the age-related increase of inflammatory cytokines, which in turn reduces the severity of autoimmune disorders (Muthukumar, Jolly, Zaman, & Fernandes, 2000). It must be noted that this study provided vitamin and mineral supplements, which may have influenced their results.

Calorie restriction and the immune system functioning

It has been demonstrated that 40% of the genes that increase in their expression with age are associated with inflammation, which suggests that the dysfunction of these inflammation genes may be involved with aging (Cao, Dhahbi, Mote, & Spindler, 2001). Further, it has been postulated that the immune systems' efficiency declines with age (Murasko, Gold, Hessen, & Kaye, 1990). CR has been shown to slow this age-related decline and inhibit the age-related increase in autoimmune diseases, as mentioned above (Weindruch & Walford, 1988).

A number of researchers have demonstrated that CR or food deprivation (food deprivation will be mentioned here as the majority of the following research investigating the immune system has used this approach) can influence the immune response after an infection. This includes demonstrating that certain behavioural aspects of the immune response can be altered (Kleitman & Satinoff, 1981; Mrosovsky, Molony, Conn, &

Kluger, 1989), CR and food deprivation can decrease mortality after infection (Murray & Murray, 1979; Peck, Babcock, & Alexander, 1992; Sun et al., 2001; Wing & Young, 1980), and cytokine and macrophage function can be altered (Dong et al., 1998; Stapleton, Fujita, Murphy, Naama, & Daly, 2001; Sun et al., 2001; Vega, de Cabo, & De Maio, 2004). There are inconsistencies with the research as to what effect CR or food deprivation may have on the immune response; however, the majority of research has demonstrated an immunosuppressive effect. In contrast, there have also been limited suggestions that CR or food deprivation can increase the sensitivity of the immune system (Stapleton et al., 2001).

Calorie restriction and behavioural aspects of the immune response

To our knowledge, there has been limited research investigating the effects of food deprivation on any aspect of sickness behaviour. For example, one-to-two day old rabbits who were food deprived for two days demonstrated an attenuation of the rise in T_b compared to their freely fed counterparts after exposure to a pyrogen (*Pseudomonas lipopolysaccharide*) (Kleitman & Satinoff, 1981). Interestingly, the behavioural heat seeking of these rabbits was intact. The pups injected with the pyrogen, although they demonstrated an attenuated febrile response, chose to settle at a warmer ambient temperature in a thermal gradient compared to saline injected controls (Kleitman & Satinoff, 1981). These findings suggest that although the physiological response was affected by food deprivation, the behavioural response to the infection was intact in these rabbit pups. Further, hamsters exposed to a 20% CR had significantly higher circulating cortisol levels after 20 days of CR; however, after LPS cortisol and IL-6 activity were similar in control and CR animals (Conn et al., 1995). The CR animals T_b demonstrated

the same increase as controls post-LPS; although the CR animals had a lower initial core T_b.

It was demonstrated that AL fed rats exhibited a dose-dependent decrease in food intake in response to continuous infusion of IL-1 (Mrosovsky et al., 1989). However, rats that were food deprived for 24 hours and then provided with between 5 and 12 g of food in the light phase for seven days (i.e., a 50-75% reduction in food intake), exhibited hyperphagia once they were allowed to eat AL after the IL-1 infusion (Mrosovsky et al., 1989). Therefore, food deprivation was demonstrated to impact on a behavioural response (food intake) after exposure to IL-1 by interrupting the 'normal' anorexic response.

Calorie restriction and mortality after infection

Murray and Murray (1979) investigated the role of anorexia after infection as part of the host's immune response. They compared mice that, after being exposed to an intraperitoneal injection of *Listeria monocytogenes*, were force fed their normal caloric intake (via a feeding tube) and mice that were not force fed after exposure to *Listeria monocytogenes*. The mice that were force fed not only had a significantly higher mortality rate; they also had a significantly shorter survival time (Murray & Murray, 1979). This finding indicates that the anorexia seen during illness and after exposure to a bacterial infection is an integral part of the host's immune response and is beneficial for survival. The authors suggested that the anorexia may be the body's way of mimicking the effects of famine, therefore reducing the life of the infection, which acts as a biological mechanism designed to support survival (Murray & Murray, 1979). This suggests that animals that are exposed to a period of CR may demonstrate similar responses in relation to prolonged survival and reduced mortality.

CR has been shown to reduce mortality after exposure to an infectious agent. For example, after an intraperitoneal injection of *Salmonella typhimurium*, female mice on a 50% CR for three weeks lived longer than AL fed controls (Peck et al., 1992). Interestingly, Peck et al. (1992) also investigated the impact on recovery post-infection of reducing protein intake to 1 or 5% of the usual protein intake. The mortality rate of these protein restricted mice was higher than that of both the regular CR mice and control mice, regardless of their overall calorie consumption (Peck et al., 1992). Therefore, the conclusion that can be drawn is that the protective effect of CR comes from a restriction of overall calorie intake, and not by the reduction of one part of the calorie intake, such as protein.

Similarly, mice deprived of food for 24, 48, or 72 hours demonstrated resistance to a lethal dose of *Listeria monocytogene* infection (Wing & Young, 1980). Interestingly, the mice food deprived for 24 hours and inoculated with the bacteria at the end of deprivation period were not resistant to the infection and were similar to controls, but those inoculated 24 hours after the end of the 24 hour deprivation period (i.e., 48 hours after the start of the food deprivation period) demonstrated a resistance to the bacteria with a lower mortality rate possibly due to increased activation of macrophages (Wing & Young, 1980). There was a relationship between the length of food deprivation period and survival post-infection, with the longer deprivation period providing the largest protection against the infection. Moreover, the food-deprived mice demonstrated significantly fewer bacteria cells in their spleens two to four days after inoculation (Wing & Young, 1980). The authors attempted to investigate whether the protection of the food-deprived mice could be transferred to the control mice via transplantation of spleen cells; however, no protection was observed (Wing & Young, 1980). T lymphocyte (an integral part of cell-mediated immunity) numbers in the deprived mice were significantly lower, which

indicates that the increased protection to the bacteria was not due to increased numbers of T lymphocytes (Wing & Young, 1980). Others have found that T cell mediated immunity is markedly reduced after food deprivation and that this may be due to increased corticosteroid release due to the stress of the food deprivation which may be a suggested explanation for the findings of this study (Wing, Magee, & Barczynski, 1988). Although this investigation involved food deprived mice, not CR mice, the results are still relevant to the investigation of CR and its impact on the immune system.

In contrast, Sun et al. (2001) demonstrated that male mice exposed to 40% CR for five months were more susceptible to sepsis, and died significantly earlier of sepsis (caused by cecal ligation and puncture) than the AL fed controls (Sun et al., 2001). The levels of TNF-α and IL-6 mRNA and the transcription factor, NF-κB, in the spleen of the CR mice were also higher than the AL fed controls. The authors suggested that in these mice, that were CR from six weeks of age, macrophage function had not matured, and this possibly meant that during infection macrophages were unable to control the rise in invading pathogens (Sun et al., 2001).

Similarly, it was demonstrated that CR mice exposed to influenza in the later stages of life showed a decreased survival rate (Gardner, 2005). Mice were placed on a 40% CR regimen from three months of age, and were challenged with influenza at 23-months of age. The rate of mortality in the older CR mice was significantly steeper compared to AL fed mice of a comparable age, and younger mice (three months old) that were fed AL. All the CR mice died within five to eight days post challenge and were not as efficient in clearing the virus from their lungs as the older and younger AL fed mice (Gardner, 2005). This indicates that age is a factor in determining the role CR plays in relation to immune system functioning.

There is some question over the comparability of the studies mentioned above. Firstly, there were opposite findings from a number of studies, namely, CR enhancing survival after infection (Peck et al., 1992; Wing & Young, 1980), and CR decreasing survival (Gardner, 2005; Sun et al., 2001). However, in these studies there were a number of methodological variables that differed, making the comparison of their findings difficult. Some researchers have used vitamin and mineral supplements (Gardner, 2005; Peck et al., 1992), and others have not (Sun et al., 2001; Wing & Young, 1980). Furthermore, there were differences in duration and severity of CR, the use of food deprivation, the strain and sex of animal used, age of the animals, and the stimuli used to evoke an immune response.

Calorie restriction, macrophages and cytokines

One potential explanation for the protective effect of CR against infection is that CR alters the levels of some cytokines after stimulation by a bacterial compound. Rats exposed to a 25% CR for three weeks displayed enhanced phagocytic activity of alveolar macrophages (first cellular defence against respiratory pathogens), and consequently demonstrated a reduction in the time taken to clear a bacterial infection (*Streptococcus zooepidemicus*) from the lungs (Dong et al., 1998). Moreover, both basal and LPS-stimulated levels of TNF-α and IL-6 mRNA in the alveolar macrophages were significantly lower in the CR group (Dong et al., 1998).

Furthermore, nine-week old mice who were restricted to 40% of their AL fed counterparts demonstrated reduced cytokines levels (Vega et al., 2004). The mice were sacrificed at 18 months of age and their peritoneal macrophages incubated with LPS *ex vivo* for five hours. Levels of IL-1β produced by the peritoneal macrophages of the CR

mice were significantly lower than controls; however, the levels of IL-6, TNF-α, and IL-10 were similar in the CR and control animals (Vega et al., 2004).

Similarly, peritoneal macrophages from mice exposed to a graduated CR (gradually reduced calories until they were reduced by 40% of AL fed mice) for five months and then exposed to LPS *ex vivo* for 24 hours demonstrated significantly less IL-6 and IL-12 compared to controls (Sun et al., 2001). In addition, the mRNA levels of TNF-α, IL-6 and IL-12 were lower in peritoneal macrophages from the CR mice compared to control mice. Further investigation revealed that the level of expression of the CD14 receptor (critical in mediating the effects of LPS) was significantly reduced in the CR mice exposed to LPS (Sun et al., 2001). The CR mice demonstrated a shorter survival time after polymicrobial sepsis induced by cecal ligation and puncture. It was concluded by the authors that the CR mice were less likely to respond to a bacterial pathogen, and could not adequately prevent the reproduction of an invading pathogen, which was suggestive of a selective immunosuppressive effect due to the CR (Sun et al., 2001). Interestingly, Sun et al. (2001) only CR their animals for five months and found reduced levels of IL-6 and IL-12; however, Vega et al. (2004) used a CR period of roughly 15 months and only found a reduction in IL-1β at the same level of CR as Sun et al. (2001). Both studies exposed peritoneal macrophages *ex vivo* to LPS, Sun et al. (2001) chose a period of 24 hours and Vega et al. (2004) used an exposure period of five hours. These small discrepancies in methodology may account for the differences between the two studies findings.

Another example of CR's impact upon the functioning of the immune system comes from the investigation of the effect CR had on an allergic reaction in rats (Dong, Kari, Selgrade, & Gilmour, 2000). Eleven-week old rats were fed a diet that was reduced to 75% of AL fed controls' food intake and were sensitized to a house dust mite antigen

after three weeks of CR. Two weeks later they were challenged again with the dust mite. The CR rats demonstrated attenuated pulmonary inflammation as evidenced by reduced numbers of white blood cells and reduced secretion of TNF-α in bronchoalveolar lavage fluid compared to controls (Dong et al., 2000). Interestingly, CR did not alter the initial antigen priming after the sensitization to the house dust mite, as evidenced by antigen specific antibody production (Dong et al., 2000). Although this experiment was concerned with the specific immune response not the innate immune response, the allergic reaction seen after exposure to the house dust mite is similar to that of LPS, provoking the production of cytokines and inflammation.

Stapleton et al. (2001) investigated the effect of a 21.9% CR over seven days and a 5.1% CR over 21 days in female mice, on the function of peritoneal macrophages in response to LPS. After the CR regimen, the mice were sacrificed and the peritoneal macrophages removed and incubated *ex vivo* for 24 hours with LPS. In contrast to Dong et al., (2000), the authors found that there was no difference in the number of macrophages produced in the peritoneal cavity in response to LPS in both CR groups compared to the AL fed mice (Stapleton et al., 2001). However, peritoneal macrophages in both groups of CR mice produced significantly more PGE_2 than the AL controls, with the CR group restricted for seven days at 21.9% demonstrating the highest levels (Stapleton et al., 2001). The authors suggested that the increase in PGE_2 suggests a more sensitive response of the peritoneal macrophages from the CR animals to produce PGE_2 in response to LPS (Stapleton et al., 2001). Although these findings contradict the abovementioned investigations, Stapleton et al. (2001) used a low (5.1%), and mild (21.9%), CR regimens. Whereas the previous investigations cited used a more moderate restriction regimen (40%), which may indicate there is a threshold that needs to be achieved before the macrophages switch from being overly sensitive to less sensitive.

Rats fasted for 48 hours before injection of LPS demonstrated a significantly attenuated fever compared to AL fed controls (Inoue, Somay, Poole, & Luheshi, 2008). In addition to the attenuated fever, plasma levels of TNF-α and IL-1ra were reduced in concentration; however, not all cytokine levels were reduced (IL-1β and IL-6 were elevated to similar levels as controls). Interestingly, levels of IL-1β, COX-2, and PGE_2 in the hypothalamus of fasted animals were at levels normally seen during a febrile response (Inoue et al., 2008); which suggests that the mechanism involved in the attenuation of fever in the fasted rats is peripherally driven.

The evidence presented above demonstrates that CR may have a slightly negative impact upon the functioning of the immune system in some species (Gardner, 2005; Sun et al., 2001). In contrast, other findings have also suggested that CR may provide a protective enhancement of the sensitivity of the immune response (Stapleton et al., 2001). These examples highlight the many inconsistencies within the research investigating the effect of CR on macrophage and cytokine production. Reduced cytokine levels in the long-term may help reduce the occurrence of autoimmune diseases (Muthukumar et al., 2000); however, it may be a disadvantage in the short-term, causing higher susceptibility to infection (Sun et al., 2001).

Possible Mediators of the Immune Response after Calorie Restriction

Evidence presented above suggests that CR affects the immune response and cytokine levels; however, the mechanisms behind these effects have yet to be fully determined. A number of immune mediators including leptin, neuropeptide Y (NPY), glucocorticoids, and preferred selection of T_a are all altered by CR and are also involved in the immune response, and thus, may serve as the mechanism.

Leptin

Leptin plays a key role in regulating energy intake and expenditure and it can also induce a fever and anorexia (Sachot, Poole, & Luheshi, 2004). Leptin levels correlate with body weight and percentage body fat and leptin administration can acutely reduce food intake (Campfield, Smith, Guisez, Devos, & Burn, 1995; Luheshi, Gardner, Rushforth, Loudon, & Rothwell, 1999). This reduction in food intake also occurs in dose-dependent manner, with higher doses of leptin leading to larger decreases in food intake (Luheshi et al., 1999). Leptin levels are increased after administration of LPS (Faggioni et al., 1998; Grunfeld et al., 1996) and mice lacking the leptin receptor have been shown to only show a partial anorexic effect post-LPS (Faggioni, Fuller, Moser, Feingold, & Grunfeld, 1997) suggesting leptin plays a large role in the anorexic response post-LPS. Further, neutralising endogenous leptin using an anti-serum has been shown to significantly reverse the anorexic effect LPS has on reducing food intake and reduction of body weight (Harden, du Plessis, Poole, & Laburn, 2006; Sachot et al., 2004).

In respect to CR, the majority of evidence suggests that leptin is decreased following CR (Chin-Chance, Polonsky, & Scholeller, 2000; Jiang, Liebman, Lucia, Phillips, & Levi, 2005; Miyawaki et al., 2002; Wadden et al., 1998; Wolfe, Jimerson, Orlova, & Mantzoros, 2004). This is not surprising due to the link between leptin levels and body fat. For example, male rats exposed to a 44% CR for four weeks demonstrated a decrease in plasma leptin (Chacon et al., 2005). Our own research group has demonstrated that a 50% CR for five weeks resulted in serum leptin levels that were almost half that of controls (Govic et al., 2008). In humans it has been shown that females exposed to a period of 4 weeks on a 40-50% CR demonstrated a 60% reduction in plasma leptin levels (Wolfe et al., 2004). In males it was also demonstrated that only three days of a 30% CR resulted in a 16% decrease in serum leptin levels (Chin-Chance et al., 2000).

Leptin is produced in the periphery by adipocytes and is then transported across the BBB (Golden, Maccagnan, & Pardridge, 1997), and acts on hypothalamic receptors to suppress appetite and increase EE (Jequier, 2002). Leptin can act as a pyrogenic substance, inducing a dose-dependent increase in core T_b and hypothalamic IL-1β (Luheshi et al., 1999). However, on the other hand others have shown that administration of leptin can lead to increases in anti-inflammatory cytokines and decreases in pro-inflammatory cytokines (Gabay, Dreyer, Pellegrinelli, Chicheportiche, & Meier, 2001; Jaworek et al., 2002; Xiao, Xia-Zhang, Vulliémoz, Ferin, & Wardlaw, 2003). Further, leptin infusion in rats at an ambient temperature of 22 °C and 28 °C failed in not only increase T_b but also failed to increase circulating levels of IL-6 and TNF-α even when the leptin infusion resulted in excessive levels of circulating plasma leptin levels (Steiner, Krall, & Lui, 2009). It was suggested that the non-stressful method of leptin administration in this study (intravenous infusion) compared to other studies that have used more stressful methods of leptin administration (injection) and the use of extremely high doses of leptin may account for the increased T_b seen in previous studies (Steiner et al., 2009). Although there are some discrepancies in the precise role of leptin during inflammation it remains that leptin plays a key role in innate immunity. Furthermore, central administration of IL-1ra inhibits the pyrogenic actions of leptin which included no increase in core T_b, and no reduction in food intake (Luheshi et al., 1999). These authors concluded that the actions of leptin on T_b and food intake may be mediated by the release of IL-1 in the brain. Others have reported that leptin may be responsible for the attenuated fever seen in fasted rats, whereby administration of an exogenous dose of leptin can reinstate food deprived-induced attenuated fever (Inoue & Luheshi, 2010).

Leptin not only acts as a pyrogenic substance, but plasma levels are also induced in a dose-dependent manner by LPS (Francis, MohanKumar, MohanKumar, & Quadri,

1999). Furthermore, pre-treatment with IL-1ra attenuated the LPS-induced increase in plasma leptin levels, again indicating the involvement of IL-1 in leptin release (Luheshi et al., 1999). These findings suggest that the anorexic effects seen after LPS may be mediated through leptin. However, other researchers have found that the ob/ob strain of mice that are genetically deficient in leptin, LPS still causes anorexia (Faggioni et al., 1997); suggesting that leptin may not be the only mediator in the anorexic effect seen after LPS.

Administration of LPS in combination with leptin antiserum results in the attenuation of LPS-induced anorexia in rats (Sachot et al., 2004). This reduction in the LPS-induced anorexia was not due to reduced levels of cytokines in the periphery as plasma levels of IL-6 in the rats exposed to LPS and leptin antiserum were not significantly different from rats only exposed to LPS (Sachot et al., 2004). However, hypothalamic levels of IL-1β and IL-1ra mRNA were significantly reduced in the rats exposed to LPS and leptin antiserum (Sachot et al., 2004), demonstrating the reduction in the LPS-induced anorexia was most likely initiated centrally. Therefore, with consideration of the evidence presented above, leptin may play a key role in the suppression of the inflammatory response observed in CR animals after exposure to LPS.

Neuropeptide Y

Another compound involved in appetite, NPY, has also been suggested to modulate the immune response. NPY is a neuropeptide in the hypothalamus that is involved in the regulation of energy balance; increased NPY results in increased food intake and decreased physical activity (Brady, Smith, Gold, & Herkenham, 1990). Leptin and NPY have been shown to mediate each other in combination with glucocorticoids; an injection of leptin results in the down-regulation of NPY in the arcuate, paraventricular,

ventromedial, and dorsomedial nucleus of the hypothalamus in the absence of glucocorticoids. However, when glucocorticoids are present, the effect leptin has on NPY is attenuated, suggesting an interaction between these three compounds (Jang, Mistry, Swick, & Romsos, 2000).

Pre-treatment with peripheral intravenous NPY before LPS attenuates the febrile response normally seen after LPS possibly by reducing thermogenesis in brown adipose tissue (Felies, von Horsten, Pabst, & Nave, 2004). It has been demonstrated that NPY, central administered in conjunction with IL-1β, can block and even reverse the anorexic effect normally seen after IL-1β administration (Sonti, Ilyin, & Plata-Salamán, 1996). In addition, NPY has been shown to reverse the anorexic effect normally seen after exposure to LPS in sheep (McMahon et al., 1999). Together, these latter two studies suggest that the reduced appetite after LPS may be due to a down-regulation of a NPY-mediated mechanism.

In terms of CR, NPY mRNA in the arcuate nucleus of female rats increased 10-fold compared to controls after two months of a 40% CR (McShane, Wilson, & Wise, 1999). Moreover, a 40% CR for 10 days in rats resulted in increased release of NPY and reduced brown adipose tissue thermogenesis (Widdowson, Upton, Buckingham, Wilson, & Williams, 1997). The increased release of NPY resulted in an enhanced drive to eat, and therefore, an attempt to try and restore the normal caloric intake. Reducing thermogenesis in brown adipose tissue helps conserve fat reserves. This was described as an energy imbalance, and in times of negative energy balance (for example, during CR) there is an increased release of NPY, and during times of a positive energy balance (hyperphagia) there is decreased release of NPY (Widdowson et al., 1997).

Glucocorticoids

Glucocorticoids are steroid hormones (of which cortisol is the most important for humans and CORT for rodents) and are released via the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Dallman, 1993). The HPA axis is a major part of the neuroendocrine system and is partially responsible for reactions to stress and the control of many bodily processes including metabolism. Glucocorticoids can alter metabolic pathways by stimulating gluconeogenesis (a metabolic pathway resulting in the production of glucose from non-carbohydrate sources in the liver) (Sistare & Haynes, 1985).

The HPA axis involves a complex set of feedback interactions between the hypothalamus, pituitary gland, and the adrenal glands (Dallman, 1993; Kirnap et al., 2008). Briefly, when activated, the HPA axis releases corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, which in turn induces the release of adrenocorticotropin (ACTH) from the anterior pituitary gland, which induces the release of CORT from the cortex of the adrenal gland (Patel & Finch, 2002). Elevated plasma CORT acts to inhibit the release of CRH and ACTH via activation of the glucocorticoid receptors in the hippocampus, hypothalamus, and the pituitary (Patel & Finch, 2002). CORT binds to glucocorticoid receptors which have a large role in the negative feedback during inflammation and control the IL-1 and IL-1ra system during an inflammatory process, by decreasing IL-1 expression and increasing IL-1ra expression (Sauer et al., 1996).

Glucocorticoids have potent anti-inflammatory effects and exert these effects by firstly, increasing the transcription of anti-inflammatory cytokines and decreasing the transcription of pro-inflammatory cytokines; and secondly, inhibiting the release of cytokines from macrophages (Barnes, 1998). Glucocorticoids achieve this by binding to the glucocorticoid receptor, and then either stimulate or inactivate gene expression

(Smoak & Cidlowski, 2004). Furthermore, it has been demonstrated that adrenalectomised rats (and therefore produce no glucocorticoids) produced far more IL-6 and exhibit much larger increases in T_b following LPS compared to controls (Morrow, McClellan, Conn, & Kluger, 1993). Pre-treatment with a glucocorticoid attenuates LPS-induced fever (Coelho, Souza, & Pela, 1992).

CR has often, but not always, been demonstrated to increase the levels of glucocorticoids (Han et al., 1995; Levay et al., 2010; Sabatino, Masoro, McMahan, & Kuhn, 1991). Interestingly, circulating CORT levels increase with age and increased CORT is associated with impairments in cognitive function (Endo, Nishimura, & Kimura, 1996; Luine, Villegas, Martinez, & McEwen, 1994). However, the increase in CORT seen after CR does not have a negative impact on cognitive performance, quite the opposite. It is thought that this is due to CR creating a neuroprotective effect, due to a number of mechanisms, which overrides the deleterious actions of increased CORT (Patel & Finch, 2002). This increase in glucocorticoids during CR has been thought to occur for two reasons. Firstly, elevations in circulating glucocorticoids would be expected in times of food scarcity to increase gluconeogenesis. Secondly, levels of glucocorticoids increase to regulate energy metabolism during a life-threatening period (including psychological and physiological stressors such as shortage of food) (Patel & Finch, 2002).Therefore, the question that is occasionally asked in terms of glucocorticoids is whether CR acts as an energy regulator or as a stressor or both.

Evidence from male rats exposed to a 40% CR from six-weeks of age until 31 months of age demonstrated significantly increased levels of plasma CORT (Sabatino et al., 1991). These authors hypothesised that this increase in CORT was attributable to the animals experiencing stress during the short periods of fasting between the times they were fed (Sabatino et al., 1991). In addition, evidence from a shorter CR period, six

weeks, produced similar findings to Sabatino et al. (1991); increased CORT in 40% CR rats (Han et al., 1995). Interestingly, ACTH was not elevated in these CR animals (Han et al., 1995; Levay et al., 2010). An elevation in ACTH might be expected if the HPA axis was activated, which therefore would indicate that the rats were experiencing stress from the CR, as was implied by Sabatino et al. (1991), which may not be the case. The reason as to why glucocorticoids are increased during CR is yet to be fully elucidated; however, the higher levels of glucocorticoids seen in response to CR may account for reductions in pro-inflammatory cytokines, and subsequently an altered immune response in CR animals.

Other possible mediators

It has been demonstrated that old rats do not develop a fever after exposure to LPS when they are housed at a normal laboratory temperature of 21 ^{o}C, but do display a fever when housed in warmer T_as (31 ^{o}C) (Peloso, Florez-Duquet, Buchanan, & Santinoff, 2003). Further, oxygen consumption decreased by about 15% in old rats following LPS administration at ambient temperatures of 21 ^{o}C, but at 31 ^{o}C they showed an increase in oxygen consumption similar to that of young rats (Buchanan, Peloso, & Santinoff, 2008). In an attempt to understand this difference these authors found that the passage of IL-1β into the brain of old rats was decreased at ambient temperatures of 21 ^{o}C (Buchanan et al., 2008). From this, the authors speculated that reduced oxygen consumption (after LPS) would likely result in reduced cerebral blood flow, resulting in less IL-1β present in the blood supply to the brain and hence less passage across the BBB (Buchanan et al., 2008). CR has been shown to lead to reduced blood pressure (Wright, Mc Murtry, & Wexler, 1981; Young, Mullen, & Landsberg, 1978), which could alter the CR animals' capacity to effectively transfer the cytokine message to the brain. As core T_b, and presumably metabolic rate and oxygen consumption, was reduced in CR animals in the

abovementioned study, it is possible that the attenuation of fever was a result of reduced cerebral blood flow in the cool T_a and thus, a decrease of IL-1β influx into the brain.

Comparison issues with adult onset calorie restriction research

As within many research disciplines, methodological limitations and inconsistencies exist with CR research. Many researchers differ on the species and strain of animals chosen for experimentation, the age of animals, and the severity, duration, and nutritional content of the CR regimen and therefore comparisons between these investigations should remain cautious if different conditions have been used. For example, it has been shown that severity, duration, and age of onset can all modify the consequences of CR (Niemann, Silber, & Rohrbach, 2008). In addition, comparisons made between CR and other procedures thought to produce similar effects as CR, such as food deprivation, restriction of one element of the diet, or even excessive exercise, need to also be made with caution. For example, comparisons between a 15% CR for three weeks in mice and a 40% CR for 6 months in primates need to remain guarded.

Strain, species and age differences of calorie restriction

It was demonstrated in a study investigating the biomarkers of aging that different strains of mice and rats demonstrated different patterns of weight loss and survival rates after CR (Turturro et al., 1999). After a 40% CR B6D2F1 mice survived until 50 months of age, whereas D2 mice only survived until 40 months of age on the same diet. B6-EM mice only survive until 30 months of age, almost two years less than that of the B6D2F1 mice (Turturro et al., 1999). A similar inconsistency was found in rats with F344 × BN F1 rats surviving until 50 months of age on a 30% CR, and F344-PUR rats surviving until 35 months of age (Turturro et al., 1999).

The age of onset of CR produces differing results (Kubo et al., 1984; Niemann et al., 2008; Weindruch & Walford, 1988). Initially it was believed that CR increased life span by retarding growth and development by increasing the age at which sexual and skeletal maturity arises, and therefore CR could only be initiated before this maturity to achieve these effects (McCay et al., 1935). However, this was subsequently shown not to be the case, with other researchers discovering that placing animals on CR after sexual and skeletal maturity still increased maximum life span compared to AL fed controls (Yu, Masoro, & McMahan, 1985). Furthermore, mice at 12 and 14 months of age who were placed on a CR diet also demonstrated an increase in life-span, although this effect was not as marked in mice that were exposed to CR after weaning (Weindruch & Walford, 1988).

A 50% CR in an autoimmune prone strain of mice produced slightly differing results when early and late onset of CR was compared (Kubo et al., 1984). CR initiated at six weeks of age resulted in a slowing of the development of autoimmune disease processes, and ultimately, renal disease. In the mice that were exposed to the CR regimen from 12 weeks of age, when most mice have already begun to show signs of autoimmune disease, CR still improved the length of life (Kubo et al., 1984), but not to the same extent.

Severity, duration, and nutritional supplementation

There is a dose-dependent increase in CORT with a CR of only 12.5% for three weeks eliciting a significant elevation in CORT levels (Levay et al., 2010). Severity of the CR regimen has been shown to impact on the weight of adrenal glands, kidneys, gonads, heart, and spleen, which differed among male rats exposed to a 20% or 40% CR (Martin et al., 2007a). Furthermore, there were differences in levels of CORT (higher in the 40%

CR group), cholesterol (lower in the 40% CR group), insulin (lower in the 20% CR group), and leptin (lower in the 40% CR group) between the two severities of dietary manipulation (Martin et al., 2007a).

Differing durations of CR have resulted in differing effects. Body-mass adjusted oxygen consumption in rats was not significantly affected by a 40% CR for two weeks or two months; however, a six month period reduced oxygen consumption by 40% (Bevilacqua, Ramsey, Hagopian, Weindruch, & Harper, 2004). The same study revealed that mitochondrial production of ROS was reduced by 53% compared to controls after two weeks on a 40% CR and a 74% reduction at six months (Bevilacqua et al., 2004). In contrast, it was found that when comparing a short and long term (precise length not specified) 44% CR, these CR durations were homologous in both direction and level of change in the expression of genes that change with age (Cao et al., 2001). Furthermore, the short term CR reproduced 100% of the same effects as long term CR on urinary protein and stress response gene expression and 67% of the effects on inflammatory response gene expression (Cao et al., 2001). These findings provide mixed messages as to the precise difference between short versus long term CR. It may be that depending on the variables measured that there is a threshold that needs to be reached (in terms of severity and duration of CR) to see changes in different variables. The timing of the CR period needs to be optimised for specific investigations.

Calorie restriction versus other forms of dietary manipulation

Food deprivation and fasting are acute forms of dietary manipulation, whereas CR is chronic and it has been demonstrated that food deprivation elicits differing impact upon hypothalamic NPY compared to CR (Bi, Robinson, & Moran, 2003; Brady et al., 1990). For instance, mRNA levels in the arcuate nucleus of the hypothalamus of NPY and

agouti-related protein were significantly higher in rats food deprived for 48 hours compared to rats CR by 30% for 14 days (Bi et al., 2003). However, in another study rats CR (given 10 g of food per day, no actual % CR given) for 14 days demonstrated significantly higher levels of NPY in the arcuate nucleus compared to rats food deprived for 4 days (Brady et al., 1990). The discrepancies here may relate to the differences in the level of CR and the length of the food deprivation.

Food deprivation has also been shown to induce a state of immunodeficiency (Faggioni, Moser, Feingold, & Grunfeld, 2000); hence, comparisons between the two should be carefully considered. Furthermore, other procedures that have been aimed at mimicking the effects of CR, such as excessive exercise (Huffman et al., 2008), often do not demonstrate the same results as CR. It has been shown that CR can prolong life; however, exercise fails to slow the aging process in the same manner as CR, and that this may be due to the exercise not fully mimicking the hormonal and/or metabolic responses seen after CR (Huffman et al., 2008).

Calorie restriction and sex differences

Sex differences have been observed after CR, for example, the optimal value and its effect on life span for male and female *Drosophila* (Magwere et al., 2004). The males' median life span peaked with a CR of 40%, whereas the females' median life span peaked at a CR of 60% (Magwere et al., 2004). In addition the male flies on a 40% CR only survived 30% longer than their fully-fed counterparts, while the female flies on a 60% CR regimen lived up to 60% longer than that of their fully-fed counterparts (Magwere et al., 2004).

In addition, AL fed female rats demonstrated greater oxygen consumption and carbon dioxide production, coupled with a reduced EE compared to male rats on the same

diet (Valle et al., 2005). After a 40% CR for 100 days, the females' oxygen consumption, carbon dioxide production and EE markedly decreased; however, these parameters did not change in the males on the same CR diet (Valle et al., 2005). The weight loss in male and female rats was similar; however, the females demonstrated a larger decrease in adipose tissue (Valle, et al., 2005). In contrast, and although in another species, it was found that male rhesus monkeys demonstrated a more pronounced weight loss after a 30% CR for approximately 10 years compared to female monkeys (Mattison et al., 2003). However, the male rhesus monkeys lost more total body weight, including lean muscle mass, whereas the females rats lost more fat mass, and retained their lean muscle mass (Valle et al., 2005). This discrepancy may be the product of females naturally having more fat mass compared to males coupled with the fact that fat is preferentially lost before muscle mass (Robergs & Robert, 1997).

In addition, CR has been shown to induce differing results in brown adipose tissue functional proteins (Valle, García-Palmer, Oliver, & Roca, 2007), and differing effects on activity and cognitive performance (Martin et al., 2007a). In female rats, a 40% CR for six months elicited more spontaneous activity, significantly increased CORT, and they performed better on cognitive tasks (as measured by a 14-unit T-maze) compared to the males on the same dietary manipulation (Martin et al., 2007a). There were also differences between the females and males on levels of growth hormone; dopamine and serotonin in the hippocampus; norepinephrine in the cerebral cortex, cerebellum, and striatum; and circulating brain-derived neurotrophic factor (Martin et al., 2007a).

Conclusion

This literature review has comprehensively assessed the impact that CR s has on behavioural, physiological, molecular, and metabolic variables, focussing for the majority

of the review on the immune system. CR has been shown to positively affect a number of health markers, including improving cardiovascular health (Lane et al., 1999), increasing the survival of animals after the development of certain cancers (Matsuzaki et al., 2000); and attenuating the progression of autoimmune diseases (Kubo et al., 1984). Furthermore, CR has been shown to increase the mean and maximum life span of a variety of animals (Ingle, Wood, & Banta, 1937; Mattison et al., 2003; McCay et al., 1935; Turturro et al., 1999); and also decrease metabolic rate (Duffy et al., 1989; Heilbronn et al., 2006). Although there has been some investigation of cytokine responses after exposure to pathogens in CR animals (Dong et al., 1998; Inoue et al., 2008; Sun et al., 2001; Vega et al., 2004), the behavioural immune response after CR has not been well investigated. Chronic inflammation has been cited as a characteristic of numerous diseases such as cancer (Coussens & Werb, 2002; Mantovani, Allavena, Sica, & Balkwill, 2008), obesity-related insulin resistance (Xu et al., 2003), polycystic ovarian syndrome (Kelly et al., 2001), inflammatory bowel disease (Itzkowitz & Yio, 2004), rheumatoid arthritis (McInnes & Schett, 2007), and Alzheimer's Disease (Sokolova et al., 2009) to name a few. We believe that this avenue of research, investigating sickness behaviour and possible mediating factors in CR animals, would provide valuable insight and further clarification into the role CR plays in relation to the immune system. It may be possible to develop a mimetic of the effects of CR to help control the chronic inflammation seen in the abovementioned diseases, which potentially could be of great benefit.

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CHAPTER 2

DOSE-DEPENDENT EFFECT OF CALORIE RESTRICTION DURATION IN RATS ON BODY WEIGHT, CORE BODY TEMPERATURE, AND LOCOMOTOR ACTIVITY

Abstract

Calorie restriction (CR) has been widely studied in many species and has been shown to elicit many health-promoting benefits. CR reduces core body temperature (T_b), which has been thought to play a significant role in the life-extension properties of CR. This study aimed to characterise the effect of three different durations of CR on body weight, T_b, and locomotor activity. Male Sprague-Dawley rats were CR to 50% (CR50%) of ad libitum (AL) animals food intake for 14, 21, or 28 days to explore how different durations of CR impact upon the abovementioned variables. There was a dose-dependent response of body weight loss, reduction in T_b, and alteration of locomotor activity with the rats CR for the longest period of time (28 days) demonstrating the most weight loss, largest reduction in T_b, and largest change in locomotor activity. T_b was gradually reduced and by day 14 the CR animals demonstrated a reduction in core T_b compared to the AL rats during both the light and dark phases. The locomotor activity of the CR rats was similar to the AL rats up until the dark phase of day 14. The CR rats then demonstrated a flattening out of their locomotor activity 24-hour rhythm, similar to their T_b. This was characterised by an increase in their light phase activity and a decrease in their dark phase activity. The current study may enable future researchers to adapt their research when using CR to investigate the impact on T_b and locomotor activity accordingly in respect to the duration of the CR period in order to elicit the desired results. Reduced T_b has been suggested to play a major role in anti-aging mechanisms and the reduction in occurrence and/or delayed maturation of diseases such as Alzheimer's, Parkinson's, diabetes, and autoimmune diseases.

Calorie restriction (CR) has been widely studied in yeast, rodents, and other mammals, and can be achieved by reducing the average unrestricted food intake. Traditionally, the reduction in food intake includes a balanced decrease in calories, protein, vitamins, and minerals, while avoiding malnutrition (Weindruch & Walford, 1988). CR has been established to prolong the lifespan (Lin, Ford, Haigis, Liszt, & Guarente, 2004; Weindruch, Walford, Fligiel, & Guthrie, 1986), reduce the occurrence of age-related diseases such as cancer (Matsuzaki et al., 2000) and Alzheimer's Disease (Halagappa et al., 2007), lessen the severity of the neurochemical deficits and motor dysfunction seen in primate models of Parkinson's Disease (Maswood et al., 2004), and attenuate the 'normal' immunosenscence seen with advanced age (Mascarucci et al., 2002). Further, CR has been shown to reduce core body temperature (T_b), and consequently has been shown to reduce metabolic rate (Blum et al., 1985; Forsum, Hillman, & Nesheim, 1981; Hill, Sparling, Shields, & Heller, 1987).

There have been a number of studies that have investigated reduced core T_b following a period of CR. For instance, the average daily core T_b in rats has been shown to be reduced by an average of 0.9 °C after a 40% CR for 12 months (Duffy et al., 1989). In non-human primates, rectal temperature has been shown to be significantly reduced by 0.5 °C in rhesus monkeys restricted to a 30% CR; however, this reduction was not evident until two-years after the initiation of the CR regimen (Lane et al., 1996). This suggests there are thresholds in terms of the duration of the CR regimens in reducing core T_b.

Limited research exists on the impact CR has on general locomotor activity; however, it has been established that a period of restricted access to food alters the circadian rhythm of locomotor activity when the food is provided during the light cycle (Challet, Pevet, Vivien-Roels, & Malan, 1997; Mistlberger, 2009). This results in anticipatory behaviour in the hour or two prior to food being provided to the animal (Lane et al., 1996; Weed, Lane, Roth,

Speer, & Ingram, 1997). However, it has also been shown that even though the pattern of CR animals' locomotor activity changes throughout the 24 period, the overall activity of the animal does not (Boyle, Storlien, Harper, & Keesey, 1981; Lane et al., 1996). In contrast other have shown that activity can increase after a period of CR in rodents (Duffy et al., 1989; Chen, Steele, Lindquist, & Guarente, 2005) and 30% CR regimen in rhesus monkeys found that general locomotor activity actually increased in the CR animals compared to controls, most notably during the time surrounding meal presentation (Weed et al., 1997).

The duration of the CR regimen has been shown to have a significant impact upon the investigatory outcomes in some investigations. For example, the investigation of different CR durations was conducted in rotifers (a microscopic pseudocoelomate animal). These rotifers were shown to exhibit the largest increase in lifespan after the longest dietary restriction period (Weithoff, 2007). The period of restriction ranged from 3 to 15 days in length, with the rotifers restricted for 15 days surviving for the longest period of time, some for up to 24 days compared to the average lifespan of 10 days (Yoshinaga, Kaneko, Kinoshita, Tsukamoto, & Watabe, 2003). This study used a microorganism and not mammals, and investigated lifespan and not T_b or locomotor activity changes; however, the possibility that differing durations of CR may elicit differing consequences can be noted.

Another example of how the duration of a CR regimen has an impact on research outcomes comes from bulls. Belgium Blue bulls were maintained on a diet that restricted the amount of growth to .5 kg/day for 115, 239, or 411 days (Hornick, Eenaeme, Clinquart, Diez, & Istasse, 1998). After the respective CR periods it was seen that the bulls restricted for the longest period of time (411 days) demonstrated the lowest levels of saturated fatty acid in their adipose tissue compared to the control bulls and the other CR bulls. Interestingly, after two months of re-feeding all of the CR groups were similar in weight; however, they were all also still lighter than controls (Hornick et al., 1998). Although these examples are not directly

relevant for the characterisation of T_b and locomotor activity after different durations of CR, they do serve as an example of how the duration of a dietary regimen does impact upon investigatory outcomes.

The aim of the current study was to characterise changes in body weight, core T_b, and locomotor activity in rats after CR and to characterise the dose-dependent effect of CR on the abovementioned variables in terms of the duration of the CR period. It is expected that CR will lead to a reduction in body weight and T_b, and lead to a change in the 24 hour pattern of locomotor activity. Further, it is hypothesised that there would be a graded response in relation to the length of the CR regimen. CR for 14 days will elicit the smallest changes in the abovementioned variables and the longest period of CR (28 days) will produce the largest changes in these variables.

Methods

Animals

Forty-one male Sprague-Dawley rats were procured from Monash SPF animal services (Clayton, Vic, Australia) and allowed to acclimate to the facility for at least one week. During this period, standard rodent chow (Barastoc, Melbourne) and water were available ad libitum. At the beginning of experimentation the rats were aged between 9 and 16 weeks old. Rats (30 × 50 × 15 cm) were individually housed in polypropylene basin cages with sawdust and tissues provided as bedding. The animals were maintained at an ambient temperature of 26 ± 1 °C, which is within the thermoneutral zone of this species (Poole & Stephenson, 1977). The animals were maintained on a 12:12 light/dark cycle (0500 – 1700 hours). Animal care and experimentation was performed in accordance with protocols of the La Trobe University Animal Ethics Committee.

Surgery

Following acclimation, all of the rats were surgically implanted in the peritoneal cavity with a biotelemetry device (SubCue Datatlogger, Calgary, Alberta, Canada: 15 mm × 5 mm, 4.2 g; or E-4000, Mini-mitter®, Bend, OR, USA: 23 × 8 mm, 1.6 g) under anaesthesia (ketamine 61 mg/kg and xylazine 9 mg/kg). Before the beginning of surgery 9 mg/kg of Carprofen, a non-steroidal anti-inflammatory, was injected subcutaneously to reduce post-operative discomfort. The animals' abdomen was shaved and a 1 cm incision was made left of the midline through the skin, and a further incision was made through the peritoneal muscle wall; the biotelemetry device was then inserted into the peritoneal cavity, as described previously (Begg, Kent, McKinley, & Mathai, 2007; Weiland, Voudouris, & Kent, 2004). The muscle wall was sutured using absorbable suture material (Polysorb) and the skin sutured with non-absorbable suture material (Supramid). All surgeries were completed under aseptic technique. All animals were returned to their home cages, which were placed on heating mats to maintain optimal T_b, until they regained consciousness. The animals were allowed one to two weeks recovery before the initiation of the CR regimens. Body weight was monitored, and visual inspections were carried out daily following surgery to ensure the animals did not develop an infection or post-operative complications.

Dietary regimens

Rats were divided into one of four CR regimens matched for weight, food intake, and age: ad lib (AL; n = 14) fed ad libitum (on average 24 g per day) and CR50% (n = 27) rats all received 50% of the amount consumed by AL rats for 14, 21, or 28 days respectively (on average 12 g per day). The recommended daily allowance (*Institute for Laboratory Animal Research. Nutrient requirements of laboratory animals*, 1995) of the diets given to the AL and CR50% rats are listed in Table 1 and have been published previously (Levay, Govic,

Penman, Paolini, & Kent, 2007). The only elements in which the CR diet was deficient were fat and vitamin B-12. The intake of the CR groups was determined weekly based on the average daily food intake of the AL rats for three consecutive days. Water was continuously provided to all groups. The dietary manipulation continued for 14, 21, or 28 days depending on the experimental group. Food was provided daily, approximately one hour before the dark phase onset. The group sizes varied across dependent variables and precise numbers used for each variable are stated in the results section.

Table 1. *The dietary composition of each feeding regimen and recommended dietary allowance (RDA) for adult male rats*

		Diet		
	Unit	RDA	AL	CR50%
Protein	g/kg	50	203	102
Fat	g/kg	50	31.1	15.6
Starch	g/kg	-	382	191
Calcium	g/kg	5	10	5
Phosphorous	g/kg	3	6.5	3.3
Sodium	g/kg	0.5	2.8	1.4
Chloride	g/kg	0.5	4.8	2.4
Potassium	g/kg	3.6	7.8	3.9
Lysine	g/kg	1.1	10.7	5.4
Magnesium	g/kg	0.5	1.9	1
Iron	mg/kg	35	198	99
Manganese	mg/kg	10	134	67
Zinc	mg/kg	12	198	99
Copper	mg/kg	5	18	9
Iodine	µg/kg	150	1460	730
Selenium	µg/kg	150	577	288
Vitamin A	mg/kg	0.7	12.5 IU/g	6.3 IU/g
Vitamin D	mg/kg	0.03	2.8 IU/g	1.4 IU/g
Vitamin E	mg/kg	18	75.5	37.8
Niacin	mg/kg	15	154.4	77.2
Vitamin B-6	mg/kg	6	12.7	6.4
Riboflavin	mg/kg	3	16	8
Thiamine	mg/kg	4	19.9	10
Vitamin K	mg/kg	1	11.3	5.7
Folic Acid	mg/kg	1	3.4	1.7
Biotin	mg/kg	0.2	0.5	0.3
Vitamin B-12	µg/kg	0.05	0.05	0.03

Determination of body weight, core T_b, and locomotor activity during CR

Animals were weighed once a week approximately three hours after lights-on during dietary manipulation using top loading scales (± 0.1 g). T_b and locomotor activity were determined by placing each animal, housed singly, on a receiver with each individual biotelemetry device generating a continuous frequency signal proportional to the animal's T_b (± 10^{-1} °C), as described previously (Weiland et al., 2004). The receiver sampled this frequency at 1-minute intervals and this sample was decoded by VitalView software (Mini Mitter Co., Inc., Bend, OR) and stored on a hard drive. The receiver for each cage was equipped with a matrix of antennas that were continuously signalled by the biotelemetry device. The receiver scanned the matrix in a sequential order to locate the position and orientation of the biotelemetry device, thus making it possible to detect the global activity of the animal. The receiver tallied the number of matrices crossed by the animal during 1-minute intervals and this information was recorded using VitalView software.

Data Analysis

Mean ± SEM body weight of each group prior to the onset of CR and weekly throughout the CR period were analysed using a two-way mixed design analysis of variance (ANOVA). A two-way mixed design ANOVA compared groups during both the light and dark phases of each day and for the 24 hours period on each day during the CR period. Data for T_b and locomotor activity in rats were collapsed into hourly means for days 0, 7, 14, 21, and 28 of the CR period to determine changes in basal T_b and locomotor activity throughout the CR period and analysed by a two-way mixed design ANOVA. To overcome violations of sphericity, the Greenhouse-Geisser statistic corrected degrees of freedom were reported. Where appropriate, post hoc pairwise comparisons were performed using the Least

Significant Difference (LSD) method. Where required, between-group simple main effects analyses were performed using LSD method. When several comparisons were required, as in the case of T_b and locomotor activity data, a Bonferroni adjustment was used to protect against inflated type 1 error rate. Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$ as the criterion for significance.

Results

Effect of a 50% CR on body weight in rats

The CR50% rats lost weight during the CR period, whereas the AL rats demonstrated a large weight gain of just over 20% of their initial body weight (Table 2). The CR rats lost a steady amount of weight up until day 14; however, stabilise from this time, even putting on weight between day 21 and day 28. The CR rats lost a total of just over 10% of their initial body weight by the end of the CR period. The ANOVA was significant for week [$F(1.48,14.78) = 22.36$, $p < .001$, partial $\eta^2 = .69$], group [$F(1,10) = 223.32$, $p < .001$, partial $\eta^2 = .96$], and their interaction [$F(1.48,14.78) = 175.07$, $p < .001$, partial $\eta^2 = .95$]. Post hoc comparisons revealed that the AL rats gained significantly more weight compared to the CR50% rats on each day measured during the CR period ($p < .001$ for all). The AL rats were heavier compared to day 0 on each day of the CR period ($p < .001$ for all) and the CR rats were lighter compared to day 0 on each day of the CR period ($p < .001$ for all).

Table 2. *Mean (± SEM) percentage body weight change from baseline for AL (n = 14) and CR50% (n = 8 - 20) rats weekly throughout the CR period*

	Day 0	Day 7	Day 14	Day 21	Day 28
AL	0	5.6% (0.9)	9.9% (1.2)	14.5% (1.0)	20.9% (1.4)

CR50%	0	-5.3%	-9.2%	-11.2%	-10.2%
		(0.7)***	(0.9)***	(1.1)***	(1.4)***

Note: (***) denotes a significant difference from the AL group at $p < .001$

Changes in light and dark phase T_b after a 50% CR in rats

The CR50% and AL rats demonstrated similar T_b in both the light and dark phases of day 0 and day 7 of the CR period (Figure 1). By the dark phase of day 14 the CR50% rats demonstrated a reduced T_b compared to the AL rats; however, were still similar to the AL rats during the light phase. This pattern continued for the remainder of the CR period. In other words the amplitude of the CR50% rat's T_b circadian rhythm decreased from day 14 of the CR period. The ANOVA found a significant effect for week [$F(1.55,12.36) = 13.53$, $p < .001$, partial $\eta^2 = .63$], group [$F(1,8) = 6.77$, $p = .032$, partial $\eta^2 = .46$], and their interaction [$F(1.55,12.36) = 6.35$, $p = .017$, partial $\eta^2 = .44$]. Post hoc tests revealed that the CR50% rats demonstrated a reduced core T_b compared to the AL animals during the dark phase on days 14 ($p < .001$), 21 ($p = .002$), and 28 ($p < .001$). The only time in which the AL group's T_b differed from day 0 was during the dark phase of day 21, in which it was lower compared to day 0 ($p = .035$). The CR50% group demonstrated a significantly lower T_b during the light phase compared to day 0 on day 14 ($p = .005$) and day 21 ($p = .015$). During the dark phase the CR50% group demonstrated a lower T_b compared to day 0 on day 14 ($p = .003$), 21 ($p = .004$), and 28 ($p = .002$).

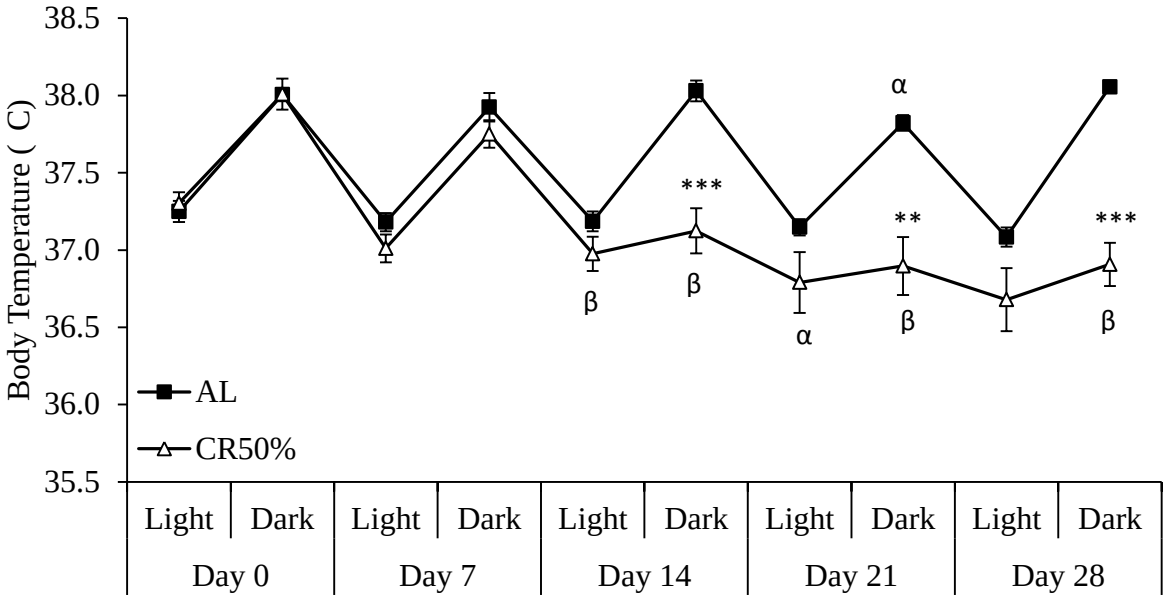

Figure 1. Mean T_b (± SEM) for AL (n = 14) and CR50% (n = 8 – 20) rats during the light and dark phases of day 0, 7, 14, 21, and 28 of the CR period. (**) denotes a significant difference from the AL group at $p < .01$, (***) denotes a significant difference from the AL group at $p < .001$, (α) denotes a significant difference from the same phase within the same group compared to day 0 at $p < .05$, and (β) denotes a significant difference from the same phase within the same group compared to day 0 at $p < .01$.

T_b throughout a 24 hour period on day 0, 7, 14, 21, and 28 of CR in rats

On day 0 the AL and CR50% rats demonstrated an almost identical pattern of T_b during the 24 hour period (Figure 2). This was apart from the exception at 1600 h when the CR50% rats received their first allotment of food for the CR period. The rise in T_b at between 0900 h and 1000 h was when the rats were weighed. There were no significant differences between the AL and CR50% rats at any hour on day 0 of the CR period.

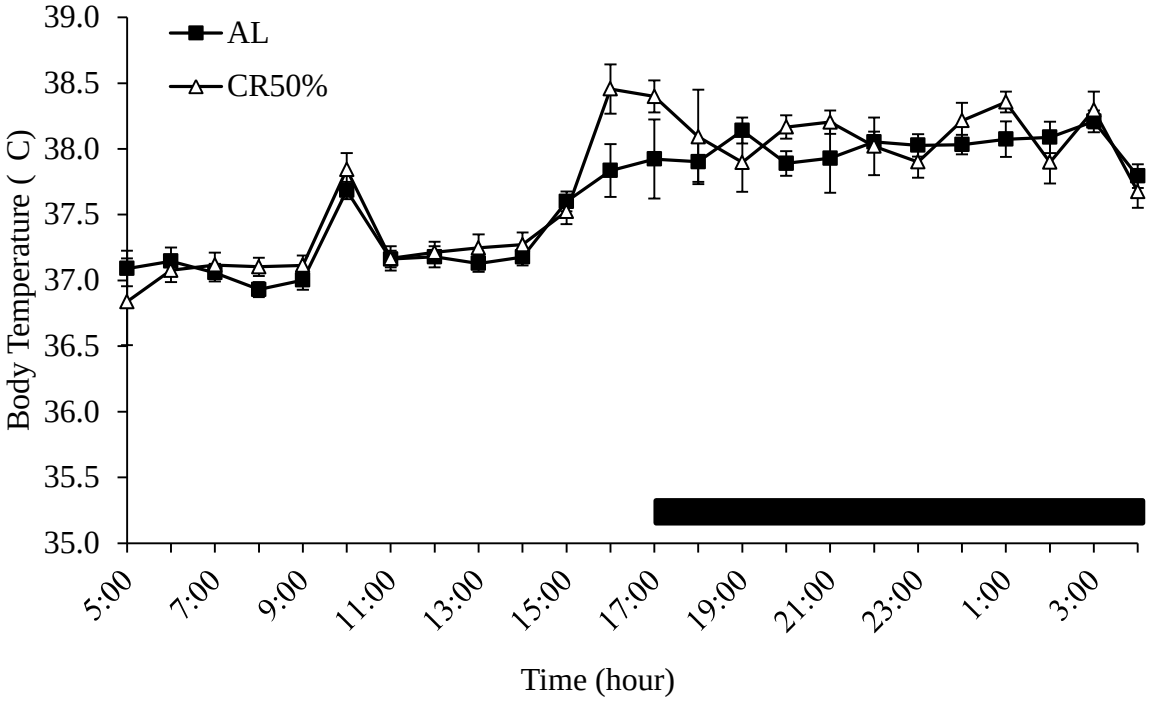

Figure 2. Mean T_b (± SEM) for AL (n = 6) and CR50% (n = 14) rats during day 0 of the CR period, with the black box representing the dark phase.

During day 7 of the CR period the AL and CR50% rats were still remarkably similar in their basal T_b during the 24 hour period, with a couple of exceptions (Figure 3). Again, similar to day 0, there was a spike in basal T_b for the CR50% rats at 1600 h when they were fed. The ANOVA only found a significant effect for hour [$F(4.41,44.10) = 26.72$, $p < .001$, partial $\eta^2 = .73$]. The only differences between the AL and CR50% rats occurred at 0700 h ($p = .026$), 0800 h ($p = .003$), 0900 h ($p = .023$), and 0300 h ($p = .018$).

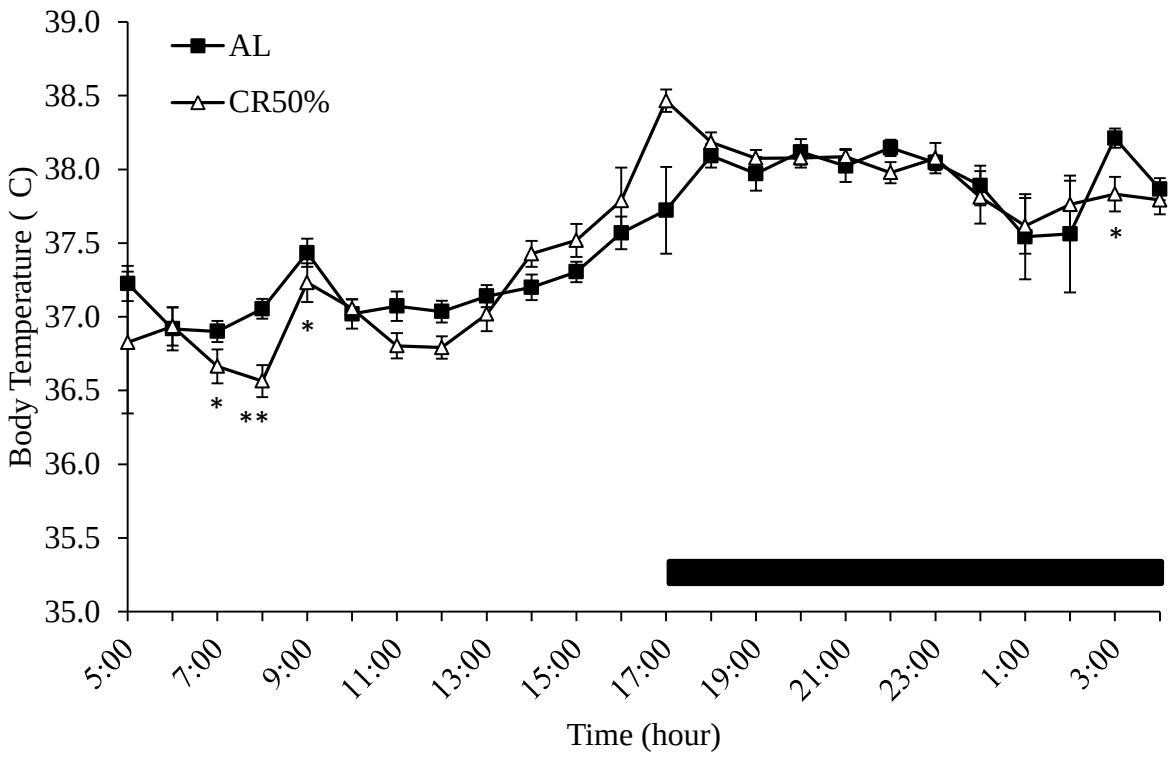

Figure 3. Mean T_b (± SEM) for AL (*n* = 6) and CR50% (*n* = 14) rats during day 7 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$ and (**) denotes a significant difference from the AL group at $p < .01$.

By day 14 of the CR period the CR50% rats had begun to demonstrate a different pattern of T_b compared to the AL rats, namely a reduction in their dark phase T_b (Figure 4). The ANOVA was significant for time [$F(4.86,126.48) = 11.55$, $p < .001$, partial $\eta^2 = .31$], group [$F(1,26) = 9.25$, $p = .005$, partial $\eta^2 = .26$], and their interaction [$F(4.86,126.48) = 4.29$, $p < .001$, partial $\eta^2 = .14$]. The CR50% rats had a lower T_b compared to the AL rats at all hours during the 24 hour period on day 14 except for the hours between 0900 h and 1700 h (ranging from $p = .034$ to $p < .001$).

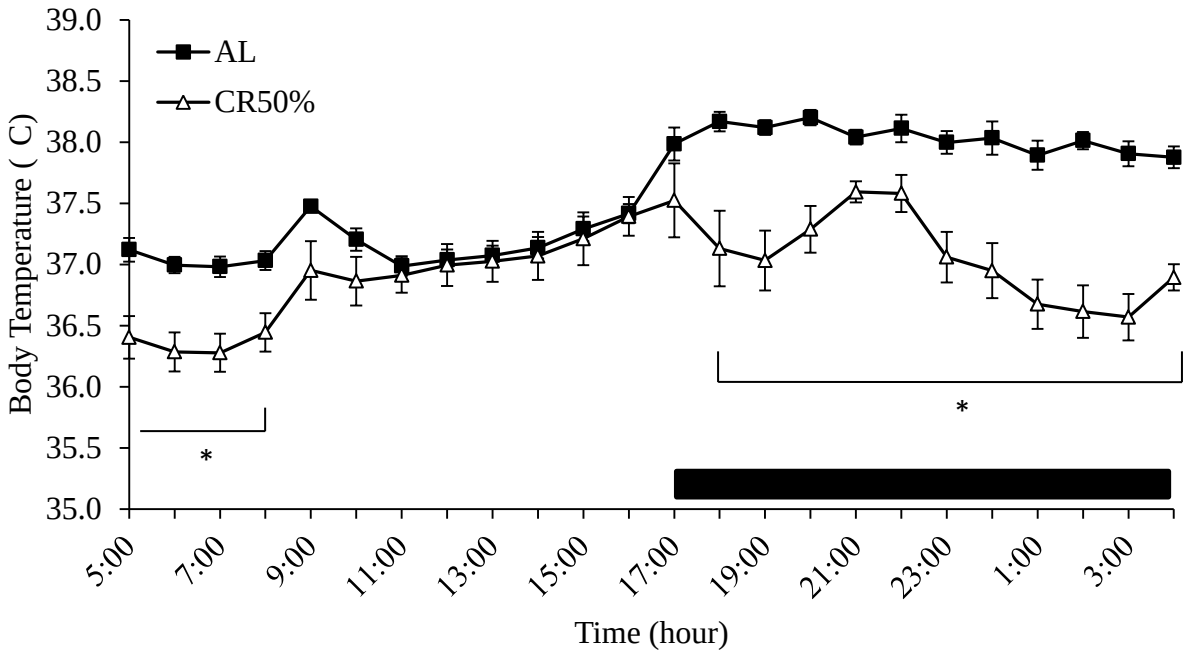

Figure 4. Mean T_b (± SEM) for AL (n = 6) and CR50% (n = 14) rats during day 14 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$.

The T_b pattern during day 21 of the CR period was very similar to that of day 14 (Figure 5). The CR50% rats demonstrated a lower T_b during the majority of the dark phase compared to the AL rats. The ANOVA found a significant effect for hour [$F(2.48,22.35) = 3.72$, $p = .032$, partial $\eta^2 = .29$] and group [$F(1,9) = 8.66$, $p = .016$, partial $\eta^2 = .49$]. The CR50% rats had a lower T_b compared to the AL rats at all hours during the 24 hour period on day 21 except for the hours between 0900 h and 1900 h (ranging from $p = .032$ to $p < .001$).

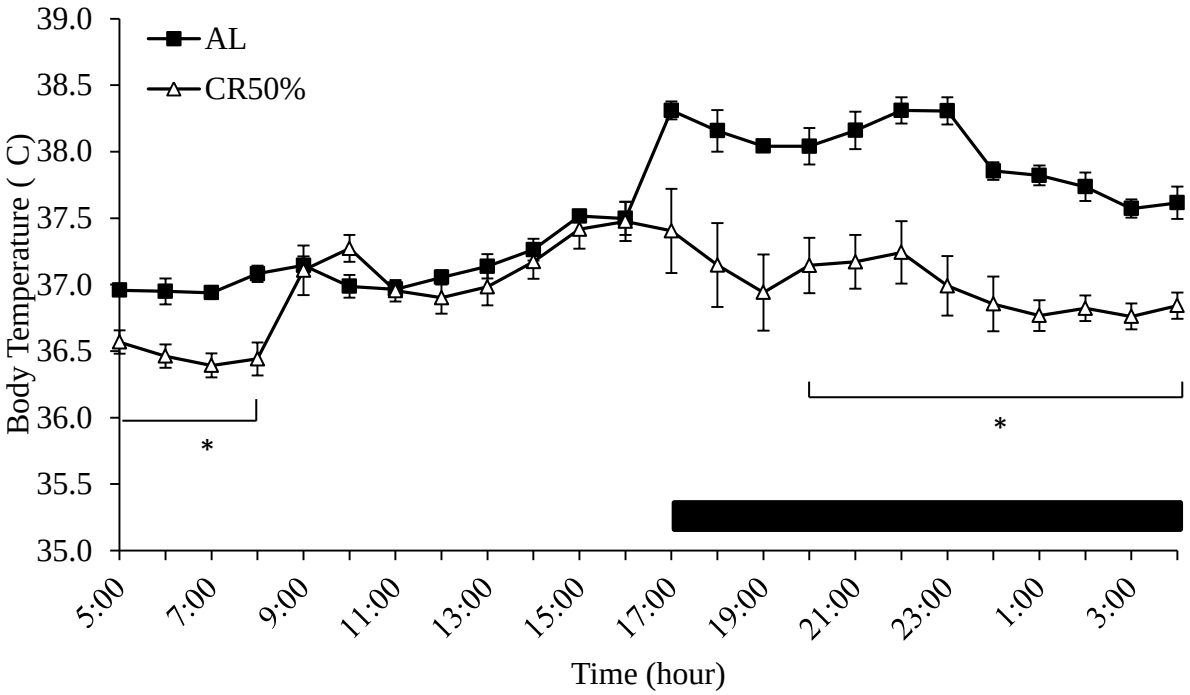

Figure 5. Mean T_b (± SEM) for AL (n = 6) and CR50% (n = 14) rats during day 21 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$.

On day 28 of the CR period the CR50% rats continued to demonstrate a lower T_b during the dark phase and were similar to the AL rats during the majority of the light phase (Figure 6). The ANOVA found a significant effect for hour [$F(2.54,25.37) = 6.98$, $p = .002$, partial $\eta^2 = .41$], group [$F(1,10) = 25.03$, $p < .001$, partial $\eta^2 = .71$], and their interaction [$F(2.54,25.37) = 4.02$, $p = .023$, partial $\eta^2 = .29$]. The CR50% rats differed from the AL rats at all hours during the 24 hour period on day 28 except for the hours between 0600 h and 1600 h (ranging from $p = .045$ to $p < .001$).

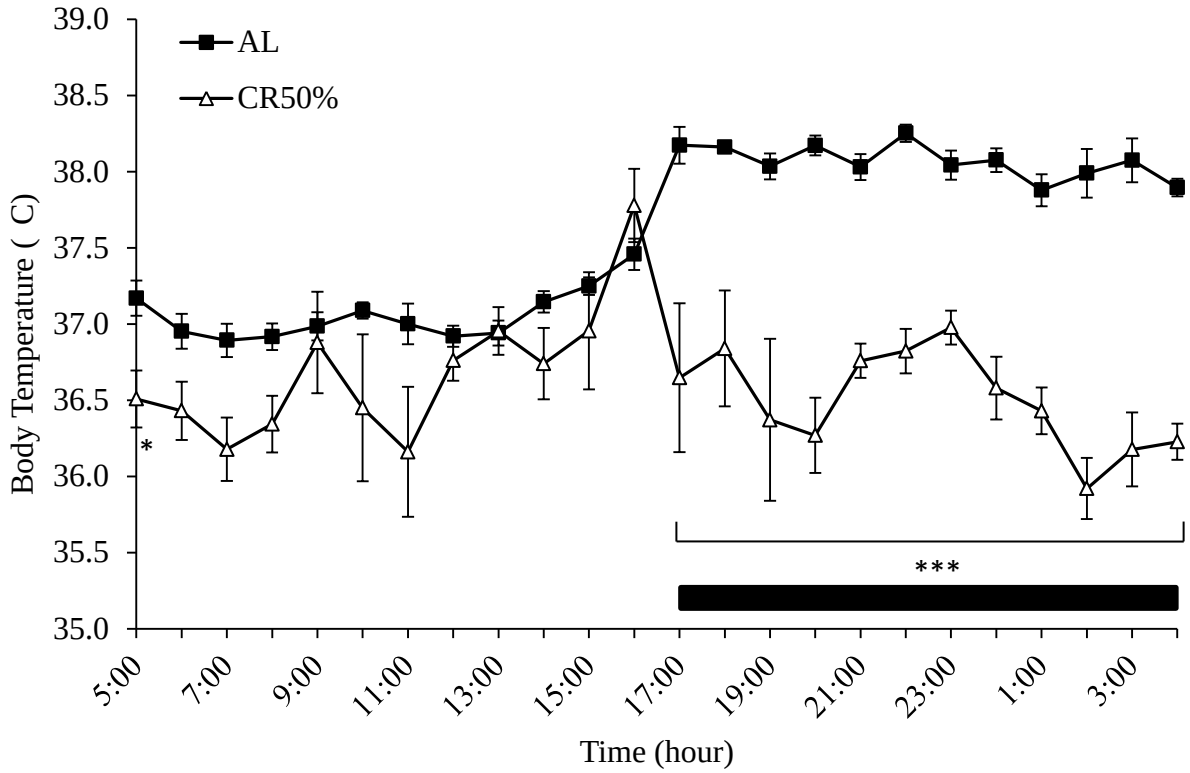

Figure 6. Mean T_b (± SEM) for AL (n = 6) and CR50% (n = 14) rats during day 28 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$, (***) denotes a significant difference from the AL group at $p < .001$.

Changes in total locomotor activity after a 50% CR in rats

Total locomotor activity was similar for the first 14 days of the CR period; however, on day 21 and day 28 the CR rats were overall less active compared to the AL rats (Figure 7). The ANOVA found a significant effect for time [$F(3.12,37.49) = 25.48$, $p < .001$, partial $\eta^2 = .68$] and group [$F(1,12) = 17.08$, $p = .001$, partial $\eta^2 = .59$]. The AL rats were more active compared to the CR50% rats on days 21 and 28 ($p < .001$ and $p = .003$ respectively).

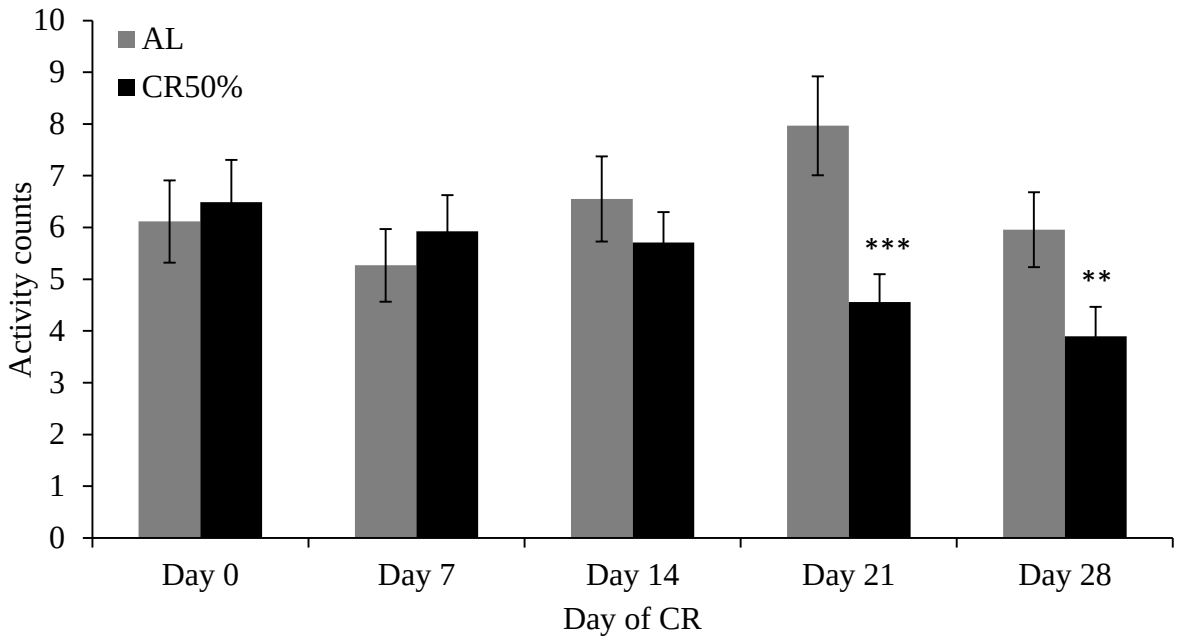

Figure 7. Mean total locomotor activity counts (± SEM) for AL and CR50% (*n*'s ranging from 5 to 20 rats per group) rats during day 0, 7, 14, 21, and 28 of the CR period. (**) denotes a significant difference from the AL group at $p < .01$ and (***) denotes a significant difference from the AL group at $p < .001$.

Changes in light and dark phase locomotor activity after a 50% CR in rats

Light and dark phase locomotor activity was determined for AL and CR50% rats. The CR50% rats demonstrated a similar pattern of locomotor activity to the AL rats on day 0 of the CR period, with a large increase in locomotor activity during the dark phase. By day 7 the CR50% rats had begun to show an altered locomotor activity pattern, whereby their light phase locomotor activity was slightly increased. On day 14 of the CR period the CR50% rats were showing reduced activity levels during the dark phase, which remained for the rest of the CR period (Figure 8). The ANOVA found a significant effect for week [$F(2.28,25.13) = 31.03$, $p < .001$, partial $\eta^2 = .74$], group [$F(1,11) = 25.04$, $p < .001$, partial $\eta^2 = .69$], and their interaction [$F(2.25,25.13) = 19.18$, $p < .001$, partial $\eta^2 = .64$]. The CR50% rats were less

active compared to the AL rats during the dark phase of day 14 ($p < .001$), 21 ($p < .001$), and 28 ($p < .001$). During the dark phase the CR50% rats were less active compared to day 0 on day 14 ($p < .001$), 21 ($p < .001$), and 28 ($p < .001$). The CR50% rats were more active compared to the AL rats during the light phase of day 7 ($p = .006$) and the light phase of day 28 ($p = .001$). The AL rats were less active than baseline during the light phase of day 7 ($p = .007$) and day 28 ($p = .002$). The CR50% animals were more active during the light phase of day 21 ($p = .007$) and day 28 ($p = .034$) compared to day 0.

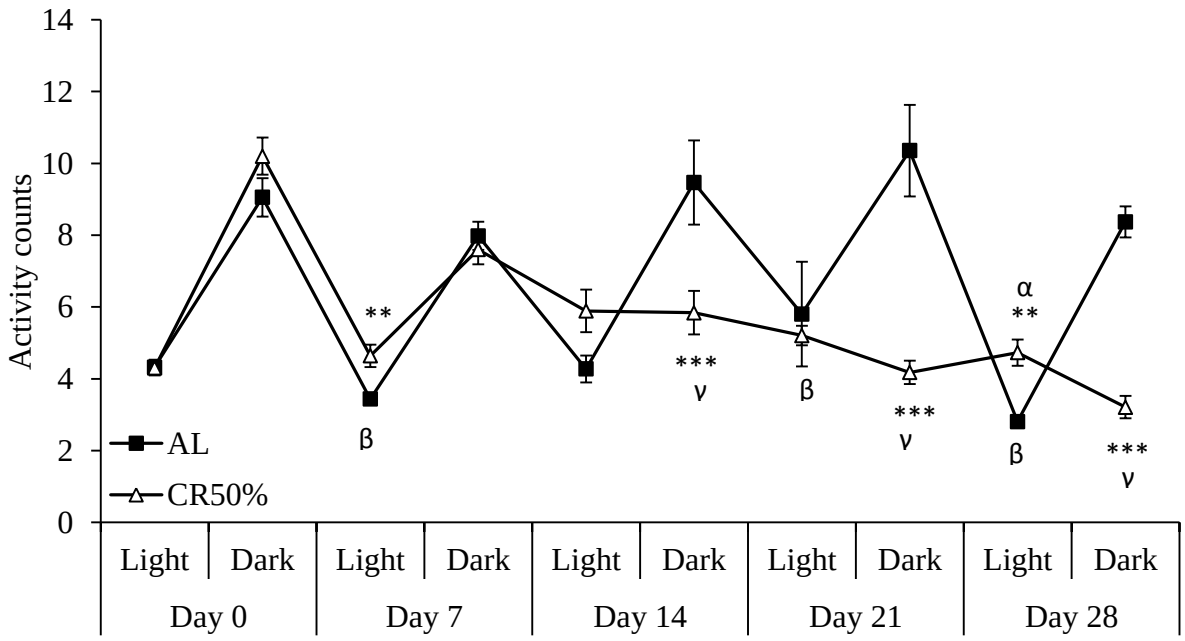

Figure 8. Mean locomotor activity counts (± SEM) for AL ($n = 14$) and CR50% ($n = 8 – 20$) rats during the light and dark phases of days 0, 7, 14, 21, and 28 of the CR period. (**) denotes a significant difference from the AL group at $p < .01$, (***) denotes a significant difference from the AL group at $p < .001$, (α) denotes a significant difference from the same phase within the same group compared to day 0 at $p < .05$, and (β) denotes a significant difference from the same phase within the same group compared to day 0 at $p < .01$, and (γ) denotes a significant difference from the same phase within the same group compared to day 0 at $p < .001$.

Locomotor activity throughout a 24 hour period on days 0, 7, 14, 21, and 28 of a 50% CR in rats

During day 0 of the CR period both the AL and CR50% groups were similar in their locomotor activity pattern over the 24 hours period (Figure 9). Similar to the T_b data, the rise in locomotor activity after 9am is due to the rats being weighed. There were a number of time points in which the AL and CR50% rats differed slightly and the ANOVA found a significant effect for hour [$F(4.63,83.33) = 23.95$, $p < .001$, partial $\eta^2 = .57$] and the interaction with group [$F(4.63,83.33) = 2.39$, $p = .049$, partial $\eta^2 = .12$]. The CR50% rats were less active compared to the AL rats at 0900 h and 0400 h, and were more active compared to the AL rats during the hours of 1200 h, 1400 h, 1800 h, 2000 h, and 2300 h (ranging from $p = .035$ to $p = .014$).

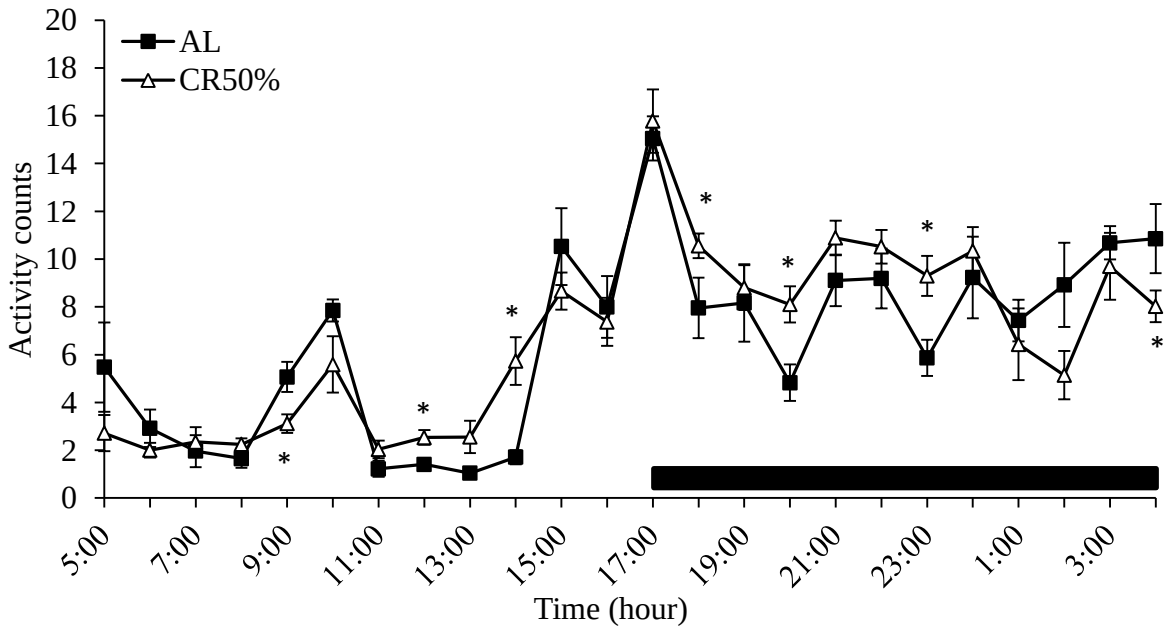

Figure 9. Mean locomotor activity counts (± SEM) for AL ($n = 6$) and CR50% ($n = 14$) rats during day 0 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$.

By day 7 of the CR period the CR50% rats demonstrated slight anticipitory behaviour to food availability, which was chacterised by the increase in locomotor activity just before the onset of the dark phase (Figure 10). The ANOVA found a significant effect for hour [$F(5.75,57.54) = 33.47$, $p < .001$, partial $\eta^2 = .77$] and group [$F(1,10) = 8.67$, $p = .015$, partial $\eta^2 = .46$]. Similar to day 0 of the CR period the CR50% rats were less active compared to the AL rats at 0900 h and were more active during the hours of 1300 h to 1500 h, 1800 h, and 2300 h (ranging from $p = .042$ to $p < .001$).

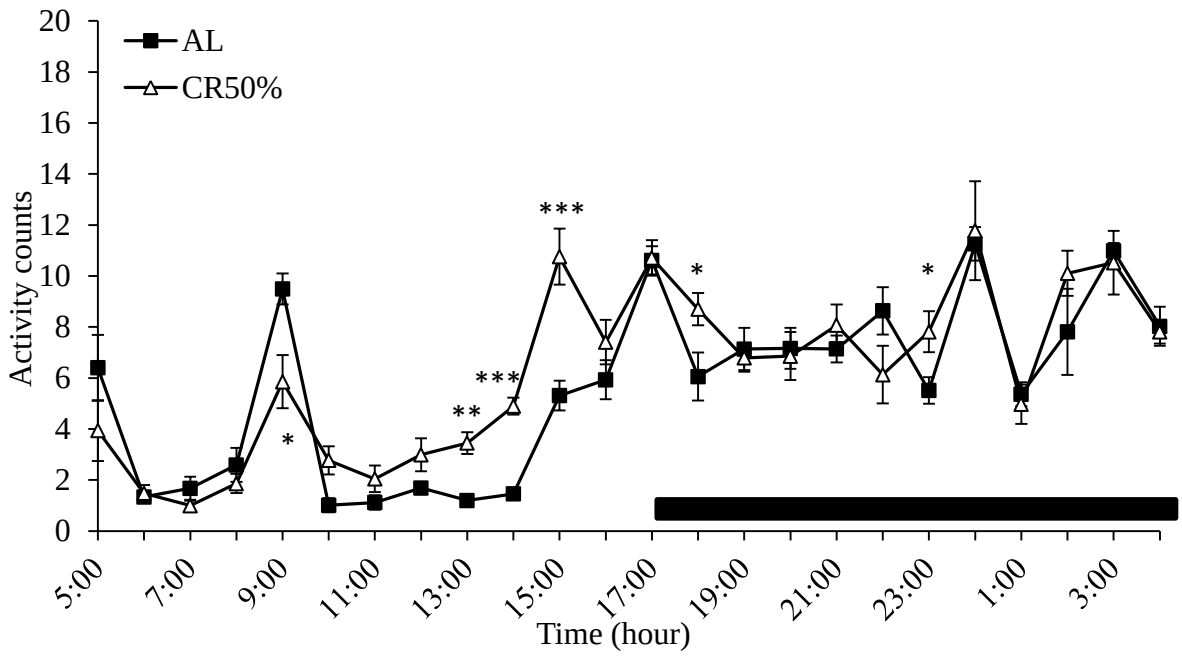

Figure 10. Mean locomotor activity counts (± SEM) for AL ($n = 6$) and CR50% ($n = 14$) rats during day 7 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .05$, and (***) denotes a significant difference from the AL group at $p < .05$.

By day 14 the CR50% rats deomstrated a slight reduction in locomotor activity during the dark phase compared to the AL rats (Figure 11). The ANOVA found a significant effect

for hour [$F(6.48,168.58) = 24.62$, $p < .001$, partial $\eta^2 = .48$] and the interaction [$F(6.48,168.58) = 8.13$, $p < .001$, partial $\eta^2 = .24$]. During day 14 of the CR period the CR50% rats were less active compared to the AL rats during the hours of 0500 h, 2000 h, 2100 h, and from 0100 h until 0400 h, and were more active during the hours of 1100 h, 1400 h, and 1600 h (ranging from $p = .022$ to $p < .001$).

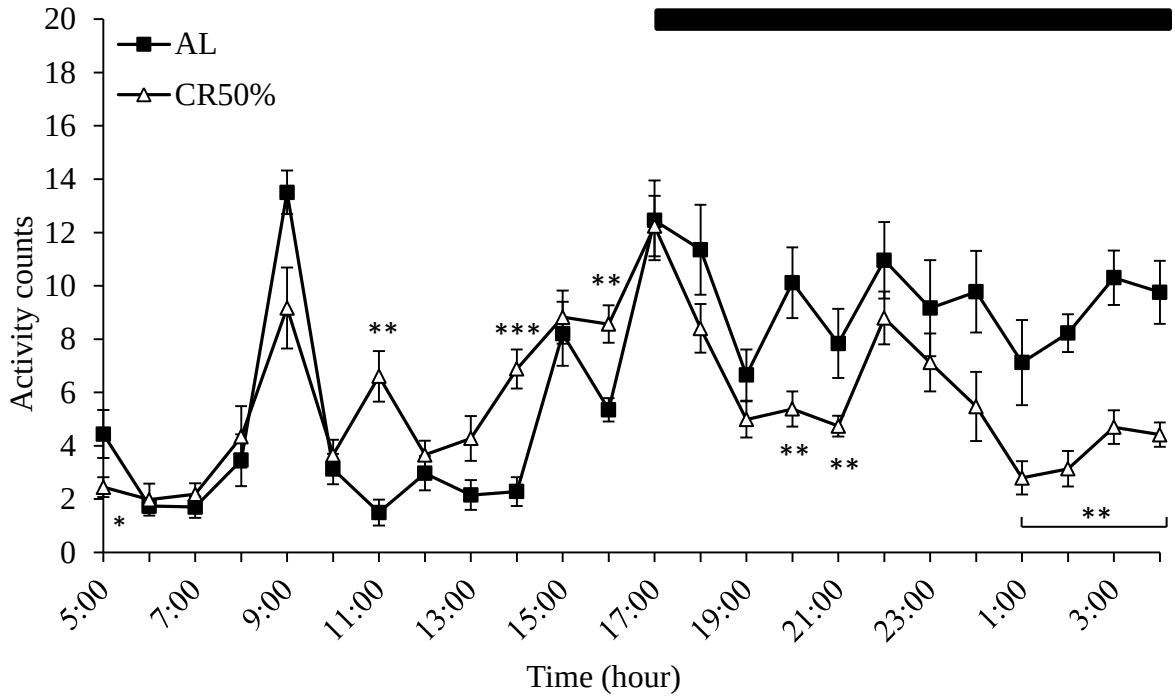

Figure 11. Mean locomotor activity counts (± SEM) for AL ($n = 6$) and CR50% ($n = 14$) rats during day 14 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .05$, and (***) denotes a significant difference from the AL group at $p < .05$.

During the dark phase of day 21 of the CR period there was a clear difference in locomotor activity between the AL and CR50% rats (Figure 12). The ANOVA found a significant effect for hour [$F(7.52,142.82) = 30.65$, $p < .001$, partial $\eta^2 = .62$], group [$F(1,19)$

= 20.02, $p < .001$, partial $\eta^2 = .51$], and their interaction [$F(7.52,142.82) = 13.18$, $p < .001$, partial $\eta^2 = .41$]. By day 21 the CR50% rats displayed a different 24 hour locomotor activity pattern to the AL rats at all hours except for between 0900 h and 1600 h and at 0200 h (ranging from $p = .030$ to $p < .001$).

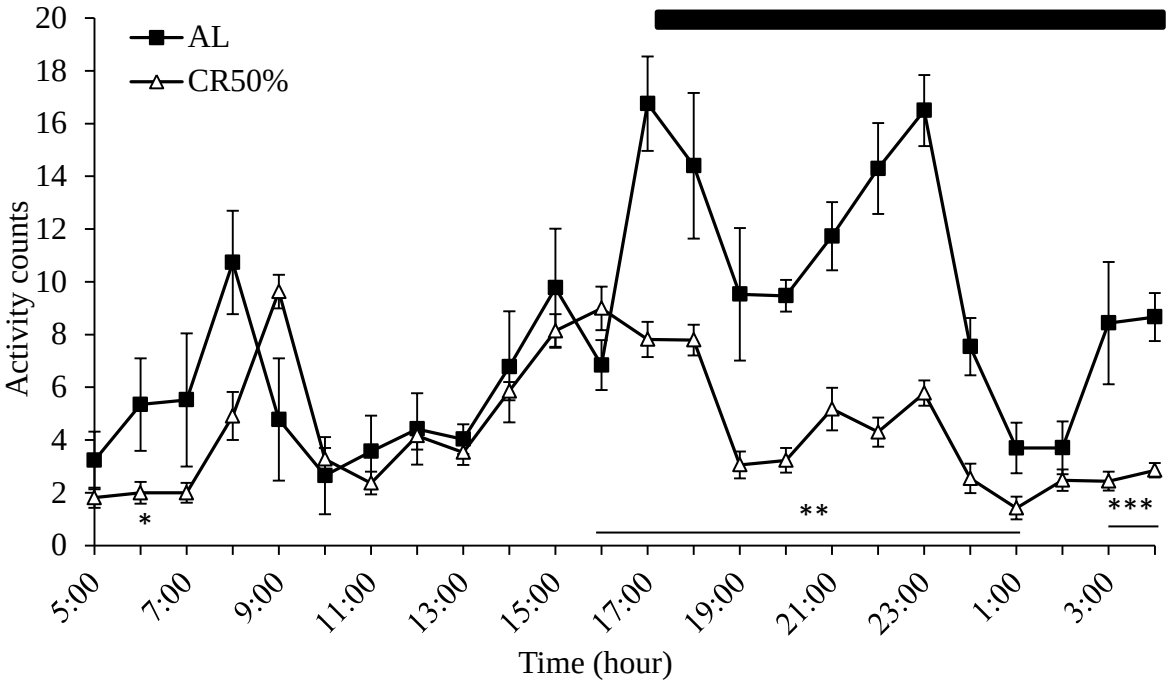

Figure 12. Mean locomotor activity counts (± SEM) for AL ($n = 5$) and CR50% ($n = 14$) rats during day 21 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .05$, and (***) denotes a significant difference from the AL group at $p < .05$.

Similar to day 21 of the CR period, during day 28 the CR50% rats were less active during the dark phase (Figure 13). The ANOVA found a significant effect for hour [$F(6.68,80.12) = 24.86$, $p < .001$, partial $\eta^2 = .67$], group [$F(1,12) = 13.60$, $p = .003$, partial $\eta^2 = .53$], and their interaction [$F(6.68,80.12) = 19.93$, $p < .001$, partial $\eta^2 = .62$]. The CR50% rats were more active during the hours of 0900 h, 1100 h, and 1300 h to 1500 h and were less

active during the hours of 0800 h, 1700 h, 1900 h, 2000 h, 2200 h, and from 2400 h until 0500 h (ranging from $p = .024$ to $p < .001$).

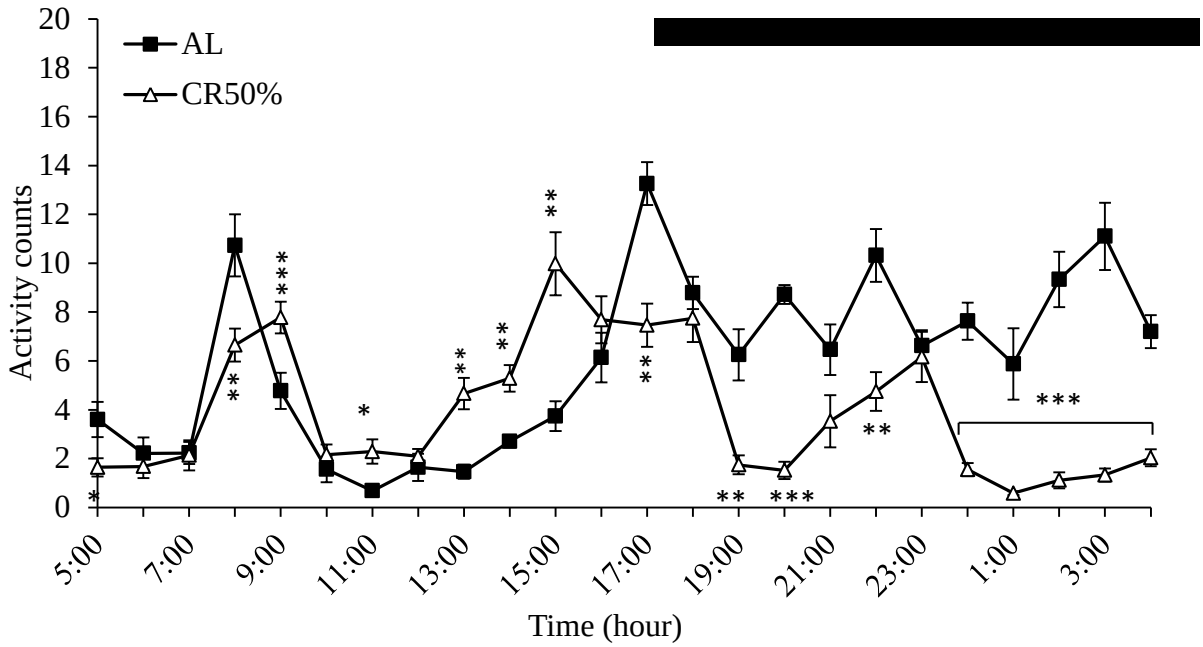

Figure 13. Mean locomotor activity counts (± SEM) for AL ($n = 6$) and CR50% ($n = 6$) rats during day 28 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .05$, and (***) denotes a significant difference from the AL group at $p < .05$.

Discussion

As expected, the CR group lost weight after the initiation of the CR regimens and the AL animals steadily put on weight. By day 14 of the CR period the weight loss of the CR rats had begun to plateau. Consistent with previous research (Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996), all of the CR groups demonstrated a reduction in core T_b following CR onset and the different periods of dietary regimens elicited differing results consistent with previous research (Hornick et al., 1998; Weithoff, 2007). Some cohorts of CR rats

demonstrated a reduction in core T_b by day 7 of the CR period; however, this finding was not consistent, with most cohorts reducing their T_b by day 14 of the CR period. During the dark phase of day 14 and day 21 they still demonstrated a lower T_b compared to the AL rats. During the light phase on day 7, 14, and 21 the CR rats did not display a different T_b compared to the AL rats. Rats CR for 28 days were slightly different again, demonstrating a significantly reduced T_b compared to the AL rats during the dark phases of days 14, 21, and 28. The only time in which the CR50% rats demonstrated a reduced T_b during the light phase was on day 14.

Further characterisation of the change in T_b profile during CR was seen in the figures representing a 24 hour period on days 0, 7, 14, 21, and 28 of the CR period. At the beginning of CR, on day 0, the AL and CR50% rats demonstrate an almost identical T_b profile. By day 7 the CR animals demonstrate a slight increase in T_b at the time in which they were fed, just before the onset of the dark phase. More notable differences between the AL and CR50% rats can be seen by day 14 of the CR period. The CR rats demonstrate a significantly reduced T_b during most of the dark phase and also a portion of the light phase immediately after the end of the dark phase. A similar pattern is observed on day 21 and 28 of the CR period, with the CR50% rats on day 28 displaying the lowest T_b during the dark phase of all the other days during the CR period. Mammals use significant amounts of energy to maintain a relatively high and constant core T_b; therefore, it would be expected that reducing energy consumption (such as via CR) would result in a lowered T_b and reduced activity as the organism lowers its metabolic rate to conserve energy (Blanc et al., 2003; Duffy et al., 1989; Gonzales-Pacheco, Buss, Koehler, Woodside, & Alpert, 1993; Heilbronn et al., 2006; Lane et al., 1996).

By the dark phase of day 14 the CR rats had begun to alter their locomotor activity. Namely, the pattern of locomotor activity of the CR rats flattens out by increasing slightly during the light phase and reducing during the dark phase. By day 21 and 28 of the CR period

the CR rats significantly reduce their activity during the dark phase compared to the AL rats. In regards to the representation of locomotor activity during the 24 hour period on each day of the CR period it could be seen that on days 0 and 7 the CR and AL rats were quite similar over the 24 hours. On day 14 the CR rats began to demonstrate a reduction in their dark phase activity during the hours between 0100 h and 0500 h. This is increased on day 21 by the CR rats becoming less active compared to the AL rats during the majority of the dark phase; however, demonstrating a similar pattern of activity during the majority of the light phase. During day 28 of the CR period the CR rats were less active compared to the AL rats for most of the dark phase and were slightly more active compared to the AL rats for most of the light phase. Total locomotor activity was similar between the groups on days 0, 7, and 14; however, the CR groups demonstrated less total locomotor activity on days 21 and 28 of the CR period.

Locomotor activity has been shown to either increase (Duffy et al., 1989) or remain the same after a period of CR (Boyle et al., 1981; Lane et al., 1996); however, the CR rats in the current study did not support this. This may be due to a multitude of methodological differences, such as the species of animal and the level and duration of CR period. In the CR rats there was a marked increase in locomotor activity surrounding the time of day in which the animals were presented with their daily allotment of food. These findings are consistent with previous findings that CR animals demonstrated anticipatory behaviour before feeding time (Duffy et al., 1989; Lane et al., 1996; Mistleberger, 2009; Weed et al., 1997). Although we demonstrated slightly reduced locomotor activity in rats after 21 days of CR others have shown that locomotor activity can actually increase (Chen et al., 2005; Duffy et al., 1989). For instance, mice that were CR by 40% for a period of 9 months, which is a dramatically different time frame than in the current study, demonstrated increased activity. Further, in addition to general movement within their home cage, they also took measures of when the

mice displayed jumping, hanging in a vertical position, and hanging in a cuddle position behaviours to calculate overall activity (Chen et al., 2005). These methodological discrepancies between this study and our own study may account for the differences seen.

It has been suggested that there is an adaptation phase and steady state phase during CR (Koubova & Guarente, 2003). The adaptation phase is said to represent the body reorganising itself after being exposed to a reduction in food intake; whilst the steady state phase indicates that the body has adapted to its lower energy input and can remain in this state for the remainder of the life span (Koubova & Guarente, 2003). One possibility is that the CR animals in this study are still in the adaptation phase, and thus T_b, locomotor activity, and presumably metabolism were reduced. Whereas the animals mentioned above with higher locomotor activity may have entered the steady state phase, having been CR for a longer period (Chen et al., 2005).

Interestingly, the variables measured in the current study were shown to be altered by the CR regimen at different time points. Namely, the CR animals continued to lose weight until day 14 of the CR regimen; however, between day 14 and 28 begun to stabilise and even put a small although non-significant amount of weight back on. In terms of the animals T_b the AL and CR rats demonstrated similar light and dark phase T_b profiles until day 14 (except for one cohort that demonstrated reductions by day 7), during which the CR rats demonstrated a reduced dark phase T_b compared to the AL animals and day 0, which continued for the remainder of the CR period. Similar to T_b the CR rats' locomotor activity began to demonstrate a reduction during the dark phase of day 14, which again remained for the rest of the CR period. However, the CR rats' locomotor activity during the light phase did not begin to increase until day 28 of the CR period. The T_b and locomotor activity of the CR animals appear to be very closely linked, as evidenced by their similar profile of changes during the CR period. It is also possible that this reduced T_b and locomotor activity which begins on day

14 may lead to reduced body weight loss a week or so later due to the CR animals becoming less active and conserving energy with a lower T_b.

A reduction in oxidative stress has been postulated to be one of the mechanisms by which CR extends the lifespan (Barja, 2004; Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; Shigenaga, Hagen, & Ames, 1994). Oxidants are produced as a by-product of oxygen consumption and energy expenditure (Shigenaga et al., 1994); therefore, it is thought that lower oxygen consumption would lead to lower oxidative damage. For example, male rats CR to 47% for 12 months demonstrated a significant reduction in oxidative damage to mitochondrial DNA (Lopez-Torres, Gredilla, Sanz, & Barja, 2002); however, this was not due to a decrease in mitochondrial oxygen consumption (which would be a consequence of lower locomotor activity and energy expenditure), but was due to the mitochondria releasing less reactive oxygen species (Lopez-Torres et al., 2002). In agreement with this other investigators have found that CR animals continue to maintain lower oxygen consumption rates even though their locomotor activity is not different from control animals (Boyle et al., 1981). It has been suggested that evolutionarily a reduction in oxygen consumption and the heat increment (expenditure of energy whilst consuming food) that follows ingestion of food during a period of reduced food availability means the animal can limit weight loss and conserve energy (Boyle et al., 1981).

CR has consistently been shown to reduce the occurrence and/or delay the onset of a number of diseases in animal models such as autoimmune diseases, cardiovascular diseases, and insulin sensitivity (Escriva et al., 2007; Kubo, Gajjar, Johnson, & Good, 1992; Lane, Ingram, & Roth, 1999). CR has also been demonstrated to impact upon animal models of Parkinson’s Disease (Bruce-Keller, Umberger, McFall, & Mattson, 1999; Duan & Mattson, 1999; Halagappa et al., 2007; Maswood et al., 2004) and Alzheimer’s Disease (Wang et al., 2005; Zhu, Guo, & Mattson, 1999) by reducing neuronal loss associated with these diseases.

The mechanism by how CR impacts upon the above mentioned diseases is not fully understood; however, one theory is that CR has been shown to attenuate the aging process. It has been postulated that organisms age due to the production of reactive oxygen species via respiration (Harman, 1988), creating oxidative damage to DNA, RNA, proteins and lipids (Liu et al., 2002; Stadtman, 2006; Yakes & Van Houten, 1997). Therefore, with CR demonstrated to reduce oxidative stress (Chung, Kim, Kim, Choi, & Yu, 2002) it has been considered that this may be the means by which CR extends the life span. However, it has been established that after a period of CR the metabolism of some mammals does not necessarily slow down as would be expected (Duffy et al., 1989; McCarter, Masoro, & Yu, 1985), and it has been shown that a reduction in oxidative damage was not due to a decrease in mitochondrial oxygen consumption (or respiration), but was due to the mitochondria releasing less reactive oxygen species (Lopez-Torres et al., 2002). Therefore, there is still some uncertainty around the processes that change as a result of a period of CR, and how these changes may impact upon extension of life span and age-related diseases.

Interestingly, the thermogenic ability of the CR rats was intact, as evidenced by the locomotor activity induced increase in T_b prior to being fed during the CR period. On day 28 of the CR period the CR50% rats demonstrated roughly a 1 °C rise in T_b in the two hours prior to the onset of the dark phase and food delivery. The rats in the current study reduced their core T_b to approximately 36.5 °C during the light phase on day 28. Others have shown that rats consistently reduce their core T_b to between 35.5 and 36.2 °C after CR (Duffy et al., 1989; Severinsen & Munch, 1999). However, the core T_b of the animal may depend heavily upon the strain of rat, severity and duration of CR regimen, and the ambient temperature the rats are housed in.

Conclusion

It was found that CR results in a dose-dependent reduction in body weight, T_b, and an alteration in the 24 hour pattern of locomotor activity depending on the duration of the CR period. Rats CR to 50% for 28 days demonstrated the largest changes in the abovementioned variables, with the rats CR for 14 and 21 days demonstrating dose-dependent alterations in body weight, T_b, and locomotor activity. These findings support previous research in regards to the impact CR has on lowering core T_b.

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CHAPTER 3

DOSE-DEPENDENT EFFECT OF CALORIE RESTRICTION SEVERITY IN MICE ON BODY WEIGHT, CORE BODY TEMPERATURE, AND LOCOMOTOR ACTIVITY

Abstract

This study aimed to characterise the effect two different severities of CR on body weight, core body temperature (T_b), and locomotor activity. Male C57BL/6 mice were CR to 25% (CR25%) or 50% (CR50%) of AL animals' food intake for 28. Mice CR to 50% of the AL mice's food intake for 28 days demonstrated the largest weight loss and reduction in T_b and the most pronounced alteration in locomotor activity. There was a dose-dependent response of core T_b with the CR50% mice demonstrating the largest decline in light and dark phase T_b. The CR25% mice demonstrated an intermediate response, only consistently reducing their dark phase T_b compared to controls. The CR mice did not demonstrate an overall change in locomotor activity; however, both CR groups demonstrated an increase in light phase activity and the CR50% mice demonstrated a reduction in dark phase activity on day 28 of the CR period. Both CR groups demonstrated anticipatory increases in their T_b in the two hours preceding food delivery, which was also reflected in an increase in locomotor activity during the same period on day 28. These results support previous findings in which CR has been demonstrated to reduce T_b. The larger magnitude of CR (50%) elicited the most dramatic changes in core body weight, T_b, and locomotor activity. The findings of this work will be informative for subsequent investigations of CR in mice.

Calorie restriction (CR) has been consistently shown to increase the mean and maximum lifespan (Lin, Ford, Haigis, Liszt, & Guarente, 2004; Weindruch, Walford, Fligiel, & Guthrie, 1986). Further, CR has been shown to reduce metabolic rate and consequently core body temperature (T_b) (Blum et al., 1985; Forsum, Hillman, & Nesheim, 1981; Hill, Sparling, Shields, & Heller, 1987). Different severities of CR have been shown to elicit differing results. For example, rectal temperature in rhesus monkeys has been shown to be reduced by 0.5 °C when they are exposed to a 30% CR (Lane et al., 1996). However, the animals CR to 10% and 20% demonstrated no difference compared to controls.

Rats CR to 90, 80, or 60% of the control rats' food intake for 25 days demonstrated a dose-dependent decrease in body weight, with the 90% CR rats losing the least weight and the 60% CR rats losing the most (Zhu, Haegele, & Thompson, 1997). However, the comparison between and AL and CR90% diet may not elicit differing results and needs to be considered with caution. These authors also demonstrated that there was a dose-dependent effect of CR severity on latency to first mammary tumor and a dose-dependent reduction in cancer incidence (Zhu et al., 1997). This was evidence by the largest CR (60% of controls food intake) eliciting the longest latency to first tumor and lowest cancer incidence. Finally, this study also indicated that there was a dose-dependent increase in corticosteroid, with the rats CR to 60% demonstrating the largest increase which was proportional to the inhibition of tumor occurrence (Zhu et al., 1997).

In a similar study to the above investigation it was demonstrated that rats CR to 90%, 80%, or 70% of control rats' food intake also demonstrated a dose-dependent decrease in colon cancer incidence (Kumar, Roy, Tokumo, & Reddy, 1990). Evidence from *Drosophila* has demonstrated that there are optimum magnitudes of CR that increase the life span (Magwere, Chapman, & Partridge, 2004). Although not in mammals this study demonstrated that male and female flies required different magnitudes of CR in order to maximise their

increase in life span. The male flies' life span peaked when they were exposed to a 60% reduction in food intake compared to controls, whereas the female flies' life span peaked at a 40% reduction (Magwere et al., 2004). A suggestion was made that the females had higher nutrient demands due to fertility and reproduction. Interestingly, at these optimised CR magnitudes the female flies lived 60% longer than fully fed or restricted female flies; however, the males only lived 30% longer than their fully fed or restricted counterparts. The discrepancy in this extension of life by CR was suggested by the authors to occur due to sex differences in insulin-like growth factor-1 signalling, which is responsible for growth and development in *Drosophila* (Garofalo, 2002).

Within our own research group we have demonstrated that different magnitudes of CR lead to different serum concentrations of corticosterone (CORT) after three weeks of CR (Levay, Tammer, Penman, Paolini, & Kent, 2010). Rats were CR to 12.5%, 25%, 37.5%, and 50% of the control rats food intake, and although all of the CR regimens led to an increase in CORT, it was the 37.5% and 50% CR groups that demonstrated that largest increases. Serum testosterone was also reduced in a dose-dependent manner, with the 37.5% and 50% CR groups demonstrating the largest decline (Levay et al., 2010).

The above examples highlight the importance of optimising CR magnitudes depending on the area of research in question. Further, these examples also emphasise that in the process of determining these optimal CR magnitudes a multitude of variables need to be taken into consideration, such as species, strain, and sex. The aim of the current study was to characterise changes in body weight, core T_b, and locomotor activity in mice after CR. A further aim was to characterise the dose-dependent effect of CR on the abovementioned variables in terms of the severity of the CR period. It is expected that CR will lead to a reduction in body weight and T_b, and lead to a change in the 24 hour pattern of locomotor

activity. Further, it is hypothesised that the largest magnitude of CR (50%) will produce the largest changes in these variables.

Methods

Animals

Forty-four male C57BL/6 mice were procured from Monash SPF animal services (Clayton, Vic, Australia) and allowed to acclimate to the facility for at least one week. During this period, standard rodent chow (Barastoc, Melbourne, Australia) and water were available ad libitum. At the beginning of experimentation the mice were aged between 9 and 16 weeks old. Mice (38 × 27 × 15 cm) were individually housed in polypropylene basin cages with sawdust and tissues provided as bedding. Mice were maintained at an ambient temperature of 30 ± 1 °C (Gordon, 1985); which is within the thermoneutral zone for this species. Mice were maintained on a 12:12 light/dark cycle (0500 – 1700 hours). Animal care and experimentation was performed in accordance with protocols approved by the La Trobe University Animal Ethics Committee.

Surgery

Following acclimation, 27 of the mice were surgically implanted in the peritoneal cavity with a biotelemetry device (E-4000, Mini-mitter®, Bend, OR, USA: 23 × 8 mm, 1.6 g; or G 2 E-mitter, Mini-mitter®, Bend, OR, USA: 15.5 mm × 6.5 mm, 1.1 g) under anaesthesia (ketamine 61 mg/kg and xylazine 9 mg/kg). These mice were allowed one to two weeks to recover before the initiation of the CR regimens (more detail in *Chapter 2*).

Dietary regimens

Mice were divided into one of three CR regimens again matched for weight, food

intake, and age: ad lib (AL; *n* = 14) fed ad libitum (average 2.6 g per day); CR25% (*n* = 10) mice received 75% of the amount consumed by AL mice (average 1.8 g per day); and CR50% (*n* = 8) mice received 50% of the amount consumed by AL mice (average 1.3 g per day). All three groups of mice remained on the dietary regimens for 28 days. The recommended daily allowance (*Institute for Laboratory Animal Research. Nutrient requirements of laboratory animals*, 1995) of the diets given to the AL, CR25% and CR50% rats and mice are listed in Table 1. The only elements of the CR animals' diet that was noted to be insufficient was fat, and vitamin B-12 and also vitamin B-6 in the CR50% only. The intake of the CR groups was determined weekly based on the average daily food intake of the AL groups of each species for three consecutive days. Water was continuously provided to all groups. The dietary manipulation continued for 28 days. Food was provided daily, approximately one hour before the dark phase onset. The group sizes varied across dependent variables and precise numbers used for each variable are stated in the results section.

Table 1. *The dietary composition of each feeding regimen and recommended dietary allowance (RDA) for adult male mice*

		Diet			
	Unit	RDA	AL	CR25%	CR50%
Protein	g/kg	50	203	152	102
Fat	g/kg	50	31.1	23.3	15.6
Starch	g/kg	-	382	287	191
Calcium	g/kg	5	10	7.5	5
Phosphorous	g/kg	3	6.5	4.9	3.3
Sodium	g/kg	0.5	2.8	2.1	1.4
Chloride	g/kg	0.5	4.8	3.6	2.4
Potassium	g/kg	2	7.8	5.9	3.9
Lysine	g/kg	4	10.7	8	5.4
Magnesium	g/kg	0.5	1.9	1.4	1
Iron	mg/kg	35	198	149	99
Manganese	mg/kg	10	134	100	67
Zinc	mg/kg	10	198	149	99
Copper	mg/kg	6	18	13.5	9
Iodine	µg/kg	150	1460	1095	730

Selenium	µg/kg	150	577	432	288
Vitamin A	mg/kg	0.72	12.5 IU/g	9.4 IU/g	6.3 IU/g
Vitamin D	mg/kg	0.025	2.8 IU/g	2.1 IU/g	1.4 IU/g
Vitamin E	mg/kg	22	75.5	56.6	37.8
Niacin	mg/kg	15	154.4	115.8	77.2
Vitamin B-6	mg/kg	8	12.7	9.5	6.4
Riboflavin	mg/kg	7	16	12	8
Thiamine	mg/kg	5	19.9	15	10
Vitamin K	mg/kg	1	11.3	8.5	5.7
Folic Acid	mg/kg	0.5	3.4	2.6	1.7
Biotin	mg/kg	0.2	0.5	0.4	0.3
Vitamin B-12	µg/kg	0.05	0.05	0.04	0.03

Determination of body weight, core T_b, and locomotor activity during CR

Animals were weighed once a week approximately three hours after lights-on during dietary manipulation using top loading scales (± 0.1 g). T_b and locomotor activity were determined by placing each animal, housed singly, on a receiver (ER-4000, Mini-mitter®, Bend, OR, USA: 56 cm × 29 cm × 7 cm). Each individual biotelemetry device generating a continuous frequency signal proportional to the animal's T_b (± 10^{-1} °C), as described previously (Weiland, Voudouris, & Kent, 2004). The receiver sampled this frequency at 1-minute intervals and this sample was decoded by VitalView software (Mini Mitter®, Bend, OR, USA) and stored on a hard drive. The receiver for each cage was equipped with a matrix of antennas that were continuously signalled by the biotelemetry device. The receiver scanned the matrix in a sequential order to locate the position and orientation of the biotelemetry device, thus making it possible to detect the global activity of the animal. The receiver tallied the number of matrices crossed by the animal during 1-minute intervals and this information was recorded using VitalView software.

Data Analysis

Mean ± SEM body weight of each group prior to the onset of CR and weekly throughout the CR period were analysed using a two-way mixed design analysis of variance

(ANOVA). Data for T_b and locomotor activity in mice was collapsed into hourly means for day 28 and analysed by a two-way mixed ANOVA. T_b and locomotor activity hourly means were then collapsed into 12-hour mean intervals for the light and dark phases for each day during the CR period and into a 24 hour mean for each day during the CR period. A two-way mixed design ANOVA compared groups during both the light and dark phases of each day and for the 24 hours period on each day during the CR period. To overcome violations of sphericity, the Greenhouse-Geisser statistic corrected degrees of freedom were reported. Where appropriate, post hoc pairwise comparisons were performed using the Least Significant Difference (LSD) method. Where required, between-group simple main effects analyses were performed using LSD method. When several comparisons were required, as in the case of T_b and locomotor activity data, a Bonferroni adjustment was used to protect for inflated type 1 error rate. Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$ as the criterion for significance.

Results

Effect of a 25% or 50% CR on body weight in mice

The CR25% (starting weight of 27.9 g ± 0.5) and CR50% (starting weight of 28.1 g ± 0.3) groups lost weight, whereas the AL (starting weight of 27.8 g ± 0.3) group demonstrated a slight weight gain (Table 2). Although both CR groups lost weight steadily at the onset of CR, the CR50% group exhibited a more pronounced weight loss compared to the CR25% group during the final 14 days of the CR period. The ANOVA found a significant main effect for week [$F(2.03,83.13) = 78.75$, $p < .001$, partial $\eta^2 = .66$], group [$F(2,41) = 66.99$, $p < .001$, partial $\eta^2 = .77$], and their interaction [$F(2.03,83.13) = 59.04$, $p < .001$, partial $\eta^2 = .74$]. Post-hoc comparisons revealed that upon allocation to dietary groups, there were no significant differences between groups. From week 1 onwards, the AL group was significantly heavier

compared to the CR25% and CR50% groups ($p < .001$ for both CR groups on all days). The CR25% and CR50% groups only differed in their percentage weight loss on day 21 of the CR period ($p = .042$). The AL mice put on a significant amount of weight compared to day 0 of the CR period ($p < .001$ for all) and the CR25% (day 7 $p = .004$, day 14, 21, and 28 $p < .001$) and CR50% ($p < .001$ for all) mice lost a significant amount of weight compared to day 0 of the CR period.

Table 2. *Mean (± SEM) percentage body weight change from baseline for AL (n = 19), CR25% (n = 10), and CR50% (n = 15) mice weekly throughout the CR period*

	Day 0	Day 7	Day 14	Day 21	Day 28
AL	0	2.0 (0.5) γ	2.9 (0.5) γ	3.4 (0.6) γ	5.2 (0.9) γ
CR25%	0	-6.1 (1.6) ***β	-10.4 (1.9) ***γ	-11.2 (2.4) ***γ	-11.9 (2.2) ***γ
CR50%	0	-5.4 (1.1) ***γ	-11.8 (1.2) ***γ	-14.3 (1.1) ***γ	-15.4 (1.5) ***γ

Note. (***) denotes a significant difference from the AL group at $p < .001$, (β) denotes a significant difference from day 0 at $p < .01$, and (γ) denotes a significant difference from day 0 at $p < .001$.

Changes in light and dark phase T_b after a 28 day 25% or 50% CR in mice

After the onset of CR, the CR25% group demonstrated an intermediate reduction in T_b and the CR50% group demonstrated the largest reduction in T_b during both the light and dark phases (Figure 1). By day 21 of CR, the reduction in basal T_b for both groups appeared to plateau. The ANOVA found a significant main effect for time [$F(3,71.66) = 117.49$, $p < .001$, partial $\eta^2 = .83$], group [$F(2,24) = 43.94$, $p < .001$, partial $\eta^2 = .79$], and their interaction [$F(6,71.66) = 10.03$, $p < .001$, partial $\eta^2 = .46$]. Post-hoc comparisons revealed no significant differences on day 0, but significant treatment effects on all other days during both the light

and dark phases. Within 7 days the T_b of the CR50% mice had reduced during the light phase and they remained below the AL mice for the duration of the CR period (p = .007 for day 7 and p < .001 for days 14, 21, and 28). It took until day 14 of the CR period for the CR25% mice to demonstrate a significant reduction T_b during the light phase compared to the AL mice (p = .040), and they remained slightly below the AL mice for the remainder of the CR period (p = .051 for day 21 and p =.049 for day 28). It was not until day 21 of the CR period when the CR25% and CR50% mice started to differ from each other during the light phase. The CR50% mice demonstrated a reduced T_b compared to the CR25% mice on day 21 and 28 (p = .005 and p = .018, respectively).

The dark phase elicited different results and by day 7 of the CR period the CR25% mice (p = .001) and CR50% mice (p < .001) demonstrated significantly reduced core T_b compared to the AL mice. The CR50% group demonstrated a lower T_b compared to the CR25% group also during the dark phase of day 7 (p = .023). During the dark phase of day 14, 21 and 28 AL mice were significantly different from CR25% and CR50% groups (both p < .001 for all 3 days) and the CR groups significantly differed from each other (p = .013, p < .001, and p < .001, respectively).

The AL animals T_b did not differ at all during the CR period compared to day 0; however, the CR25% and CR50% animals did (for simplicity these differences were not indicated on the figure). The CR25% animals demonstrated a reduced light (day 7 p = .010, day 14 p = .006, day 21 p = .002, and day 28 p = .005) and dark phase (day 7 p = .003 and day 14, 21, and 28 p < .001) T_b on each day of the CR period compared to day 0. Similarly, the CR50% animals demonstrated a reduced T_b compared to day 0 for the light (day 7 p = .005 and day 14, 21, and 28 p < .001) and dark phase (p < .001 for all) of each day.

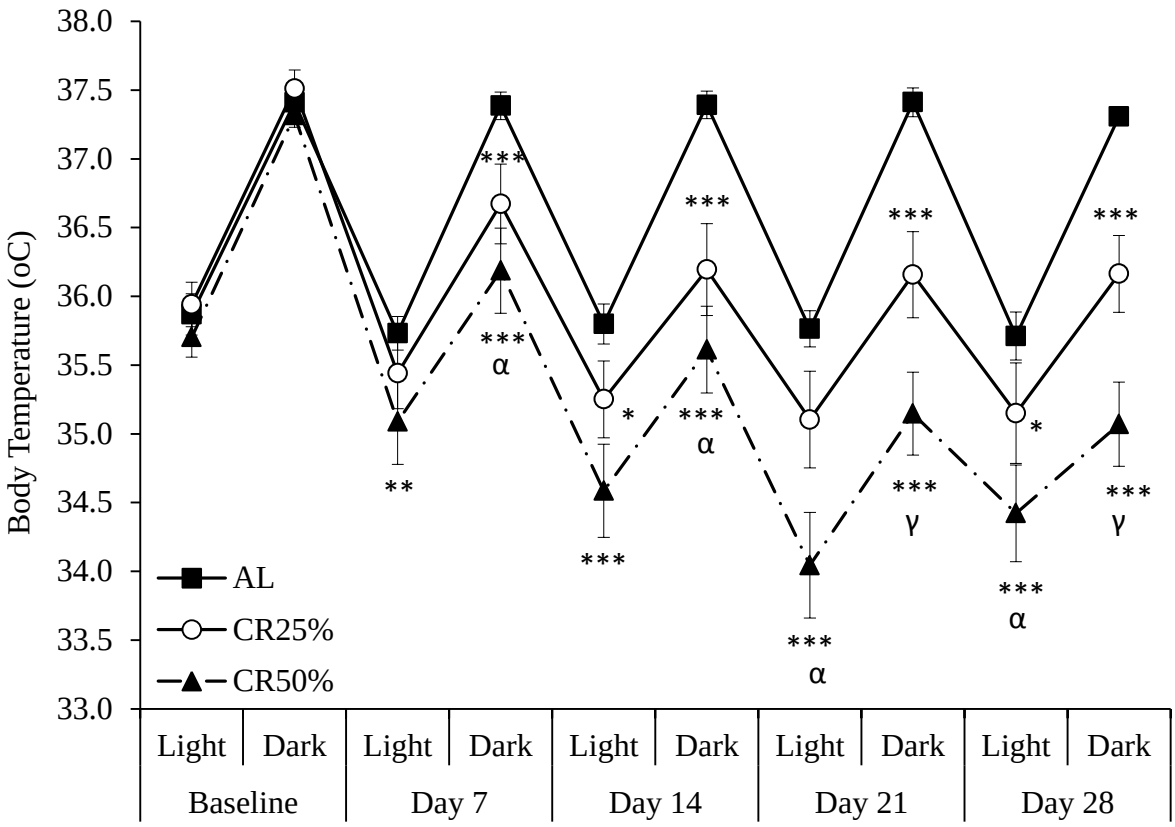

Figure 1. Mean T_b (± SEM) of AL (n = 10), CR25% (n = 9), and CR50% (n = 8) mice during the light and dark phases of day 0, 7, 14, 21, and 28 of the CR period – also presented in Figure 2, Chapter 4. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .01$, (***) denotes a significant difference from the AL group at $p < .001$, (α) denotes a significant difference between the CR50% group and the CR25% group at $p < .05$, (γ) denotes a significant difference between the CR50% group and the CR25% group at $p < .001$.

Changes hourly T_b after a 28 day 25% or 50% CR in mice

During the 28th day of the CR period it was evident that both of the CR groups displayed a different pattern of T_b over the 24 hour period (Figure 2). All three groups begin the 24 hour period at a different T_b in a dose-dependent manner, with the CR50% displaying the lowest T_b. Both CR group's T_b increases at feeding time (1600 h) and steadily decreases

during the dark phase, whilst the AL animals T_b remains at an elevated level throughout the dark phase. The ANOVA found a significant effect for hour [$F(4.27,102.54) = 67.29$, $p < .001$, partial $\eta^2 = .74$], group [$F(2,24) = 52.30$, $p < .001$, partial $\eta^2 = .81$], and their interaction [$F(8.55,102.54) = 12.11$, $p < .001$, partial $\eta^2 = .50$]. There were significant differences between the groups at all time points except for the hours of 1300 h and 1400 h and a full list of all the significant differences can be seen in Appendix A.

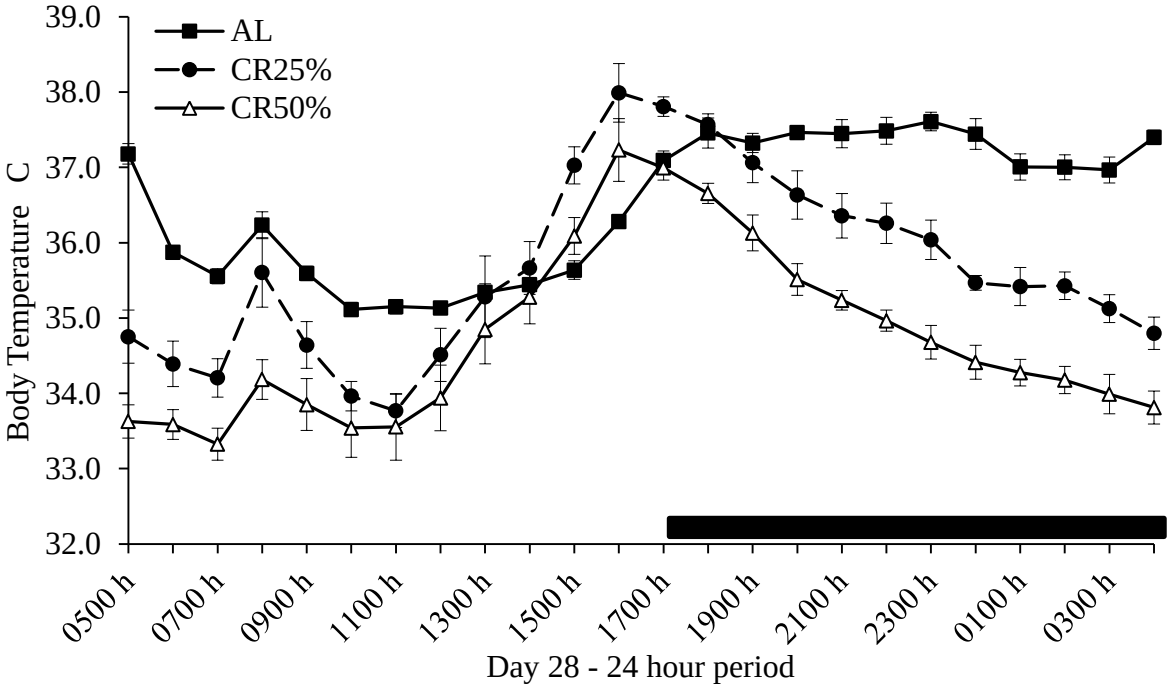

Figure 2. Mean T_b (±SEM) for AL (n = 10), CR25% (n = 9), and CR50% (n = 8) mice in hourly blocks on day 28 of the CR period, with the black box representing the dark phase.

Changes in locomotor activity after a 25% or 50% CR in mice

Over the CR period all of the treatment groups exhibit similar total locomotor activity levels on each day of the CR period (Figure 3). There were no significant differences between all groups over the CR period.

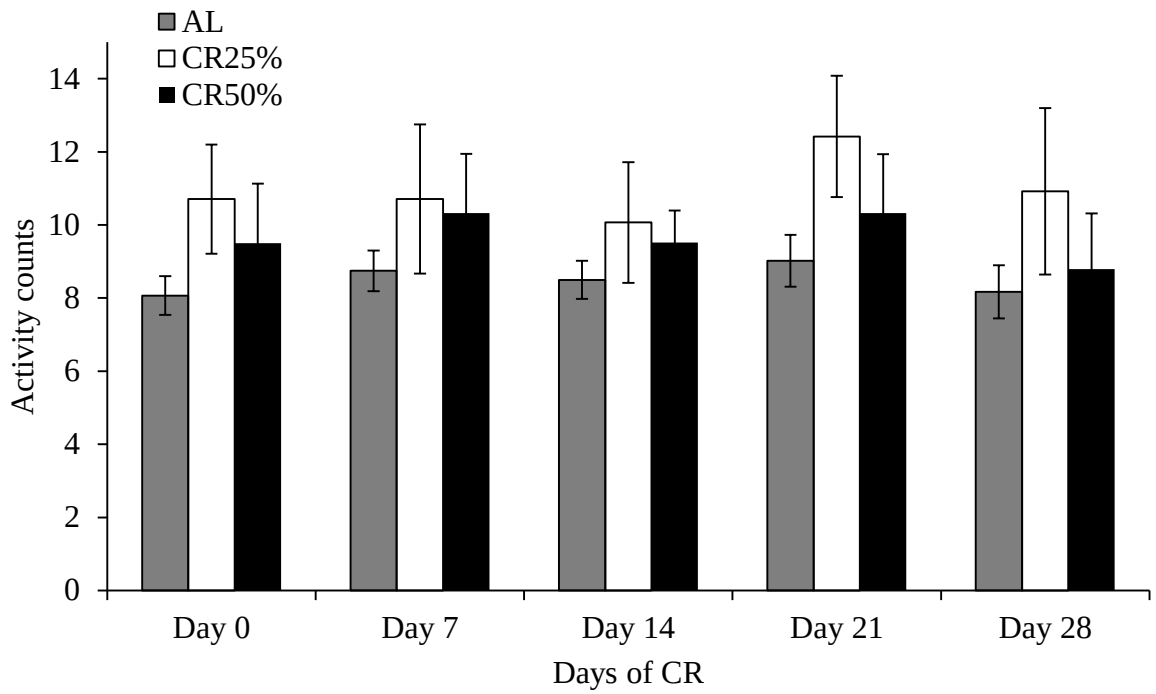

Figure 3. Mean total locomotor activity counts (± SEM) for AL (n = 10), CR25% (n = 9), and CR50% (n = 8) mice during day 0, 7, 14, 21, and 28 of the CR period.

On day 0, 7, and 14 of the CR period all mice demonstrated similar locomotor activity; however by day 21 of CR both the CR25% and CR50% groups demonstrated clear changes in their locomotor activity (Figure 4). Namely, both groups demonstrated an increase in their light phase locomotor activity and a reduction in their dark phase locomotor activity. The ANOVA found a significant main effect for time [$F(2.8, 63.9) = 15.38$, $p < .001$, partial $\eta^2 = .39$] and the interaction with group [$F(5.6, 63.9) = 2.86$, $p = .035$, partial $\eta^2 = .17$]. Post-hoc comparisons revealed no group differences on day 0 or day 7, but significant treatment effects on days 14, 21, and 28 during the light phase and on day 28 during the dark phase. During the light phase of day 14, 21, and 28 there was a significant difference between AL mice and CR25% mice ($p = .027$, $p < .001$, and $p = .024$ respectively), and during the light phase of day 21 and 28 there was a significant difference between the AL and CR50% mice ($p = .004$ and $p < .001$ respectively).

The only time in which the AL mice differed from their day 0 activity levels was during the dark phase of day 7 (p = .040) when they were more active. The CR25% mice demonstrated similar light and dark phase activity compared to day 0 except for when they were more active during the light phase of day 21 (p = .025). The CR50% mice differed from their day 0 activity levels when they were more active during the light phase on day 21 (p = .024) and less active during the dark phase of day 28 (p = .032).

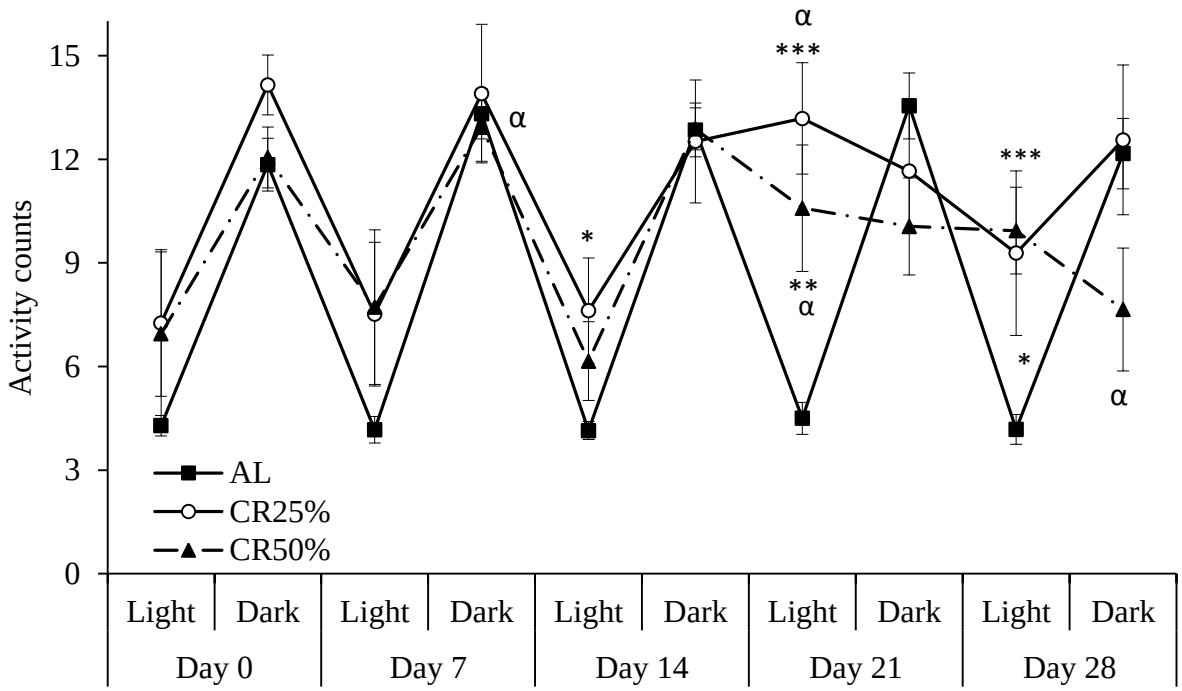

Figure 4. Mean locomotor activity counts (± SEM) of AL (n = 10), CR25% (n = 9), and CR50% (n = 8) mice during the light and dark phases of day 0, 7, 14, 21, and 28 of the CR period. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .01$, (***) denotes a significant difference from the AL group at $p < .001$, and (α) denotes a significant difference from day 0 during the same phase within the same group at $p < .05$.

As can be seen in Figure 5, the locomotor activity of the CR50% mice changes from being the most active during the dark phase (as the AL mice are), to being the most active during the light phase on day 28 of the CR period. This is due to a large amount of locomotor activity that occurs before CR animals are fed. The CR25% mice demonstrate the same pattern of food-related increase in locomotor activity before the onset of the dark phase, and then are almost as active as the AL mice for part of the dark phase. The CR25% mice then drop to the CR50% animals' activity levels at 2400 h for the remainder of the dark phase. The ANOVA found a significant effect for hour [$F(3.68,88.19) = 9.50$, $p < .001$, partial $\eta^2 = .28$] and the interaction [$F(7.35,88.19) = 3.89$, $p < .001$, partial $\eta^2 = .25$]. The AL mice were more active during the hours of 0500 h, 2400 h, and 0400 h compared to both CR groups (ranging from $p = .036$ to $p = .002$), whereas the CR25% and CR50% mice were more active during the hours of 1500 h, 1600 h, and 1700 h compared to the AL mice (ranging from $p = .027$ to $p < .001$). The CR25% mice were more active compared to both other groups at 2000 h (AL $p = .017$ and CR50% $p = .014$) and the CR50% mice were less active compared to both other groups at 2100 h and 2200 h (ranging from $p = .046$ to $p < .001$).

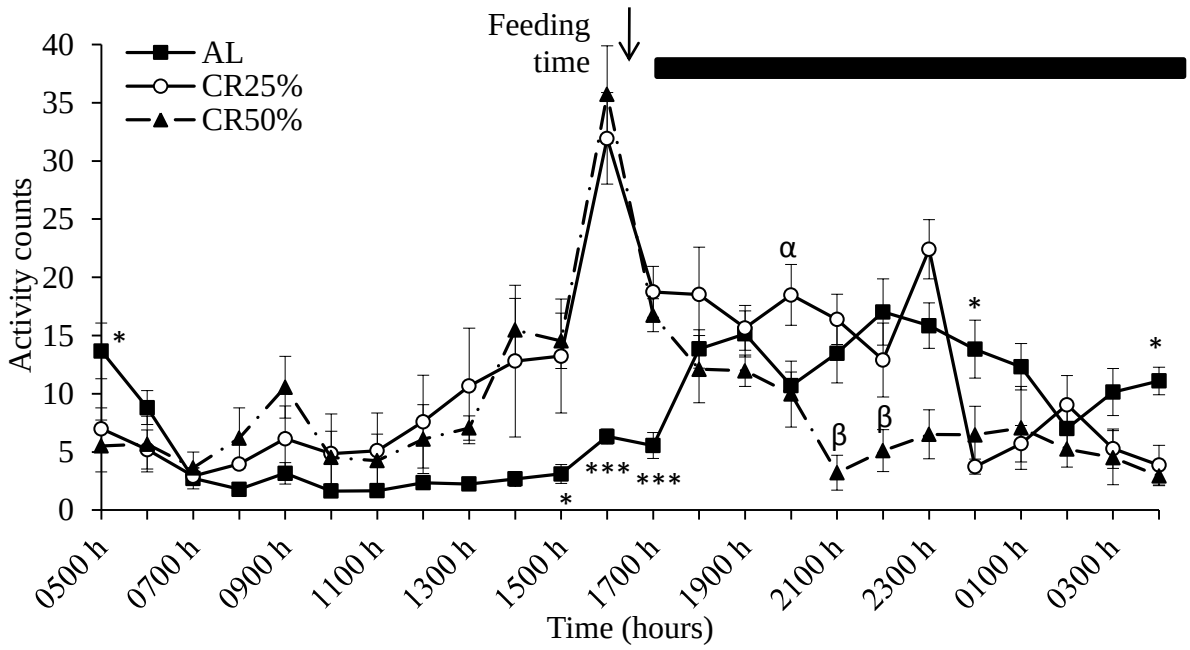

Figure 5. Mean locomotor activity counts (± SEM) for AL (n = 10), CR25% (n = 9), and CR50% (n = 8) mice in hourly blocks on day 28 of the CR period, with the black box representing the dark phase – also presented in Figure 3, Chapter 4. (*) denotes a significant difference from both the CR groups at $p < .05$, (***) denotes a significant difference from both CR groups at $p < .001$, (α) denotes a significant difference from the AL and CR50% groups at $p < .05$, (β) denotes a significant difference from the AL and CR25% groups at $p < .05$.

Discussion

Consistent with previous research (Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996), both of the CR groups demonstrated a reduction in core T_b following CR onset. As expected all the CR mice lost weight during the CR period. There was a dose-dependent response of weight loss, with the CR25% mice losing almost -12% of their initial body weight and the CR50% mice losing -15% of their initial body weight.

Both CR groups demonstrated a dose-dependent response in regards to how their T_b lowers during the light and dark phases during the CR period. The CR25% mice displayed significantly lower core T_b than AL mice during the dark phase after one week of CR and this pattern continued for the remainder of the CR period. The CR25% mice took until the light phase of day 14 to demonstrate a reduced light phase T_b compared to the AL mice, and also remained this way for the remainder of the CR period. The CR50% mice demonstrated a significantly lower core T_b than AL mice during both the light and dark phases after one week of CR. The CR50% mice also demonstrated a reduced T_b compared to the CR25% mice by day 21 during both the light and dark phases. This work supports previous literature that have investigated other variables after a period of CR with a more severe CR eliciting the

largest changes in investigatory variables (Kumar et al., 1990; Levay et al., 2010; Zhu et al., 1997)

The data from the current study supported previous research that total general locomotor activity does not change during a period of CR (Boyle, Storlien, Harper, & Keesey, 1981; Lane et al., 1996). Overall locomotor activity was not changed in either the CR25% or CR50% mice throughout the CR period, which was in contrast to the rats in *Chapter 2*. From day 0 until day 14 of CR the CR25% and CR50% animals demonstrated no alterations in their locomotor activity; however, by day 21 of CR both the CR25% and CR50% animals were more active during the light phase compared to the AL mice and were no different to the AL mice during the dark phase. During day 28 of the CR period both CR25% and CR50% mice were still more active during the light phase compared to AL, which was due to the large anticipatory behaviour that the CR animals displayed before they were fed, which has been demonstrated previously (Duffy et al., 1989; Lane et al., 1996; Mistleberger, 2009; Weed, Lane, Roth, Speer, & Ingram, 1997).

Similar to the CR50% rats in *Chapter 2*, it appeared that there was an adjustment of when the CR mice were active, moving to being more active during the light phase, and less during the dark phase. On day 28 the locomotor activity of each group was presented during the 24 hour period in a similar fashion as the T_b data. This illustrated how the CR50% mice reduced their locomotor activity for the majority of the dark phase compared to the AL and CR25% mice. Also, for the majority of the light phase both of the CR groups were more active compared to the AL mice.

As mentioned above, total activity of the mice for each week of the CR period was not reduced compared to the AL group. This is in contradiction to the CR50% rats in *Chapter 2* that did show a reduction in overall activity. It has been demonstrated that locomotor activity during the light phase in mice, and not rats, is temperature dependent (Swoap,

Overton, & Garber, 2004). It was shown that mice became more active as the ambient temperature decreased which was suggested to occur due to the mice needing to rely on increased activity as an additional source of heat production during a normally inactive period (Swoap, et al., 2004). Although the mice in that study were not exposed to a period of low food availability there are some similarities that can be drawn. For example, the CR mice in the current study may have been experiencing the ambient temperature, which is within the thermoneutral zone for ad libitum fed mice (Gordon, 1985), as below their thermoneutral zone and thus needed to increase their activity to increase heat production. The CR50% mice demonstrated an increase in light phase activity from day 21 of the CR period; however, the CR50% rats in *Chapter 2* only began to increase their light phase activity on day 28. This may be an explanation for why the total locomotor activity was not changed in the CR50% mice.

Importantly for our subsequent studies of CR and fever, the thermogenic ability of the CR mice was intact. This was shown by locomotor activity induced increase in T_b prior to being fed during the CR period. The CR25% and CR50% mice demonstrated a 2 to 2.5 °C increase in T_b in the two hours prior to the onset of the dark phase and food delivery. In comparison, in *Chapter 2* the CR50% rats demonstrated roughly a 1 °C rise in T_b during the period before food delivery. The reduction in core T_b in CR mice to approximately 34 °C during the light phase was substantially lower than the CR50% rats in *Chapter 2* core T_b of roughly 36 °C during the light phase. Others have shown that mice consistently reduce their core T_b to around 35 °C after CR (Rikke et al., 2003). A reduced food intake for mice would understandably be more metabolically costly compared to rats due to their body size and surface-to-volume ratio, and this is possibly why the mice in the current study reduce their T_b further compared to the rats.

Conclusion

It was found that CR results in a dose-dependent reduction in body weight, T_b, and an alteration in the 24 hour pattern of locomotor activity depending on the severity of the CR period. Mice CR to 25% for 28 days demonstrated a reduction in body weight and T_b and an alteration in locomotor activity. The mice CR to 50% demonstrated a larger change in body weight, T_b, and locomotor activity compared to the CR25% mice. These findings support previous research in regards to the impact CR has on lowering core T_b and can potentially provide future researchers with the optimal magnitude of CR to use in order to elicit dramatic changes in the above-mentioned variables.

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Appendix A

Table A1. *Figure 2* Mean T_b (±SEM) for AL (*n* = 10), CR25% (*n* = 9), and CR50% (*n* = 8) mice in hourly blocks on day 28 of the CR period, with the black box representing the dark phase.

Day 28				
Time	AL	Sig. value	CR25%	Sig. value
5am	CR25%	.000	CR50%	.005
	CR50%	.000		
6am	CR25%	.000	CR50%	.013
	CR50%	.000		
7am	CR25%	.000	CR50%	.005
	CR50%	.000		
8am	CR50%	.000	CR50%	.006
9am	CR25%	.013	CR50%	.046
	CR50%	.000		
10am	CR25%	.002	-	-
	CR50%	.000		
11am	CR25%	.001	-	-
	CR50%	.000		
12pm	CR50%	.001	-	-
15pm	CR25%	.000	CR50%	.004
16pm	CR25%	.000	CR50%	.001
	CR50%	.000		
17pm	CR25%	.001	CR50%	.000
18pm	CR50%	.003	CR50%	.001
19pm	CR50%	.001	CR50%	.006
20pm	CR25%	.012	CR50%	.002
	CR50%	.000		
21pm	CR25%	.001	CR50%	.002
	CR50%	.000		
22pm	CR25%	.000	CR50%	.000
	CR50%	.000		
23pm	CR25%	.000	CR50%	.000
	CR50%	.000		
24am	CR25%	.000	CR50%	.001
	CR50%	.000		
1am	CR25%	.000	CR50%	.001
	CR50%	.000		
2am	CR25%	.000	CR50%	.000
	CR50%	.000		
3am	CR25%	.000	CR50%	.001
	CR50%	.000		
4am	CR25%	.000	CR50%	.003

	CR50%	.000

CHAPTER 4

CALORIE RESTRICTION ATTENUATES LPS-INDUCED SICKNESS BEHAVIOUR AND SHIFTS HYPOTHALAMIC SIGNALLING PATHWAYS TO AN ANTI-INFLAMMATORY BIAS

This chapter has been published

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Abstract

Calorie restriction (CR) has been demonstrated to alter cytokine levels; however, its potential to modify sickness behaviour (fever, anorexia, and cachexia) has not. The effect of CR on sickness behaviour was examined in male C57BL/6J mice fed ad libitum, restricted 25% (CR25%), or restricted 50% (CR50%) in food intake for 28 days and then injected with 50 μg/kg of lipopolysaccharide (LPS) on day 29. Changes in body temperature, locomotor activity, body weight, and food intake were determined. A separate cohort of mice were fed ad libitum or CR50% for 28 days, and hypothalamic mRNA expression of IκB-α, COX-2, mPGES-1, SOCS3, IL-10, NPY, leptin, POMC, and CRH were determined at 0, 2, and 4 hours post-LPS. CR50% mice did not develop fevers, whereas the CR25% mice displayed a fever shorter in duration, but with the same peak as the controls. Both CR25% and CR50% mice showed no sign of anorexia and reduced cachexia after LPS administration. Hypothalamic mRNA expression of NPY and CRH were both increased by several fold in CR50% animals pre-injection compared to controls. The CR50% mice did not demonstrate the expected rise in hypothalamic mRNA expression of COX-2, mPGES-1, POMC, or CRH 2 hours post-LPS, and leptin expression was decreased at this time point. Increases in SOCS3, IL-10, and IκB-α expression in CR50% animals were enhanced compared to ad libitum fed controls at 4 hours post-LPS. CR results in a suppression of sickness behaviour in a dose-dependent manner, which may be due to CR attenuating pro-inflammatory pathways and enhancing anti-inflammatory pathways.

Calorie restriction (CR), a dietary regimen low in calories while avoiding malnutrition, is the only manipulation that has consistently been shown to extend the mean and maximum life span in a range of species (Austad, 1989; Lane et al., 1996; Sohal, Ferguson, Sohal, & Forster, 2009; Turturro et al., 1999). In addition to an increase in life span, CR attenuates the 'normal' immunosenscence seen with age (Mascarucci et al., 2002), along with reducing the occurrence of other age-related diseases (Chandrasekar, Nelson, Colston, & Freeman, 2001; Kubo, Day, & Good, 1984; Lane, Ingram, & Roth, 1999; Maswood et al., 2004; Matsuzaki et al., 2000; Walford, Mock, Verdery, & MacCallum, 2002).

It has been postulated that the mechanism by which CR extends the mean and maximum life span is by reducing oxidative stress caused by unregulated increases in reactive oxygen species (ROS) (Barja, 2004; Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; Shigenaga, Hagen, & Ames, 1994); as well as by limiting the age-related increase in basal pro-inflammatory cytokine levels (Spaulding, Walford, & Effros, 1997). One possible mechanism by which CR exerts its anti-inflammatory effect are glucocorticoids, which are increased after CR (Holmes, French, & Seckl, 1997; Levay, Tammer, Penman, Paolini, & Kent, 2010) and act to increase the transcription of anti-inflammatory cytokines and decrease the transcription of pro-inflammatory cytokines (Barnes, 1998). Indeed, CR reduces both basal and LPS-stimulated levels of *tumour necrosis factor- α (*TNF-α) and interleukin-6 (IL-6) mRNA in alveolar macrophages in rats (Dong et al., 1998).

The physiological and behavioural changes that occur in response to infection and inflammation, occasionally referred to as sickness behaviour, are adaptive (Hart, 1988). Further, injection of a moderate dose of a pyrogenic compound such as lipopolysaccharide (LPS) can induce a biphasic fever (Romanovsky et al., 1996). The first phase of the biphasic

fever includes a tightly regulated increase in core body temperature (T_b), while the second phase can be characterised by a further increase in T_b (Romanovsky, et al., 1996); however, in cases such as low food availability this second phase can be represented by hypothermia (Shido, Nagasaka, & Watanabe, 1989). These phases of fever are associated with differing patterns of locomotor activity. During the first phase (within an hour of injection) the animal displays a large amount of activity, whilst during the second phase (approximately 2 to 2 ½ hours post-injection) the animal displays motor depression, or reduced activity for at least 5 hours post-LPS (Romanovsky, et al., 1996). Further, metabolic rate has been shown to be increased by up to 20% in the first phase (Jansky & Vybiral, 2004); however, the second phase is characterised by energy conservation due to the cost of the first phase of fever and the increased dependence of the animals' core T_b on the ambient temperature (Romanovsky & Blatteis, 1994).

Sickness behaviour is well known to be due to the central actions of pro-inflammatory cytokines (Dantzer, 2004). The decrease in pro-inflammatory cytokines by CR should result in reduced levels of sickness behaviour, which appears to be counter-intuitive to the ability of CR to increase lifespan. To our knowledge, the effect of CR on the development of sickness behaviour has not been examined. If sickness behaviour post-LPS were to be attenuated then it's important to speculate as to why this may be occurring. The CR animals may exhibit a shift in the balance of pro- and anti-inflammatory cytokines post-LPS. Thus, if anti-inflammatory cytokines were increased this would potentially lead to reduced sickness behaviour. For example, the anti-inflammatory compound suppressor of cytokine signalling 3 (SOCS3) is known to aid IL-10's anti-inflammatory effects and reduces the production of IL-6 (Bogdan, Vodovotz, & Nathan, 1991) whilst also inducing the production of IL-1ra (Berlato et al., 2002).

The effects of acute food deprivation on sickness behaviour have been examined and may be relevant. However, it is important to note that food deprivation exerts different effects on many physiological and pharmacological measures compared to CR (Bi, Robinson, & Moran, 2003; Johansson et al., 2008). For example, hypothalamic levels of neuropeptide Y (NPY), agouti-related protein (Bi et al., 2003), and cocaine- and amphetamine-regulated transcript and plasma levels of glucose and insulin have been shown to be different between food deprived and CR animals (Johansson et al., 2008). Continuous infusion of IL-1 resulted in a dose-dependent decrease in food intake in ad libitum fed rats (Mrosovsky, Molony, Conn, & Kluger, 1989). In contrast, rats that were food deprived for 24 hours and then provided with between 5 and 12 g of food in the light phase for seven days (i.e., a 50-75% reduction in food intake), exhibited hyperphagia once they were allowed to eat ad libitum after the IL-1 infusion (Mrosovsky et al., 1989).

More recently, rats fasted for 48 hours and then exposed to an injection of LPS demonstrated a significantly attenuated fever compared to ad libitum fed controls (Inoue, Somay, Poole, & Luheshi, 2008). In addition to the attenuated fever, the rise in plasma levels of TNF-α and IL-1ra were attenuated; however, not all cytokine levels were reduced in fasted animals. Interestingly, mRNA expression of IL-1β, cyclooxygenase-2 (COX-2), and prostaglandin E_2 (PGE_2) in the hypothalamus of fasted animals exposed to LPS were at levels seen in ad libitum fed animals injected with LPS (Inoue et al., 2008).

The present study investigated the effect of a 25% and 50% CR on various indicators of sickness behaviour, including fever, anorexia, cachexia, and decreased locomotor activity, following an intraperitoneal injection of LPS. Quantitative PCR was used to assess mRNA expression of inhibitory factor kappa B alpha (IκB-α), COX-2, microsomal prostaglandin E synthase-1 (mPGES-1), SOCS3, interleukin-10 (IL-10), NPY, pro-opiomelanocortin

(POMC), leptin, corticotrophin-releasing hormone (CRH), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) to elucidate a potential central mechanism behind the attenuation of sickness behaviour after CR. All of these compounds are known to be involved in either the regulation of energy balance or are implicated in the immune response. Due to evidence from food deprivation studies we hypothesized that CR would lead to an attenuation of LPS-induced sickness behaviour in mice and that one or a combination of compounds measured through qPCR may be responsible.

Methods

Animals

Eighty-three male C57BL/6J mice (aged 10 to 16 weeks at the beginning of experimentation) were procured from either the Central Animal House at La Trobe University or Monash SPF animal services (Clayton, VIC, Australia) and allowed to acclimate to the facility for at least one week. During this period, standard rodent chow (Barastoc, Melbourne, Australia) and water were available ad libitum. Mice were individually housed in polypropylene basin cages (38 × 27 × 15 cm) with sawdust as bedding and were maintained at an ambient temperature of 30 ± 1 °C, which is within the thermoneutral zone for this species (Gordon, 1985), on a 12:12 light/dark cycle (0500 – 1700 hours). Animal care and experimentation was performed in accordance with protocols of the La Trobe University Animal Ethics Committee.

Surgery

Following acclimation, 40 of the mice were aseptically surgically implanted in the peritoneal cavity with a biotelemetry device (E-4000, Mini-mitter, Bend, OR, USA: 23 × 8 mm, 1.6 g) under anaesthesia (ketamine 61 mg/kg and xylazine 9 mg/kg; more detail in *Chapter 2*) (Weiland, Voudouris, & Kent, 2004). These mice were allowed two to three weeks to recover before the initiation of the CR regimens.

Dietary regimens

Mice were divided into one of three CR regimens matched for weight, food intake, and age: ad lib (AL; n = 39) fed ad libitum (on average 2.6 g per day); CR25% (n = 10) mice received 75% of the amount consumed by AL (on average 1.8 g per day); and CR50% (n = 34) mice received 50% of the amount consumed by AL (on average 1.3 g per day). The dietary composition of the AL, CR25%, and CR50% diets has been published elsewhere (Levay, Govic, Penman, Paolini, & Kent, 2007; Levay et al., 2010). The group sizes varied across dependent variables and precise numbers used for each variable are stated in the results section. Food intake of the CR groups was determined weekly based on the average daily food intake of the AL group for three consecutive days. Water was continuously provided ad libitum to all groups. The dietary manipulation continued for 28 days before LPS or saline challenge and for four days after LPS or saline challenge in Experiment 1; and for 28 days before LPS challenge until sacrifice in Experiment 2. Food was provided daily, approximately one hour before dark phase onset.

Experiment 1 – Effect of calorie restriction and determination of sickness behaviour

The effect of a 28 day CR on LPS-induced sickness behaviour was determined by the continuous measurement of core T_b, locomotor activity, body weight, and food intake for four

days pre- and four days post-LPS challenge. T_b, locomotor activity, and body weight were determined throughout the CR period.

T_b and locomotor activity were determined by placing each cage on a receiver with each individual biotelemetry device generating a continuous frequency signal proportional to the animal's T_b (± 10^{-1} °C) (Weiland et al., 2004). The receiver sampled this frequency at 1-minute intervals and this sample was decoded by VitalView software (Mini Mitter Co., Inc., Bend, OR) and stored on a hard drive. Locomotor activity was determined by the same system. Each receiver was equipped with a matrix of antennas which continuously signalled the biotelemetry device. The receiver scanned the matrix in a sequential order every minute in order to locate the position and orientation of the biotelemetry device. The global locomotor activity of the mouse was determined by the receiver at 1-minute intervals and recorded using VitalView software. Mice were weighed once a week approximately three hours after lights-on during dietary manipulation and daily at the same time for the four days pre- and post-LPS/saline challenge using top loading scales (± 0.1 g). Food consumption was also determined at this time and was calculated to the nearest 0.1 g by providing a set amount of food each day and weighing the remaining food 24 hours later, including the uneaten food in the bedding.

After 28 days of the dietary regimen (i.e., on the 29th day) all mice were injected intraperitoneally with 50 μg/kg of LPS from *Escherichia coli* (serotype 0111:B4; Sigma, Castle Hill, NSW) or 150 μl/25 g of saline. The LPS and saline challenge was performed approximately four hours after lights-on (ranging from 0800 hours to 1000 hours). All measures were continued for four days post-LPS.

Experiment 2 – qPCR

Thirty-nine male mice were used in Experiment 2 to determine mRNA levels of IκB-α, COX-2, mPGES-1, SOCS3, IL-10, NPY, POMC, leptin, CRH, GR, and MR, and at three time intervals (i.e., immediately before, 2 hours, or 4 hours post-LPS). Mice were handled and exposed to the same dietary manipulations as in Experiment 1; however, the CR25% group was not included. There were two levels of diet, AL and CR50%, and these animals were exposed to a dose of LPS (50 μg/kg) on the 29th day of CR.

Tissue preparation

Mice were rapidly decapitated at 0, 2, and 4 hours after LPS injection, 4 to 8 hours following light onset. The brains were rapidly removed, and the hypothalamus was located by landmarks as previously described (Weiland, Kent, Voudouris, & Shulkes, 2005) and dissected from the brain using a scalpel. Individual tissue samples were immersed in RNAlater solution (Qiagen) and kept at room temperature for 3 hours and then 4 ºC overnight to allow the solution to infiltrate the tissue, then stored at -20 ºC until processing.

Primer design

All primers were synthesised by GeneWorks (Hindmarsh, SA, Australia). Primers were 18-24 nucleotides in length with melting points between 52 and 64 ºC. Forward and reverse primers for all primers are presented in Table 1 as well as abbreviations.

Table 1. *Forward and reverse primer details used for RT-PCR*

Gene name	Forward primer	Reverse Primer
B-actin (ACT)	cac tgc cgc atc ctc ttc ct	aac cgc tca ttg ccg ata gtg
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	aca tgc cgc ctg gag aaa cct	gcg cag gat gcc ctt tag tgg

Histone 3 (H3)	att cgc aag ctc ccc ttt cag	tgg aag cgc agg tct gtt ttg
Inhibitory factor kappa B alpha (IκB-α) (Murray, 2005)	acc tgg cct tcc ctc aac ttc	gcc tgc agg acg gag tgg
Cyclooxygenase 2 (COX-2) (Ziotopoulou, Erani, Hileman, Bjørbæk, & Mantzoros, 2000)	cct ccg agc tgt gct gct ctg c	cat gat taa act tcg cag gaa ggg g
Microsomal prostaglandin E synthase-1 (mPGES-1) (Boulet et al., 2004)	cca gts tta cag gag tga ccc aga t	gga aag gat aga ttg tct cca tgt c
Suppressor of cytokine signalling 3 (SOCS3) (Ziotopoulou et al., 2000)	acc agc gcc act tct tca cg	gtg gag cat cat act gat cc
Interleukin-10 (IL-10) (Rodriguez et al., 2008)	act gca ccc act tcc cag t	ttg tcc agc tgg tcc ttt gt
Neuropeptide Y (NPY) (Ziotopoulou et al., 2000)	gct tga aga ccc ttc cat gtg gtg	ggc gga gtc cag cct agt gg
Pro-opiomelanocortin (POMC) (Ziotopoulou et al., 2000)	tgg tgc ctg gag agc agc cag tgc	tgg agt agg agc gct tgc cct cg
Leptin (Hoggard et al., 1997)	tgc tgc aga tag cca atg ac	gag tag agt gag gct tcc agg a
Corticotrophin-releasing hormone (CRH) (Muglia, Jenkins, Gilbert, Copeland, & Majzoub, 1994)	gca tcc tga gag aag tcc ctc tg	gcc cgg gcc att tcc aag ac
Glucocorticoid receptor (GR) (Abbott et al., 1999)	tgc tat gct ttg ctc ctg atc tg	tgt cag ttg ata aaa ccg ctg cc
Mineralocorticoid receptor (MR) (Ouvrard-Pascaud et al., 2005)	tca cat ttt taa cat gtg cag gc	ctt agt cag ctc agg ctt gcc

RNA extraction and cDNA synthesis

Individual tissue samples were homogenised in QIAzol lysis buffer (Qiagen) using a TissueRuptor (Daintree Scientific). RNA was extracted from the tissue using an RNeasy Lipid Tissue Mini Kit (Qiagen) in accordance with manufacturer’s instructions. Chloroform

was added to the homogenate, which was then centrifuged at 12,000 g for 15 min at 4 ºC. The aqueous phase was mixed thoroughly with 70% ethanol before being transferred to the RNeasy Mini spin column, which was then centrifuged twice at 8000 g for 15 sec at room temperature with the flow through being discarded after each spin. At this stage, on-column DNA digestion was performed using the RNase-Free DNase set (Qiagen) to remove any residual DNA. Following this, Buffer RPE was added to the column, which was then centrifuged for 15 sec at 8000 g at room temperature and the flow-through discarded. The same step was then performed with a longer centrifugation time of 2 min to ensure that no ethanol was carried over during RNA elution. The RNeasy spin column was then placed in a new collection tube and centrifuged at full speed for 1 min to eliminate any possible carryover effects of Buffer RPE. RNA was then eluted using 20-30 µl of RNase-free water and stored at -80 ºC until cDNA synthesis.

Quantification and purity of RNA samples was performed by absorbance 260 nm and 280 nm on a spectrophotometer. A 260/280 nm absorbance ratio ranging between 1.7 and 2.1 being acceptable purity. cDNA was synthesised with reverse transcriptase from Applied Biosystems (Scoresby, VIC) using random primers and following manufacturer's instructions.

Real-time quantitative PCR

Relative levels of mRNA were determined by real-time quantitative PCR (qPCR). qPCR was performed in a 20 µl final reaction volume containing 0.96 ng/µl of template cDNA, 0.8 uM of each forward and reverse primer, and 1X SYBR Green PCR Master mix (Applied Biosystems). Samples, mastermix containing primers and water were aliquoted into 96 well TWIN.TEC PCR plates (Eppendorf, North Ryde, NSW, Australia) using the

epMotion 5070 automated liquid handler (Eppendorf) to increase pipetting accuracy. qPCR was performed using a Realplex real-time detection instrument (Eppendorf). All samples were measured in duplicate and compared with a negative water control for each primer pair. A standard curve was generated on every plate with a 5 × serial dilution, with a top concentration of 1600 µg/ml, to determine efficiency and Ct values increased with decreasing concentration of template. Annealing temperature was 62 ^{o}C and 40 cycles were performed. Melting point curves were included to confirm that only one product was formed. All qPCR experiments were performed on individual samples.

Data Analysis

Analyses of measures during CR period

Locomotor activity data for day 28 was collapsed into 1-hour blocks and a two-way mixed design analysis of variance (ANOVA) was conducted to determine if CR altered the circadian rhythm of the CR mice. Where appropriate, post hoc pairwise comparisons were performed using the Least Significant Difference (LSD) method.

Analyses of sickness behaviour measures

All data collected over the 4-day pre-injection and 4-day post-injection period were averaged and means ± SEM were calculated for each group. To determine the baseline for each animal on each measure the 4-day baseline measures were averaged into one 24-hour period. To determine a change in any measure the day of LPS injection was subtracted from the averaged baseline. Locomotor activity on the day of LPS injections was averaged into one value for each group to compare the total locomotor activity of each group. Data were then analysed using ANOVA; to overcome violations of sphericity, the Greenhouse-Geisser

statistic corrected degrees of freedom were reported were appropriate. Where required, between-group simple main effects analyses were performed using the LSD method. When more than two comparisons were required, as in the case of T_b and locomotor activity data, a Bonferroni adjustment was used to protect for inflated type 1 error rate. Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$.

Analysis of qPCR data

Data analysis for qPCR data was performed using qBase software (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007). This software utilises a modified version of the delta-delta-Ct that has the capacity to take into account multiple reference genes and gene specific amplification efficiencies. mRNA levels were normalised to the levels of the reference genes ACT, GAPDH, and H3 using this software and normalised relative quantities (NRQs) were the product. Outliers were identified by box-plots and removed where appropriate. These included 1 mouse from the CR50% (2 hour group) COX-2 data, 1 mouse from the CR50% (4 hour group) mPGES-1 data, 1 mouse from the AL (2 hour group) leptin data, 1 mouse from the CR50% (4 hour group) leptin data, 1 mouse from the AL (2 hour group) IL-10 data, 1 mouse from the CR50% (4 hour group) IL-10 data, 1 mouse form the AL (4 hour group) POMC data, and 1 mouse from the AL (4 hour group) GR data. This represents less than 2% of all samples. All data are presented as mean ± SEM. A 2 × 3 ANOVA was used for determining differences between groups at $p < .05$. Post-hoc tests were conducted where deemed appropriate using the LSD method. The correlation between all of the PCR variables at each time point was evaluated statistically using Pearson product-moment correlation coefficient for each dietary group.

Results

Experiment 1 – The effect of CR on LPS-induced sickness behaviour

Effect of CR on body weight

The CR25% and CR50% groups lost weight, whereas the AL group demonstrated a slight weight gain (Figure 1). Although both CR groups lost weight steadily at the onset of CR, the CR50% group exhibited a more pronounced weight loss compared to the CR25% group. On day 28 of the CR period the AL animals weighed on average 29.2 g (± 0.5), the CR25% animals weighed on average 24.5 g (± 0.5), and the CR50% animals were similar to the CR25% mice, weighing 23.8 g (± 0.4) on average.

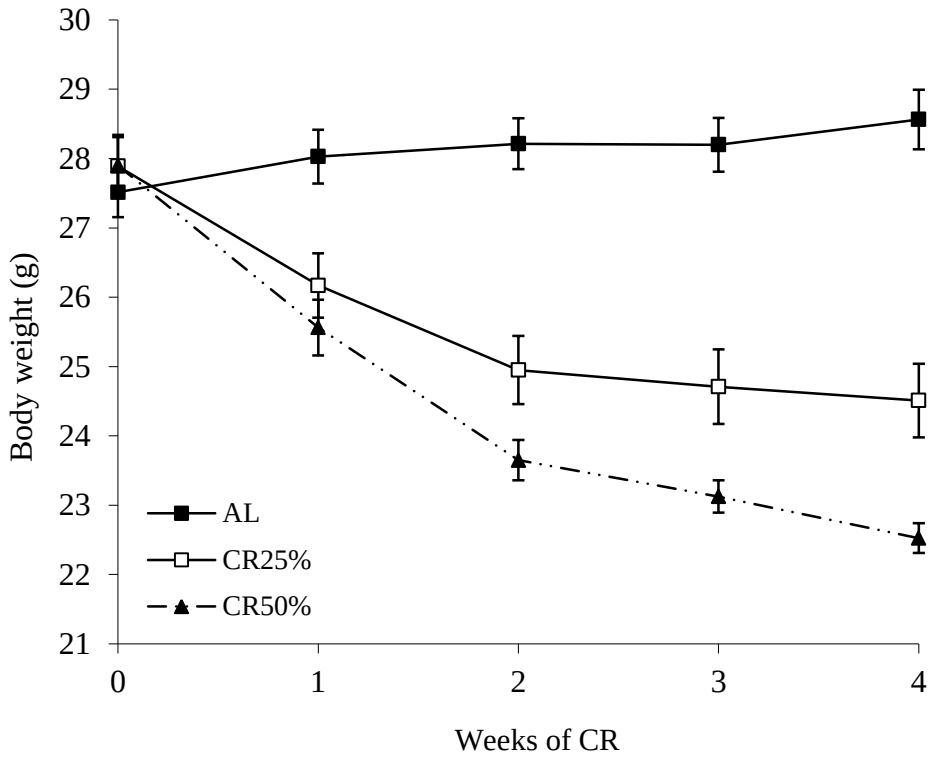

Figure 1. Mean (± SEM) body weight (g) of the AL (*n* = 10), CR25% (*n* = 9), and CR50% (*n* = 8) animals during the CR period.

Changes in basal body temperature after CR

After the onset of CR, the CR25% group demonstrated a moderate reduction in basal T_b and the CR50% group demonstrated a large reduction in T_b during both the light and dark phases. By day 21 of CR, the reduction in basal T_b for both groups appeared to plateau. During day 28 of the CR period the AL mice had an average T_b of 35.2 °C (± 0.2) during the light phase and 37.3 °C (± 0.1) during the dark phase. The CR25% mice demonstrated similar light phase T_b compared to the AL mice at 35.2 °C (±0.4), but a reduction of more than 1 °C during the dark phase (36.2 °C ± 0.3). The T_b of CR50% mice during the light phase was more than 1 °C below that of the AL mice (34.4 °C ± 0.4) and was more than 2 °C below the AL mice during the dark phase (35.1 °C ± 0.3).

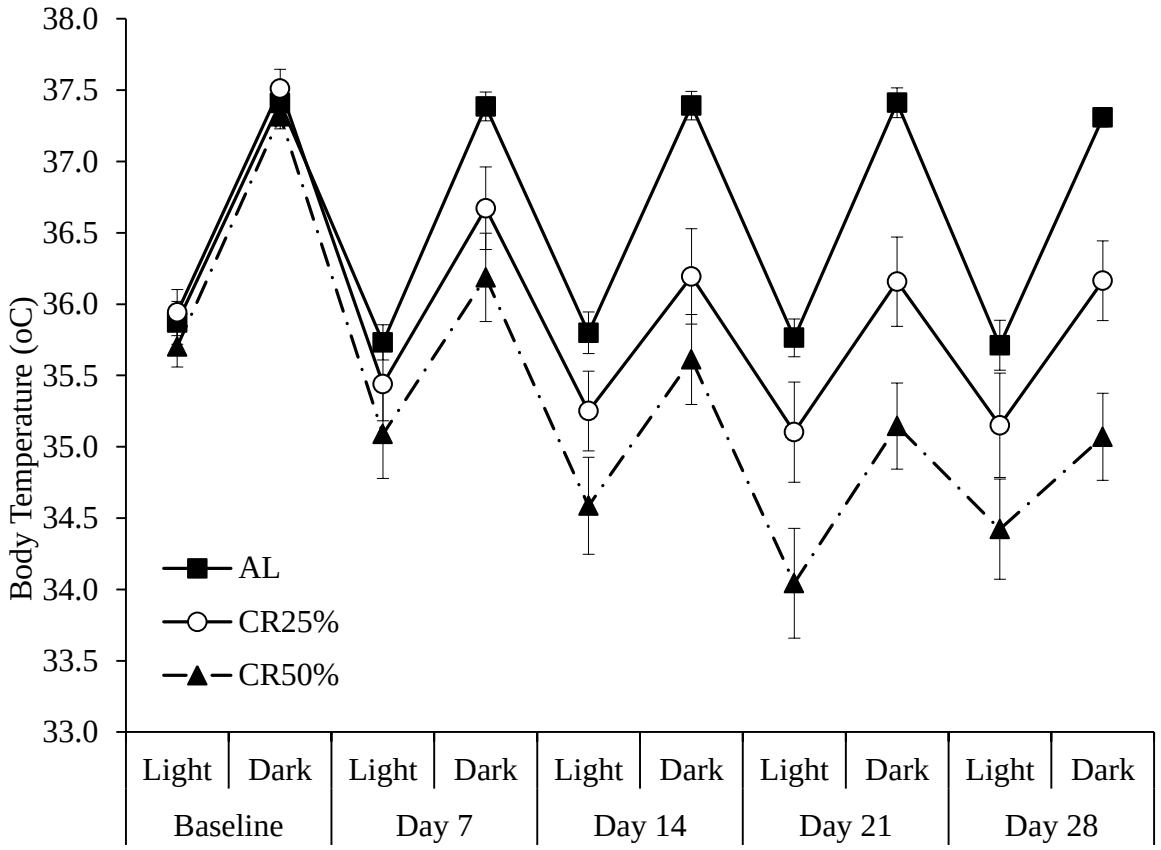

Figure 2. Mean T_b (±SEM) of AL (n = 10), CR25% (n = 9), and CR50% (n = 8) during the light and dark phases of day 0, day 7, day 14, day 21, and day 28 of the CR period – also presented in Figure 1, Chapter 3.

Changes in locomotor activity after CR

For two weeks after the onset of CR all animals demonstrated similar locomotor activity; however, by 21 days after the onset of CR both the CR25% and CR50% groups demonstrated a change in their locomotor activity. Namely, both groups demonstrated an increase in their light phase locomotor activity and a reduction in their dark phase locomotor activity. This is largely due to anticipatory feeding behaviour that occurs before the CR animals are fed (Figure 3). During the light phase of day 28 of the CR period the AL mice were only minimally active (4.2 locomotor activity counts ± 0.4/minute), and were more active during the dark phase (12.2 counts ± 1.0/minute). In comparison, the CR25% and CR50% mice were more active during the light phase compared to the AL mice (9.3 counts ± 2.4 and 9.9 counts ± 1.3 respectively). During the dark phase the CR25% mice were as active as the AL mice (12.6 counts ± 2.2); however, the CR50% mice were less active than both the AL and CR25% mice, and were less active compared to their light phase activity (7.7 counts ± 1.8). It is interesting to note that even though the distribution of the locomotor activity of the CR animals changed over the CR period, their total locomotor activity did not. AL animals averaged 8.2 (± 0.7) locomotor activity counts per minute on day 28 of the CR period, compared to 10.9 (± 2.3) counts for the CR25% animals and 8.8 (± 1.5) counts for the CR50% animals. The ANOVA was significant for hour [$F(3.67,88.19) = 9.49$, $p < .001$, partial $\eta^2 = .28$] and the interaction [$F(7.35,88.19) = 3.89$, $p < .001$, partial $\eta^2 = .25$]. The AL mice were more active compared to both the CR25% and CR50% mice during the hours of

0500 h, 2400 h, and 0400 h (ranging from $p < .001$ to $p = .036$) and were less active compared to both CR groups during the hours of 1500 h, 1600 h, and 1700 h, (ranging from $p < .001$ to $p = .027$). The CR25% group was more active compared to both other groups at 2000 h ($p = .014$ and $p = .017$) and the CR50% group was less active compared to both other groups at 2100 h and 2200 h (ranging from $p < .001$ to $p = 006$).

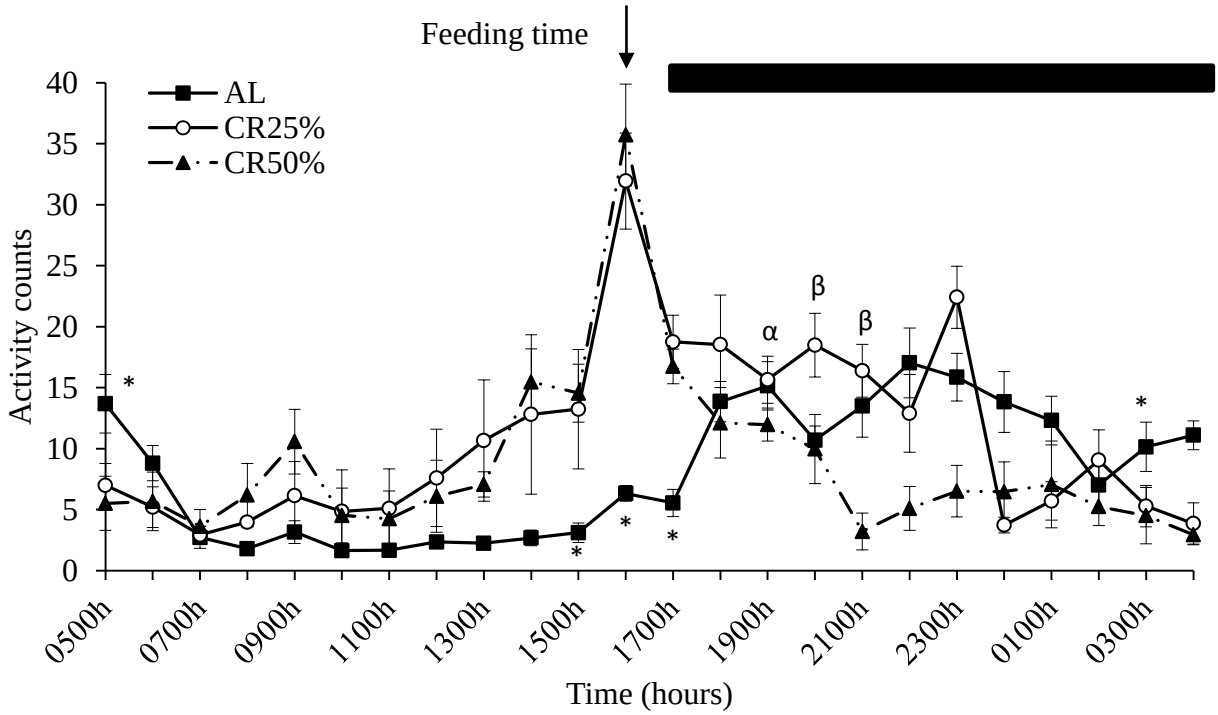

Figure 3. Mean locomotor activity counts (±SEM) for AL ($n = 10$), CR25% ($n = 9$), and CR50% ($n = 8$) animals in hourly blocks on day 28 of the CR period, with the black box representing the dark phase – also presented in Figure 5, Chapter 3. (*) denotes a significant difference from the CR25% and CR50% groups at $p < .05$, (α) denotes a significant difference from the AL and CR50% groups at $p < .05$, (β) denotes a significant difference from the CR50% group at $p < .01$.

Sickness behaviour measures

Core body temperature

LPS-induced a fever in both AL and CR25% animals, whereas in CR50% mice it did not (Figure 4). AL animals' T_b increase peaked on average at 180 minutes post-LPS challenge at 2.0 ^oC (± 0.2) and the T_b of the CR25% LPS animals had increased by 2.1 ^oC (± 0.5) at 174 minutes post-LPS challenge, whereas the largest increase in CR50% animals was only 0.3 ^oC (± 0.3) at 210 minutes post-LPS challenge. Fever in the CR25% animals was shorter lived (lasting until approximately 5 hours post-LPS) than in the AL animals who maintained a fever until 8 hours post-LPS. Both saline groups demonstrated no rise in T_b following injection (shown in Appendix A).

ANOVA found a significant main effect for hour [$F(3.46,75.53) = 6.75$, $p < .001$, partial $\eta^2 = .17$], group [$F(4,34) = 12.37$, $p < .001$, partial $\eta^2 = .59$], and their interaction [$F(13.84,75.53) = 3.72$, $p < .001$, partial $\eta^2 = .30$]. Post-hoc comparisons revealed significant treatment effects for all 8 hours post-LPS. CR50% animals displayed a significantly lower T_b than AL animals for the first 7 hours post-LPS (ranging from p .002 to $p < .001$). At hours 1 to 4, and 7, the CR50% LPS animals also showed a significantly lower T_b than the CR25% LPS animals (ranging from $p = .046$ to $p < .001$). At hours 5, 6, and 7 the CR25% LPS group displayed a significantly lower T_b than AL LPS animals ($p = .004$, $p < .001$, and $p < .001$ respectively). The AL saline and CR50% saline animals were significantly different from the AL LPS animals at every hour (ranging from $p = .022$ to $p < .001$ for both saline groups).

T_b was significantly increased compared to baseline (the average of each hour during a 24-hour period of the 4 days immediately before LPS administration) in AL animals for eight hours following LPS ($p < .001$ for all). In contrast, T_b was only significantly higher than baseline at 2 and 3 hours post-LPS in CR25% ($p = .003$ and $p = .007$ respectively), and only

at hour 8 post-LPS in CR50% animals ($p < .001$). Saline injection did not alter T_b in either group (data not shown).

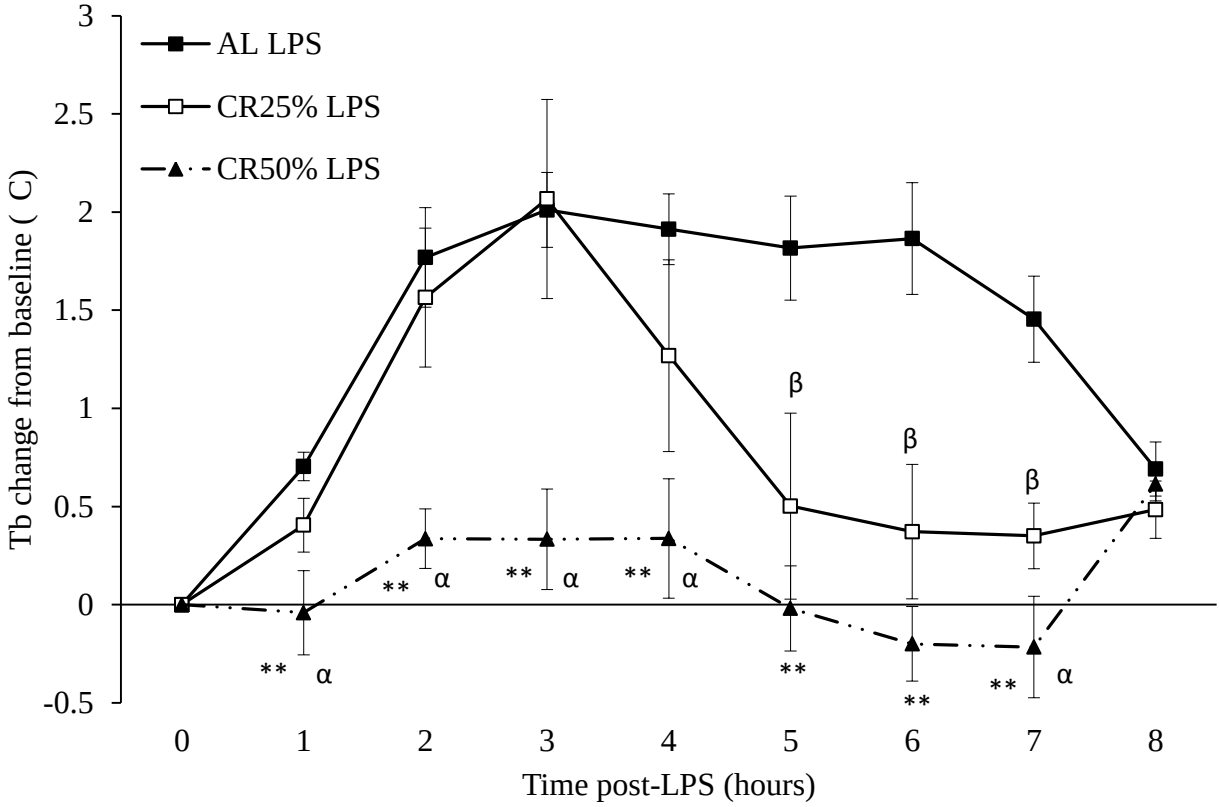

Figure 4. Mean T_b change from baseline (the average of each hour during a 24-hour period of the 4 days before LPS administration; ± SEM) for AL LPS (n = 9), CR25% LPS (n = 10), and CR50% LPS (n = 8) animals, for 8 hours post-injection. (**) denotes a significant difference from the AL group at $p < .01$, (α) denotes a significant difference from the CR25% group at $p < .05$, (β) denotes a significant difference from the AL group at $p < .01$.

Locomotor activity

During the 24 hours post-LPS the AL and CR animals demonstrate different patterns of reduced locomotor activity (Figure 5). The CR25% animals' locomotor activity decreased

53% and the CR50% groups' activity decreased 38% during the period of 4-8 hours post-LPS (-13.4 ± 2.3 counts and -10.2 ± 2.3 counts respectively); however the AL LPS animals' largest decrease in activity (54%) occurred at 16 hours post-LPS (-10.1 ± 1.5 counts). In all 3 cases this corresponds to the block of time when they were most active during baseline. Changes in activity in the AL saline and CR50% saline groups were minimal and non-significant (data not shown). The ANOVA found a significant main effect for 4-hour block of time post-LPS [$F(3.49,83.84) = 13.27$, $p < .001$, partial $\eta^2 = .36$] and the interaction between this factor and group [$F(6.99,83.84) = 11.19$, $p < .001$, partial $\eta^2 = .48$]; however the main effect for group was not significant. Post-hoc comparisons revealed significant treatment effects for all 4-hour blocks post-LPS except for the 20-24 hour block. The AL group demonstrated a significantly reduced locomotor activity compared to both of the CR groups from 8 to 20 hours post-LPS (ranging from $p = .007$ to $p < .001$ for both groups at all times). The CR25% and CR50% groups demonstrated a larger decline in locomotor activity from 0 to 8 hours post-LPS compared to the AL group (ranging from $p = .011$ to $p < .001$ for both groups at both 0-4 and 4-8 hours post-LPS).

The AL group differed from baseline at every 4-hour block, with 0-4 hours post-LPS being the only time they were more active compared to baseline ($p = .033$); they were less active compared to baseline for the remaining five 4-hour blocks (ranging from $p = .011$ to $p < .001$ for all). The CR25% animals were less active compared to baseline at every 4-hour block except for 16-20 hours post-LPS (ranging from $p = .026$ to $p < .001$), and the CR50% animals were only less active compared to baseline during the 0-4, 4-8, and 16-20 hour post-LPS blocks ($p = .011$, $p = .003$, and $p = .037$ respectively). Similar to the activity of the groups during the CR period the AL (-4.18 ± 0.44, -29.8%), CR25% (-4.60 ± 0.59, -9.9%), and CR50% (-2.66 ± 0.61, -12.7%) animals' total change in locomotor activity did not differ

during the 24 hours post-LPS for either the absolute change or the percentage change in locomotor activity.

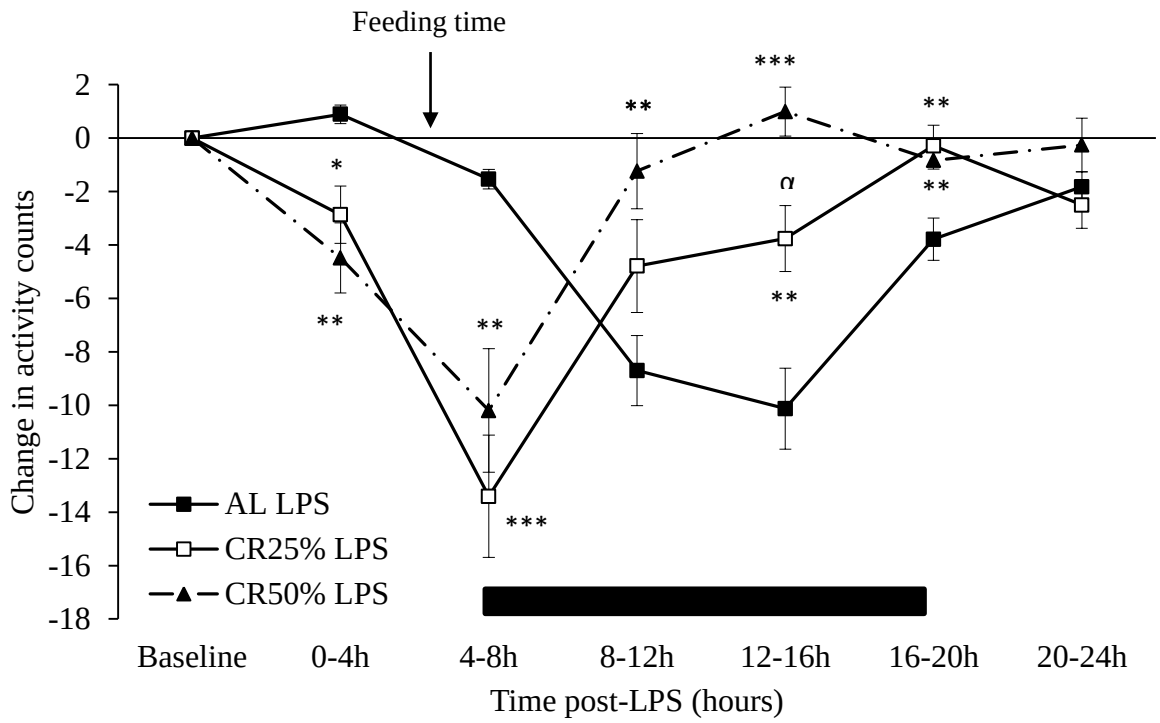

Figure 5. Mean activity change from baseline (the day of LPS subtracted from the baseline average) in 4 hour blocks (±SEM) post-injection for AL LPS (n = 9), CR25% LPS (n = 10), CR50% LPS (n = 8) animals. The black box represents the dark phase. (*) Denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .01$, (***) denotes a significant difference between the AL group at $p < .001$, (α) denotes a significant difference between the CR25% and CR50% group at $p < .05$.

Body weight

On day 1 post-LPS the AL animals demonstrated that most severe weight loss (-4.8% ± 0.6%), this was 4-fold the weight loss of the CR25% animals (-1.2% ± 0.4%) and 2½ times the loss observed in the CR50% animals (-1.9% ± 0.1%) (Figure 6A). The AL saline and

CR50% saline groups did not lose weight (data not shown). The ANOVA found a significant main effect for day [$F(2.82,90.02) = 20.48$, $p < .001$, partial $\eta^2 = .36$], group [$F(4,37) = 5.91$, $p < .001$, partial $\eta^2 = .38$], and their interaction [$F(9.73,90.02) = 6.21$, $p < .001$, partial $\eta^2 = .39$]. On day one the AL LPS showed a larger decrease compared to all other groups ($p < .001$ for all), and the CR50% LPS group lost more weight compared to both the saline groups ($p = .031$ for the AL saline group and $p = .016$ for the CR50% saline group). On day two the AL LPS group was significantly different from all groups except the CR50% LPS group (ranging from $p = .013$to $p < .001$). The only differences on day three and day four were between the AL LPS and the CR25% LPS group ($p = .013$ and $p = .021$ respectively).

Body weight was reduced in AL animals compared to baseline on day 1 ($p < .001$) and day 2 ($p = .003$) post-LPS. The CR50% group only lost a significant amount of weight compared to baseline on day 1 post-LPS ($p < .001$); however, the CR25% LPS, AL saline, and CR50% saline groups did not differ from baseline on any day post-LPS.

Food intake

AL animals have a sharp reduction in food intake during the initial 24 hours following LPS injection (-1.2 ± 0.2 g or -46.6% ± 6.5%), with a small reduction still present on day two (-0.6 ± 0.1 g or -21.3% ± 3.3%) before returning to baseline by day three (-0.1 ± 0.1 g or -2.8% ± 4.8%; Figure 6B). Food intake in all other groups was unaffected post-injection; the CR groups ate all of their allocated food. ANOVA found a significant main effect for day [$F(1.90,70.34) = 3.99$, $p = .025$, partial $\eta^2 = .10$], group [$F(4,37) = 15.06$, $p < .001$, partial $\eta^2 = .62$], and their interaction [$F(7.60,70.34) = 6.56$, $p < .001$, partial $\eta^2 = .42$]. Post-hoc comparisons revealed significant differences on all days post-LPS; however on day 3 and day 4 post-LPS this is solely due to the CR regimen. This is evidenced by within-group simple

main effects revealing that the AL mice ate significantly less than baseline only on day 1 and day 2 post-LPS ($p < .001$ for both).

Further, on day 1 post-LPS the AL LPS group ate significantly less than all other groups ($p < .001$ for all). By day 2 post-LPS the AL LPS were still eating significantly less compared to all other groups ($p < .001$ for all). On day 3 post-LPS the AL LPS group had returned to eating the amount of food they were consuming before LPS.

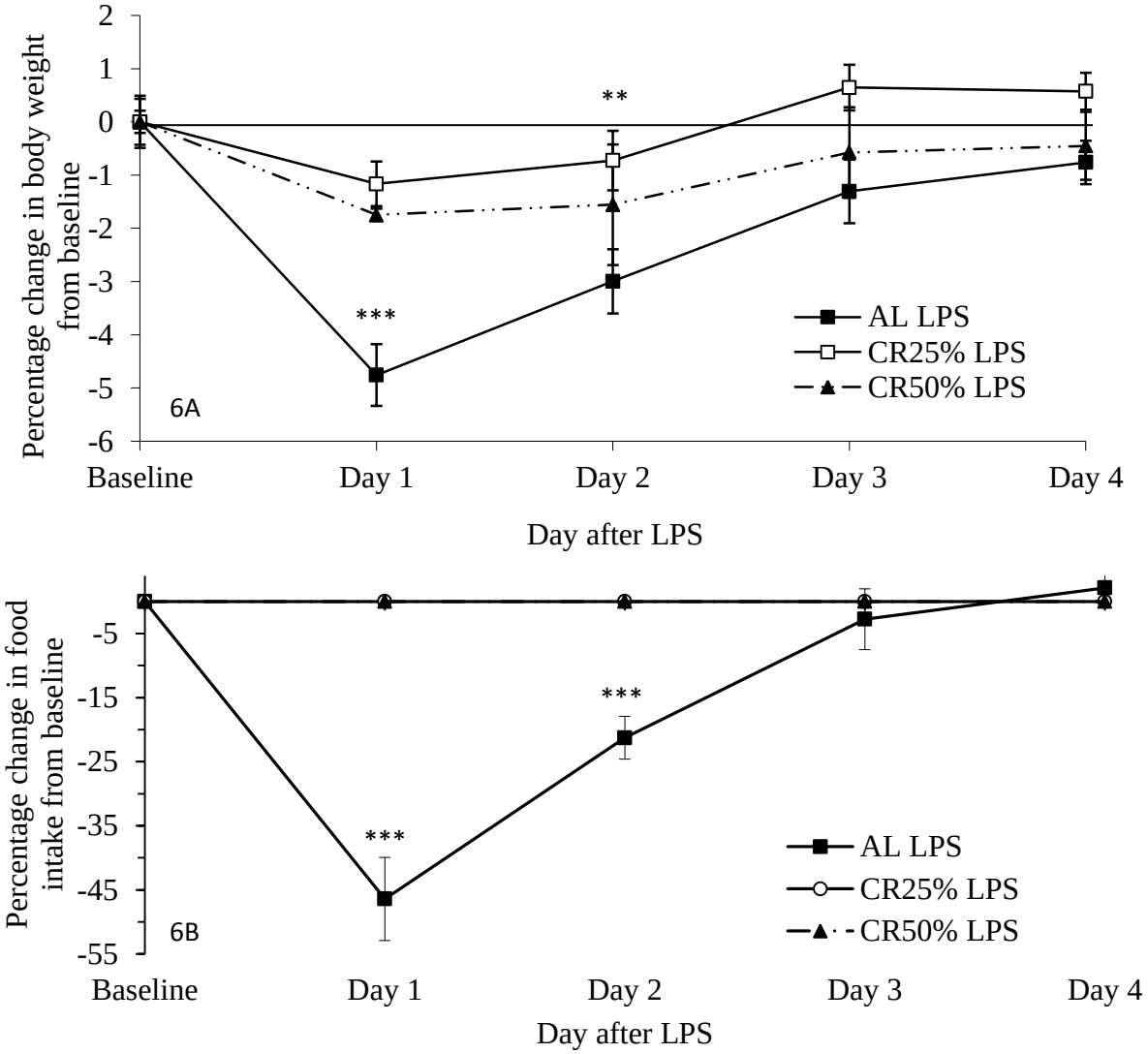

Figure 6A. Mean body weight changes (%) from baseline (weight on day of LPS injection subtracted from the day immediately prior to the LPS injection; ± SEM) for AL LPS (n = 13),

CR25% LPS (n = 10), and CR50% LPS (n = 8) animals for baseline (average of 4 days pre-LPS injection) and four days post-LPS injection. (**) denotes a significant difference between the AL LPS group and the CR25% LPS group at $p < .05$, (***) denotes a significant difference from the AL group and both CR groups at $p < .001$.

Figure 6B. Mean (± SEM) % change from baseline (the day of LPS subtracted from the baseline average) in food intake for AL (n = 10), CR25% (n = 9), and CR50% (n = 8) animals. (***) denotes a significant difference from both CR groups at $p < .001$.

Experiment 2 – mRNA levels of IκB-α, COX-2, mPGES-1 SOCS3, IL-10, NPY, POMC, leptin, CRH, GR, and MR

We compared expression levels of IκB-α, COX-2, mPGES-1, SOCS3, IL-10, NPY, POMC, leptin, CRH, GR, and MR mRNA in the hypothalamus of AL and CR50% mice at 0, 2, and 4 hours post-LPS injection. The only differences at baseline were in NPY and CRH expression with levels increased in CR50% mice (Figure 8). IκB-α hypothalamic expression mRNA was different among the groups (Figure 7), with an ANOVA revealing effects for hour [$F(2,33) = 26.49$, $p < .001$, partial $\eta^2 = .62$], group [$F(1,33) = 13.41$, $p = .001$, partial $\eta^2 = .29$], and their interaction [$F(2,33) = 3.73$, $p = .035$, partial $\eta^2 = .18$]. Expression of IκB-α was similar at baseline in the AL and CR50% animals, and both groups demonstrated a similar increase in IκB-α expression at 2 hours post-LPS ($p < .001$ for both). However, at 4 hours post-LPS the CR50% animals demonstrated significantly higher expression of IκB-α compared to the AL animals ($p < .001$).

ANOVA revealed a significant interaction effect for COX-2 expression [$F(2,32) = 3.8$, $p = .033$, partial $\eta^2 = .19$] (Figure 7). Analysis of simple main effects revealed that in the AL animals' COX-2 was significantly increased 2 hours post-LPS compared to baseline (p =

.036), and then by 4 hours post-LPS COX-2 levels were reduced to baseline levels (p = .033, compared to 2 hours post-LPS). The CR50% animals' COX-2 expression was similar at baseline and 2 hours post-LPS; however, was slightly increased at 4 hours post-LPS (p = .020). The AL animals' COX-2 expression was slightly higher than the CR50% animals' at 2 hours post-LPS (p = .057).

mPGES-1 expression was similar at baseline for the AL and CR50% animals, however, differences were seen at the other two time points (Figure 7). ANOVA revealed an effect for hour [$F(2,30) = 16.81$, $p < .001$, partial $\eta^2 = .53$], and its interaction with group [$F(2,30) = 7.55$, $p = .002$, partial $\eta^2 = .34$]. Similar to the COX-2 results, the AL animals demonstrated an increase in mPGES-1 expression at 2 hours post-LPS ($p < .001$) and a return to baseline levels by 4 hours post-LPS (compared to 2 hours post-LPS, $p = .015$). In comparison, the CR50% animals demonstrated no increase in mPGES-1 expression at 2 hours post-LPS, and displayed a significantly lower expression compared to the AL animals ($p = .008$). Again, as with COX-2, the CR50% animals' expression of mPGES-1 increased from 2 to 4 hours post-LPS ($p = .009$), and was also higher than the AL animals' expression at 4 hours post-LPS ($p = .019$).

ANOVA found an interaction effect for SOCS3 expression [$F(2,33) = 7.81$, $p = .002$, partial $\eta^2 = .32$] (Figure 7). Simple main effects revealed that AL animals had an increase in SOCS3 expression at 2 hours post-LPS compared to baseline ($p = .031$) and a further increase from 2 hours to 4 hours post-LPS ($p = .002$). The CR50% animals' expression of SOCS3 at 2 hours post-LPS was not different from baseline; however they had a 45-fold increase at 4 hours post-LPS compared to baseline ($p = .002$). This was almost 2 ½ times higher than the expression of AL animals at the same time ($p < .001$).

IL-10 mRNA expression differed between the groups as evidenced by ANOVA finding a significant effect for hour [$F(1,30) = 4.70$, $p = .038$, partial $\eta^2 = .14$] and group [$F(2,30) = 13.55$, $p < .001$, partial $\eta^2 = .48$] (Figure 7). Both AL and CR50% animals' IL-10 expression increased from baseline at 2 hours post-LPS ($p < .001$ for both). The AL animals' expression was declining by 4 hours post-LPS ($p = .016$); however, the CR50% animals' IL-10 expression remained at a heightened level, differing from the AL animals' at 4 hours post-LPS ($p = .017$).

Hypothalamic expression of NPY was significantly different between the AL and CR50% animals [$F(1,33) = 32.75$, $p < .001$, partial $\eta^2 = .50$] (Figure 8). The CR50% animals' expression of NPY was significantly higher compared to the AL expression at 0, 2, and 4 hours post-LPS ($p < .001$, $p = .030$, and $p = .007$ respectively). AL animals' expression of NPY remained consistent across the three time points, whereas the CR50% animals' expression of NPY was the highest at baseline, with slight, but non-significant, decreases at 2 and 4 hours post-LPS ($p = .053$ and $p = .074$ respectively compared to baseline).

ANOVA found a group effect for leptin expression post-LPS [$F(1,27) = 5.16$, $p = .031$, partial $\eta^2 = .16$] (Figure 8). AL animals displayed a significantly higher expression of leptin mRNA at 2 hours post-LPS compared to the CR50% animals ($p = .020$). ANOVA found an interaction between group and time for POMC expression [$F(2,33) = 4.72$, $p = .016$, partial $\eta^2 = .22$] (Figure 7). Simple main effects revealed that the AL animals had a significant increase in POMC expression from baseline to 2 hours post-LPS ($p = .017$). This was also greater than the expression levels in the CR50% animals at this time ($p < .001$). The CR50% animals' POMC expression increased from 2 hours post-LPS to 4 hours post-LPS ($p = .017$).

ANOVA found an interaction effect for CRH expression [$F(2,33) = 9.24$, $p < .001$, partial $\eta^2 = .36$] (Figure 8). CR50% animals had almost 3 times the level of CRH expression of AL animals at baseline ($p = .002$), but this was essentially reversed at 2 hours post-LPS ($p = .010$). The AL and CR50% animals displayed the opposite pattern of CRH expression after LPS. AL animals' expression increased from baseline to 2 hours post-LPS ($p = .020$), and the CR50% animals' expression decreased from baseline to 2 hours post-LPS ($p = .004$). Expression of hypothalamic GR and MR did not differ between the AL and CR50% animals either before or after LPS (data not shown).

The relationship between all of the PCR variables was investigated and it was determined that at 2 hours post-LPS the CR50% animals demonstrated a strong positive relationship between NPY and both SOCS3 ($r = .906$, $p = .034$) and IκB-α ($r = .898$, $p = .038$). AL animals demonstrated a strong negative correlation between CRH and SOCS3 ($r = -.841$, $p = .036$) at 2 hours post-LPS. Further, at 4 hours post-LPS there was a strong positive relationship between IκB-α and mPGES-1 ($r = .878$, $p = .009$) and IL-10 ($r = .786$, $p = .036$) and SOCS3 was positively related to mPGES-1 ($r = .831$, $p = .021$), IκB-α ($r = .984$, $p < .001$), and IL-10 ($r = .756$, $p = .049$) in the AL animals.

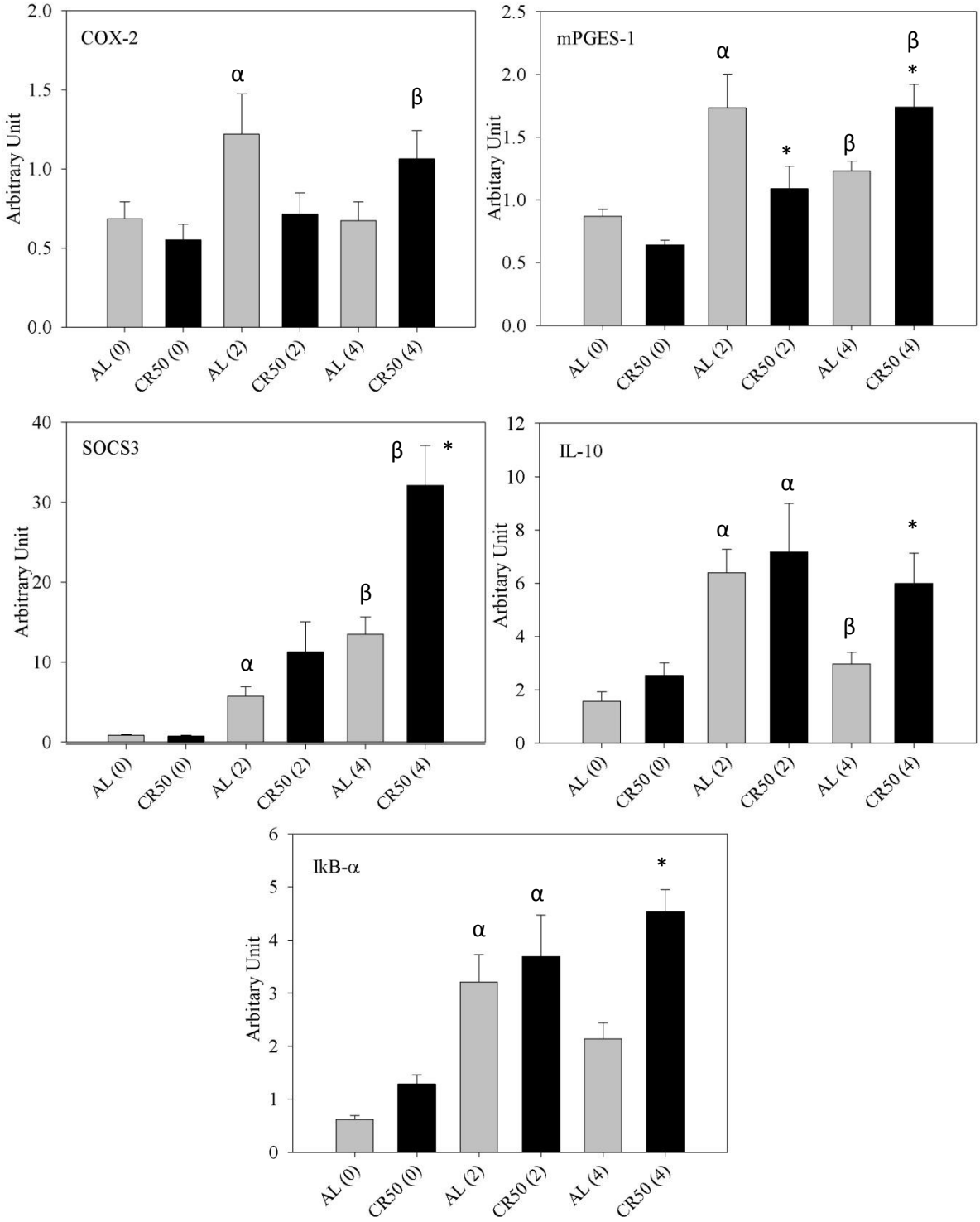

Figure 7. Mean (±SEM) hypothalamic mRNA expression levels of IκB-α, COX-2, mPGES-1, SOCS3, and IL-10 at 0, 2, and 4 hours post-LPS in AL and CR50% animals (*n*'s ranging

from 5 to 7 animals per group). (*) denotes a significant difference between the CR50% group and the AL group of the same hour, (α) denotes a significant difference within the same dietary manipulation compared to baseline, (β) denotes a significant difference within the same dietary manipulation compared to 2 hours post-LPS.

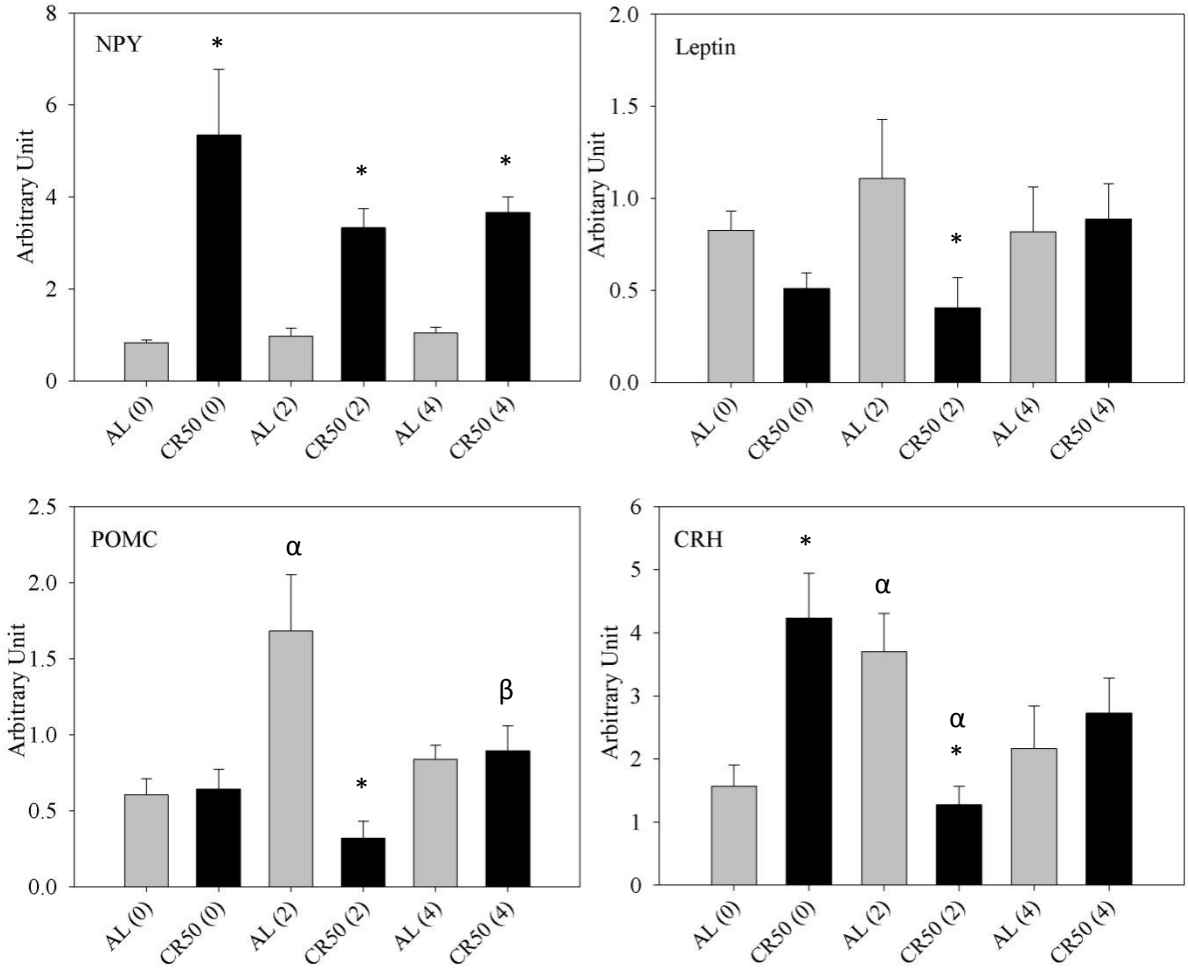

Figure 8. Mean (±SEM) hypothalamic mRNA expression levels of NPY, leptin, POMC, and CRH at 0, 2, and 4 hours post-LPS in AL and CR50% animals (*n*'s ranging from 5 to 7 animals per group). (*) denotes a significant difference between the CR50% group and the AL group of the same hour, (α) denotes a significant difference within the same dietary

manipulation compared to baseline, (β) denotes a significant difference within the same dietary manipulation compared to 2 hours post-LPS.

Discussion

The main finding of this study was that CR attenuates LPS-induced sickness behaviour. CR50% mice demonstrated a complete attenuation of sickness behaviour (fever, loss of appetite and body weight), in comparison to AL fed controls; however, the CR25% mice showed a partial attenuation of sickness behaviour. CR25% mice displayed a fever shorter in duration, with the same peak compared to the AL mice, whereas the CR50% mice did not develop fevers. The CR50% mice did not demonstrate the expected rise in hypothalamic mRNA expression of COX-2, mPGES-1, or POMC. CRH was reduced compared to baseline at 2 hours post-LPS in the CR50% animals. AL animals displayed the expected increase in leptin 2 hours post-LPS; however, CR50% animals demonstrated no increase in leptin at this time point. Further, the CR50% mice demonstrated 2 ½ times the expression of SOCS3 and an increase in IL-10 at 4 hours post-LPS compared to the AL animals. In combination these results provide a possible explanation of how CR attenuates initial pro-inflammatory pathways and enhances subsequent anti-inflammatory pathways. Further, these findings may provide a stepping stone towards further research investigating chronic inflammation and associated illnesses, and potentially aid in providing treatments for these conditions.

Effect of calorie restriction

Consistent with previous research (Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996), the CR groups demonstrated a reduction in core T_b following CR onset. The

CR50% animals had significantly lower core T_b than AL during both the light and dark phases after only one week of CR. CR25% animals displayed significantly lower core T_b than AL during the dark phase after one week of CR, however, did not differ from AL during the light phase for the entire CR period. This latter effect was most likely due to the CR animals increase in activity during the light phase. CR slows metabolic rate due to attempts to conserve energy (Martin et al., 2007), which results in a reduction in the amount of cytotoxic agents released by macrophages and ultimately reduces oxidative damage to cells (Chung, Kim, Kim, Choi, & Yu, 2002). This has implications for the proposed longevity effects of CR as reduced core T_b, metabolic adaptation, and decreased oxidative stress are proposed biomarkers of longevity in rodent and monkey CR research (Lane et al., 1996).

Locomotor activity was unaffected for the first two weeks of CR; however, during the third week both CR25% and CR50% animals were more active during the light phase compared to AL mice and were no different to AL fed mice during the dark phase. This was due to the large amount of anticipatory behaviour that the CR animals exhibit before feeding time. Food related anticipatory behaviour, the outcome of an entrainment process initiated by a set meal time, and the capacity for limited food availability to shift circadian rhythms is well known (Mistlberger, 2009). However, behavioural anticipation stands out most clearly when the food is provided to the animals in the middle of their usual rest phase, the light cycle (Challet, Pevet, Vivien-Roels, & Malan, 1997; Mistlberger, 2009), which was not the case in the current study, with food being provided just before the dark phase. The only difference during the dark phase was during the fourth week of CR when the CR50% mice were less active than the AL fed mice. Surprisingly, total activity in both CR groups was not reduced compared to the AL group. Instead, there was an adjustment of when the CR animals were active, shifting to more activity during the light phase, and less during the dark phase.

Calorie restriction and sickness behaviour

The current study is the first investigation of the effect of CR on the development of sickness behaviour and demonstrates a dose-dependent relationship between CR and sickness behaviour development. The CR50% group demonstrated a complete attenuation of sickness behaviour on all measures except for a reduction in locomotor activity. The CR50% group did not develop a fever after LPS and demonstrated a similar T_b profile to the AL and CR50% animals that received saline injections. The CR50% animals locomotor activity decreased post-LPS, however, it had returned to baseline levels by 12 hours post-LPS, which was when the AL mice were still exhibiting a significant decrease in locomotor activity. The CR25% group also displayed a partial attenuation of sickness behaviour. The CR25% animals generated a fever which had a similar peak to that seen in AL; however, it lasted only half as long. Both 25% and 50% CR mice demonstrated a full attenuation of LPS-induced body weight loss and anorexia.

It is interesting to note that the CR50% animals did not display any sickness behaviour responses apart from the reduction in locomotor activity. Voluntary locomotor activity has been reported to be the most sensitive measure of illness (Skinner, Mitchell, & Harden, 2009). Our results support these previous findings as locomotor activity is the only behavioural indicator of illness in CR animals. Further, as mentioned earlier, fever is exhibited in a biphasic manner, of which the second phase can be altered (i.e., hypothermia) in cases of low food availability (Shido et al., 1989). However, the CR50% animals in this study did not demonstrate either the first or second phase of the biphasic fever. Lococmotor activity is also differentially characterised in each phase of fever (Romanovsky et al., 1996). The AL (231.5% ± 56.5) and CR25% (288.9% ± 69.6) animals demonstrated an increase in

locomotor activity during the first hour post-LPS; however, the CR50% (62.7% ± 27.1) animals only demonstrated a slight increase compared to baseline during this time (data not shown). All of the groups then demonstrated a decrease in locomotor activity during the later phase of fever. The increase in activity during the first phase and decrease during the second phase of fever in the AL and CR25% mice is expected after an LPS injection (Romanovsky et al., 1996); however, the CR50% animals do not follow the same pattern. It has been suggested that locomotor activity acts as a thermoregulatory effector, therefore, in cases where the animals' locomotor activity is reduced, so too is their T_b (De Castro, 1980). The lack of an increase in activity in the CR50% mice may contribute to the lack of fever or it may be a reflection of changes in the pro- and anti-inflammatory pathways. In addition, the CR animals may find the development of a fever too metabolically costly due to their already reduced metabolic rate (Forsum, Hillman, & Nesheim, 1981). A limitation of the current study was the lack of measuring brown fat thermogenesis, which has been shown to be reduced in CR rats (Rothwell & Stock, 1982); there have been suggestions of a correlation between the amount of brown fat and fever intensity, with reduced levels of brown fat being linked to reduced fever intensity (Cannon, Houstek, & Nedergaard, 1998). However, it is important to note that the thermogenic ability of the CR25% and CR50% mice was intact, as evidenced by the locomotor activity induced increase in T_b prior to being fed during the CR period (data not shown). The CR25% and CR50% mice demonstrated a 2 to 2.5 °C increase in T_b in the two hours prior to the onset of the dark phase and food delivery.

The AL animals displayed an anorexic effect reducing their food intake (-47%) to roughly the same level as that of the CR50% mice. The presence of an increased drive to eat in CR animals was supported by previous research, where food deprived, IL-1 treated rats ate approximately 60% more food than ad libitum fed animals also administered with IL-1 and

approximately 40% more than AL rats not administered with IL-1 (Mrosovsky et al., 1989). Further investigations involving food-deprivation have demonstrated that LPS-induced reductions in food intake and weight loss are attenuated (Gautron, Mingam, Moranis, Combe, & Layé, 2005), as are LPS-induced increases in T_b and some cytokine levels (Inoue et al., 2008). The drive to eat in these food deprived animals, and also in the CR animals in the current study, may be more pronounced than actually displaying sickness behaviour. A possible alternative explanation for why the CR mice demonstrate an attenuation of sickness behaviour may be because of weight differences between CR and AL fed mice. This meant the total amount of LPS administered to these groups differed by more than 15% and that this difference in dosing might have accounted for at least some of the observed results. However, the CR25% mice weighed on average 24.5 g and the CR50% mice weighed on average 23.8 g, whilst the AL mice weighted on average 29.2 g on the day of LPS injections. Even though the CR animals' weight was very similar (and would therefore have received a similar dose of LPS) we still observed a 2 °C fever in the CR25% mice, albeit shorter than the fever seen in the AL mice; and no fever in the CR50% mice. Therefore, we believe that a potential 15% difference in dose (which would equate to approximately 57 μg/kg) between the AL and CR animals would not elicit a different fever profile in the CR animals.

Calorie restriction, sickness behaviour and a potential mechanism

To determine the possible central mediators of the attenuation of sickness behaviour hypothalamic mRNA levels of IκB-α, COX-2, mPGES-1, SOCS3, IL-10, NPY, POMC, leptin, CRH, GR, and MR were investigated. IκB-α mRNA expression was similar for both dietary groups at baseline and 2 hours post-LPS. However, at 4 hours post-LPS, when the AL animals' IκB-α expression was declining, the CR50% animals' expression increased. IκB-α is

known to act transiently during the inflammatory process, ensuring that the NF-κB response is shut down; this is the first component of the biphasic expression of NF-κB. The other isoform of IκB, IκB-β, is responsible for the extended expression of NF-κB expression during inflammation (Thompson, Phillips, Erdjument-Bromage, Tempst, & Ghosh, 1995). Therefore, the heightened expression of IκB-α in the CR50% animals at 4 hours post-LPS may indicate an over activation of the IκB-α response in the CR animals.

Although the AL and CR50% animals had similar baseline levels of COX-2, only the AL mice demonstrated the expected increase in COX-2 expression 2 hours post-LPS. Others have investigated COX-2 mRNA levels 2 hours after LPS in animals fasted for 48 hours and found no difference from controls (Inoue et al., 2008). The discrepancy between the fasted and CR animals may be due to the two different dietary regimens, which have been shown to induce slightly different changes in mRNA profiles in the hypothalamus (Bi et al., 2003; Brady, Smith, Gold, & Herkenham, 1990). This lack of increase in COX-2 [the rate-limiting step in PGE_2 production (Vane, Bakhle, & Blotting, 1998), thus being the main driver of fever production] is incredibly important, and may be a major element in inhibiting the CR50% mice from developing a fever to LPS. Curiously, the CR50% animals demonstrated an increase in COX-2 expression but still do not demonstrate a fever at 4 hours post-LPS. This time frame was not investigated by Inoue et al. (2008), and therefore, we are unable to make direct comparisons with their data. However, it may again indicate that the CR animals, in contrast to fasted animals, display a delayed inflammatory response at 4 hours post-LPS. Further, these CR animals still do not demonstrate the expected increase in T_b that is associated with increased COX-2 expression, which may indicate that the inhibition of the T_b increase in the CR mice may be occurring further along the COX-2 pathway such as reduced levels of PGE_2. It was shown that PGE_2 levels in spleen cells exposed to LPS were

approximately 50% lower in 40% CR rats compared to control rats, possibly due to a reduction in arachidonic acid, a precursor to PGE_2 (Venkatraman & Fernandes, 1992).

mPGES-1 expression profiles were similar to COX-2, which is not surprising given their direct involvement with each other, mPGES-1, a PGE_2 synthase enzyme, is functionally coupled with COX-2 (Murakami et al., 2000); however, mPGES-1 continues to be expressed once COX-2 has declined (Ivanov & Romanovsky, 2004). Further, mPGES-1 is increased during inflammation and is down-regulated by glucocorticoids (Ivanov, Pero, Scheck, & Romanovsky, 2002; Kudo & Murakami, 2005). AL animals demonstrated the expected rise in mPGES-1 at 2 hours post-LPS; in contrast, the CR50% animals demonstrated no increase in mPGES-1 at 2 hours post-LPS but an increase was noted at 4 hours post-LPS, which was essentially the same as COX-2 mRNA expression. Inoue et al. (2008) also investigated mPGES-1 expression in fasted and control animals, and similar to COX-2, found no difference between the dietary groups (i.e., the fasted group still demonstrated the expected rise in mPGES-1 expression at 2 hours post-LPS). This is further evidence that food deprivation and CR can produce different physiological outcomes.

SOCS3 expression was similar at baseline between the AL and CR50% animals, and both groups demonstrated an increase at 2 and 4 hours post-LPS compared to their respective baselines. The AL and CR50% animals were not significantly different at 2 hours post-LPS, which is consistent with previous research looking at SOCS3 2 hours after LPS in fasted animals (Inoue et al., 2008). However, at 4 hours post-LPS the CR50% animals demonstrated more than twice the mRNA expression levels of SOCS3 compared to the AL animals. SOCS3 is a known feedback inhibitor of cytokine signalling, and is induced by IL-6 and IL-10. SOCS3 acts to inhibit the production of IL-6 and aids in IL-10's anti-inflammatory properties [for example, suppressing the release of TNF-α (Bogdan et al., 1991)] and has

been suggested to induce the expression of IL-1ra (Berlato et al., 2002). It has been established that of all the pro-inflammatory cytokines, IL-6 most closely correlates with the magnitude of fever (Cartmell, Poole, Turnbull, Rothwell, & Luheshi, 2000; LeMay, Vander, & Kluger, 1990), and it has been demonstrated that IL-6 deficiency or treatment with IL-6 anti-serum can almost completely block fever induced by LPS (Rummel, Sachot, Poole, & Luheshi, 2006). Even more interesting in light of our findings is that IL-6 can directly induce COX-2 expression in the brain (Rummel et al., 2006), which in our study was attenuated at 2 hours post-LPS compared to the AL animals. Therefore, in the present study, an increase in expression of SOCS3 could lead to attenuated production and release of IL-6 after LPS, and contribute to the attenuated fever seen after LPS in CR animals. Unfortunately we did not measure circulating levels of IL-6, and therefore cannot make a conclusion regarding this. However, it is also interesting that this increase in SOCS3 in the CR animals again occurs at 4 hours post-LPS (as with some of the other compounds we have investigated), which points to a stunting of the immune response at 2 hours post-LPS.

Mirroring the expression of SOCS3 was IL-10, with the AL and CR50% animals demonstrating similar patterns of IL-10 mRNA expression at baseline and 2 hours post-LPS. However, at 4 hours post-LPS, as with SOCS3, the CR50% animals demonstrate a significantly higher expression level compared to the AL animals. As stated above, IL-10 is known to induce the activation of SOCS3 (Cassatella et al., 1999), which may account for the heightened expression of SOCS3 in the CR animals. Further, IL-10 has potent anti-inflammatory properties (Berlato et al., 2002; Bogdan et al., 1991; Leon, Kozak, Rudolph, & Kluger, 1999), and although COX-2 and mPGES-1 are increased at 4 hours post-LPS in the CR animals, it may be the case that the induction of IL-10 and consequently SOCS3 is enabling the CR animals to continue to display no increase in T_b post-LPS.

As expected, at all three time points post-LPS the CR50% animals had significantly higher levels of hypothalamic NPY mRNA expression compared to the AL animals. CR is well known to result in increases in NPY (Bi et al., 2003; Brady et al., 1990; Lewis et al., 1993; McShane, Wilson, & Wise, 1999). Importantly for our results, when NPY is administered centrally, it antagonises the effects of centrally administered IL-1β in a dose-dependent manner, and thus leads to an elimination of anorexia (Sonti, Ilyin, & Plata-Salamán, 1996) and stabilises and reduces the rise in T_b associated with intravenously administered LPS-induced fever (Felies, von Horsten, Pabst, & Nave, 2004). Therefore, naturally occurring high levels of NPY in the CR animals may play a contributory role in the attenuation of sickness behaviour seen in the CR animals.

In the current study hypothalamic expression of POMC was similar at baseline for both AL and CR50% animals. This finding is in agreement with previous research demonstrating no change in POMC expression after CR (Gautron et al., 2005; Han et al., 1995; Harrold, Williams, & Widdowson, 1999). However, others have found that POMC is reduced after a period of CR (Brady et al., 1990; Lindblom, Haitina, Fredriksson, & Schiöth, 2005). Even though circulating CORT levels are often increased after CR (Han et al., 1995; Holmes et al., 1997; Levay et al., 2010; Sabatino, Masoro, McMahan, & Kuhn, 1991), earlier components of the HPA axis are often unaffected such as ACTH, which is synthesised from POMC (Armario, Montero, & Jolin, 1987). This highlights that HPA axis alterations after CR have yet to be fully understood.

At 2 hours post LPS the AL animals had significantly higher POMC expression compared to the CR50% animals, which has also been seen in animals fasted for 39.5 hours before LPS treatment (Gayle, Ilyin, & Plata-Salamán, 1999). LPS increases leptin (Francis, MohanKumar, MohanKumar, & Quadri, 1999), which in turn induces POMC activation

(Elias et al., 1999). The CR50% animals in the current study demonstrated an attenuation of leptin expression at 2 hours post-LPS. Therefore we can speculate that this may be resulting in the reduction in POMC also seen in the CR50% animals at 2 hours post-LPS. In addition, it has been recognised that POMC derived melanocortin peptides reduce food intake via the activation of the melanocortin 4-receptor (MC4R), and that reduced POMC expression can lead to hyperphagia and obesity (Schwartz, Woods, Porte Jr., Seeley, & Baskin, 2000). Leptin has been widely demonstrated to have pro-inflammatory properties and has been shown to mediate the reduction in food intake following LPS (Harden, du Plessis, Poole, & Laburn, 2006; Luheshi, Gardner, Rushforth, Loudon, & Rothwell, 1999; Sachot, Poole, & Luheshi, 2004). The attenuated increase in POMC and leptin at 2 hours post-LPS seen in the CR50% animals may contribute to the CR animals' attenuated anorexia and weight loss. Further, the CR50% animals did not demonstrate a significant reduction in leptin at baseline, although there was a trend towards this being the case. This may also be why there was no reduction in baseline POMC in the current study, given the relationship between POMC and leptin.

Hypothalamic expression levels of CRH were significantly elevated at baseline in the CR50% animals compared to the AL; however, at 2 hours post-LPS the AL animals had a significant increase, and the CR50% animals had a significant decrease in CRH expression. The profile of the AL animals CRH expression during LPS-stimulation is expected, as higher levels of CRH are associated with reduced feeding and weight loss (Bradbury, McBurnie, Denton, Lee, & Vale, 2000; Spina et al., 1996). The profile of the CR50% animals CRH expression, higher expression at baseline and lower expression at 2 hours post-LPS compared to the AL may be another contributing factor to the lack of an anorexic and cachexic effect in CR50% mice. Further, it has been demonstrated that elevated plasma CORT levels, often seen in CR animals (Han et al., 1995; Levay et al., 2010), act to inhibit the release of CRH

via activation of the glucocorticoid receptors in the hippocampus, hypothalamus, and the pituitary (Patel & Finch, 2002), which may be the case for the CR50% animals post-LPS.

In the current study hypothalamic expression levels of GR and MR did not differ between the AL and CR50% animals. GR and MR control the IL-1 and IL-1ra system during an inflammatory process, by decreasing IL-1 expression and increasing IL-1ra expression (Sauer et al., 1996). As mentioned in the discussion of POMC, further investigation is of importance to further elucidate the role CR plays in altering certain compounds within the HPA axis, and more specifically, what role CR plays in altering HPA compounds during an immune response.

Of interest in relation to the correlation of the PCR variables SOCS3 expression at 4 hours post-LPS in the AL animals was positively related to mPGES-1, IκB-α and IL-10 expression and IκB-α expression was positively related to both mPGES-1 and IL-10 expression at the same time point. The positive relationship between SOCS3 and IL-10 is expected due to their known involvement with each other, namely IL-10 inducing the activation of SOCS3 (Cassatella et al., 1999). However, this was not the case with the CR50% animals, demonstrating no relationship between these variables at the same time point. The most notable relationship between the variables in the CR50% animals was a positive relationship between NPY and both IκB-α and SOCS3 at 2 hours post-LPS. These three compounds have been described above as all playing a role in the anti-inflammatory response post-LPS (Berlato et al., 2002; Bogdan et al., 1991; Sonti et al., 1996; Thompson et al., 1995) and indicates that they are potentially working in unison to attenuate sickness behaviour in CR animals.

Conclusion

The present study is the first to reveal that CR results in a suppression of sickness behaviour responses, which may be due to CR leading to a reduction in energy metabolism and/or influencing several central nervous, endocrine, and immune mechanisms. Further to this, it was found that a number of hypothalamic mRNA profiles were altered in the CR50% animals at baseline and post-LPS stimulation. COX-2 and mPGES-1 expression were attenuated at 2 hours post-LPS in the CR50% mice, indicating that these compounds play a major role in the attenuated fever seen in the CR50% animals. There was a significant increase in SOCS3 expression in the CR50% animals at 4 hours post-LPS, which was also reflected in an increase in IL-10 expression at 4 hours post-LPS. Further, there was an attenuation of both POMC and leptin expression at 2 hours post-LPS, which has implication for both weight loss and food intake post-LPS. With regard to the shortened febrile responses of the CR25% animals, the present study also hints at the possibility that CR may affect these mechanisms in a dose-dependent manner according to the level of CR that mice are subjected to. The findings provide a stepping-stone towards further research investigating the effect of CR on sickness behaviour, and further exploration of the mechanisms involved may have important implications for the management of chronic inflammation, illness, and disease.

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Appendix A

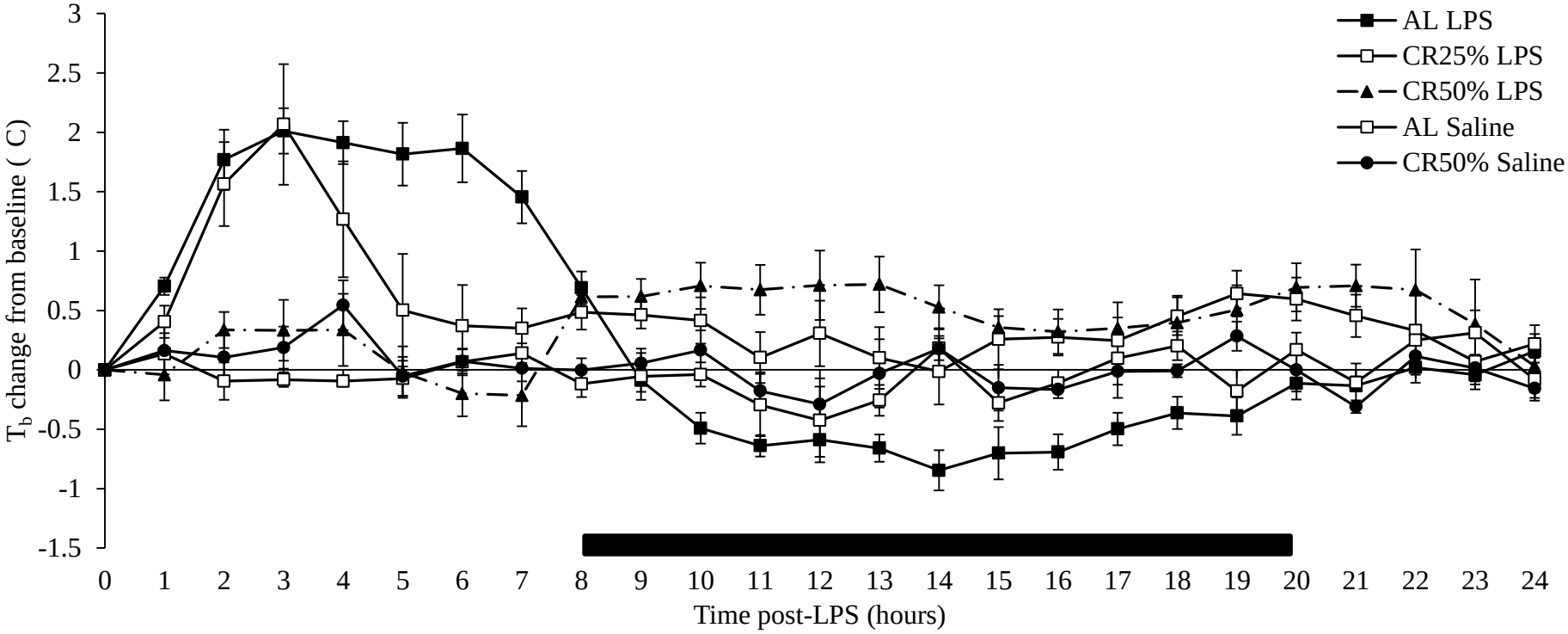

Figure A1. Mean T_b change from baseline (the average of each hour during a 24-hour period of the 4 days before LPS administration; ±SEM) for AL LPS (*n* = 9), CR25% LPS (*n* = 10), and CR50% LPS (*n* = 8) animals, for 8 hours post-injection with the black box representing the dark phase.

CHAPTER 5

CALORIE RESTRICTION ATTENUATES LPS-INDUCED SICKNESS BEHAVIOUR AND INCREASES CIRCULATING CORT, DECREASES CIRCULATING IL-6, AND DOES NOT ALTER CIRCULATING IL-10 LEVELS

Abstract

Previously we have demonstrated that sickness behaviour after lipopolysaccharide (LPS) is attenuated by a 50% calorie restriction (CR) for 28 days in mice and that CR mice demonstrate a central anti-inflammatory bias. This study examined the effect of CR on sickness behaviour (fever, anorexia, cachexia) and peripheral immune markers post-LPS. Male Sprague-Dawley rats fed ad libitum or restricted in food intake by 50% (CR50%) for 28 days were injected on day 29with 50μg/kg of LPS or saline (1mL/500g). Changes in body temperature (T_b), locomotor activity, body weight, and food intake were determined. A separate cohort of rats was fed ad libitum or CR50% for 28 days, and serum levels of corticotserone (CORT), interleukin 6 (IL-6), and IL-10 were determined at 0, 2, and 4 hours post-LPS. The CR50% rats demonstrated an attenuation of sickness behaviour, which resulted in no fever, a limited reduction in locomotor activity, no sign of anorexia, and reduced cachexia following LPS. Serum CORT levels were significantly increased at 2 hours post-LPS in both groups; however they were almost two times larger in the CR50% animals. The increase in circulating levels of IL-6 was significantly attenuated at 2 hours post-LPS in the CR50% animals. IL-10 levels were similar post-LPS in control and CR50% rats. These findings further suggest that CR results in an enhanced anti-inflammatory response in the form of increased CORT and diminished pro-inflammatory signals as evidenced by an attenuated increase in IL-6. Together these contribute to the observation of decreased sickness behaviour.

It has been well established that calorie restriction (CR) can prolong lifespan (Weindruch, Walford, Fligiel, & Guthrie, 1986), reduce the occurrence of age-related diseases such as cancer (Matsuzaki et al., 2000), lessen the severity of the neurochemical deficits and motor dysfunction seen in primate models of Parkinson's disease (Maswood et al., 2004), and attenuate the 'normal' immunosenscence seen with age (Mascarucci et al., 2002). To date there has been limited investigation on the impact CR plays on the development of sickness behaviour. We have previously demonstrated that a 50% CR for 28 days attenuates sickness behaviour in mice (MacDonald, Radler, Paolini, & Kent, 2011). The CR mice demonstrated no increase in body temperature (T_b) normally seen after lipopolysaccharide (LPS) administration, no anorexia or cachexia, and only a limited reduction in locomotor activity.

Further to this, the CR mice exhibited central anti-inflammatory bias post-LPS. This was demonstrated by an attenuated increase of the expression of hypothalamic cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) in CR mice at 2 hours post-LPS. Further, hypothalamic suppressor of cytokine signalling 3 (SOCS3) mRNA expression was increased in the CR50% mice (twice that of the controls) at four hours post-LPS, which led to the conclusion that SOCS3 may play an important role in the attenuation in sickness behaviour seen in the CR mice (MacDonald et al., 2011). Hypothalamic mRNA levels of the anti-inflammatory cytokine interleukin-10 (IL-10) were significantly increased in the CR mice 4 hours post-LPS. SOCS3 is a known feedback regulator of cytokine production; it is induced byIL-10 (Cassatella et al., 1999), inhibits the production of IL-6 (Lang et al., 2003), and aids in IL-10's anti-inflammatory actions (Bogdan, Vodovotz, & Nathan, 1991). Therefore, it is imperative to investigate peripheral levels of IL-10 in CR animals to determine if they are increased in accordance with hypothalamic IL-10 levels. IL-6 is critically important for LPS-induced fever, with IL-6

deficient mice unable to develop fevers post-LPS injection (Chai, Gatti, Toniatti, Poli, & Bartfai, 1996). In addition to IL-10, investigations of peripheral levels of IL-6 can be seen as imperative due to the previously mentioned relationship IL-6 has with SOCS3 (Lang et al., 2003). In previous work we have shown that SOCS3 is dramatically increased in CR animals after LPS (MacDonald et al., 2011) and thus may be working to inhibit the release of IL-6 during the inflammatory process.

Increases in anti-inflammatory compounds such as corticosterone (CORT) may also play a role in attenuating sickness behaviour following LPS. CORT is well established to exert anti-inflammatory effects which are achieved by firstly, increasing the transcription of anti-inflammatory cytokines and decreasing the transcription of pro-inflammatory cytokines (Smoak & Cidlowski, 2004); and secondly, by inhibiting the release of cytokines from macrophages (Barnes, 1998). CORT levels are well known to increase moderately after a period of CR (Han et al., 1995; Heiderstadt, McLaughlin, Wright, Walker, & Gomez-Sanchez, 2000), and serum CORT concentration increases dose-dependently with increasing severity of CR, with a significant increase in serum CORT seen in rats after only 21 days at a 12.5% CR (Levay, Tammer, Penman, Paolini, & Kent, 2010). A 20% CR in hamsters demonstrated that circulating cortisol and IL-6 activity were similar in CR animals as control animals after LPS administration and that the CR animals T_b demonstrated the same increase as controls; albeit the CR animals had a lower initial core T_b due to the dietary regimens (Conn et al., 1995). Given that the CR regimen used in this study was relatively low (20%) and that we have previously demonstrated no change in T_b post-LPS in mice (MacDonald et al., 2011) it would be interesting to see what effect a 50% CR would have on circulating CORT and IL-6 levels post-LPS.

Previously we have demonstrated that LPS-induced sickness behaviour can be attenuated by a 50% CR for 28 days in mice (MacDonald et al., 2011). The current study

aims to extend that finding to another species and also to investigate peripheral immune related targets. Due to our previous work it is hypothesised that a similar attenuation in sickness behaviour will be seen in rats CR to 50% for 28 days. Further, we expect that the peripheral targets investigated (CORT, IL-6 and IL-10) will further elucidate the mediating factors behind attenuated sickness behaviour after CR. Given our previous findings we expect that the LPS-induced increase in CORT will be enhanced in the, the increase in IL-6 will be attenuated, and that the increase in IL-10 will be increased in the CR50% rats.

Methods

Animals

Seventy-seven male Sprague-Dawley rats were procured from Monash SPF animal services (Clayton, Victoria, Australia) and allowed to acclimate to the facility for at least one week. During this period, standard rodent chow (Barastoc, Melbourne, Australia) and water were available ad libitum. At the beginning of experimentation the rats were aged between 9 to 12 weeks old. Rats were individually housed in polypropylene basin cages (30 × 50 × 15 cm) with sawdust and tissues provided as bedding. Rats were maintained at an ambient temperature of 26 ± 1 °C, which is within the thermoneutral zone for this species (Poole & Stephenson, 1977) on a 12:12 light/dark cycle (0500 – 1700 hours). Animal care and experimentation was performed in accordance with protocols approved by the La Trobe University Animal Ethics Committee.

Surgery

Following acclimation, 38 of the rats were surgically implanted in the peritoneal cavity with a biotelemetry device (E-4000, Mini-mitter®, Bend, OR, USA: 23 × 8 mm, 1.6 g) under anaesthesia as previously described (Begg, Kent, McKinley, & Mathai, 2007). Rats

were anaesthetised in an induction chamber using 4% isoflurane and 0.6 L/min oxygen flow and were maintained during surgery on 2.5% isoflurane and 0.4 L/min oxygen flow using a nose cone (refer to *Chapter 2* for more detail). These rats were allowed one to two weeks to recover before the initiation of the CR regimens.

Dietary regimens

Rats were divided into one of two CR regimens matched for weight, food intake, and age: ad lib (AL; n = 22) fed ad libitum (approximately 20-30g per day); or CR50% (n = 16) rats received 50% of the amount consumed by the AL animals (approximately 10-15g per day). The dietary composition of the control and CR50% diets has been published elsewhere (Levay, Govic, Penman, Paolini, & Kent, 2007; Levay et al., 2010). The group sizes varied across dependent variables and precise numbers used for each variable are stated in the results section. The intake of the CR groups was determined weekly based on the average daily food intake of the control group for three consecutive days. Water was continuously provided to all groups. The dietary manipulation continued for 28 days before, and four days after LPS/saline administration in Experiment 1; and for 28 days before LPS administration until sacrifice in Experiment 2. Food was provided daily, approximately one hour before the dark phase onset.

Experiment 1 – Determination of sickness behaviour after 28 days of CR

Sickness behaviour was determined by continuous measurement of core body temperature (T_b), locomotor activity, body weight, and food intake for four days pre- and four days post-LPS challenge. T_b, locomotor activity, and body weight were also determined throughout the CR period.

T_b and locomotor activity were determined by placing each cage on a receiver with each individual biotelemetry device generating a continuous frequency signal proportional to the animal's T_b (± 10^{-1} °C). The receiver sampled this frequency at 1-minute intervals and this sample was decoded by VitalView software (Mini Mitter Co., Inc., Bend, OR) and stored on a hard drive. Rats were weighed once a week approximately three hours after lights-on during dietary manipulation and daily at the same time for the four days pre- and post-LPS/saline injection using top loading scales (± 0.1 g). Food consumption was also determined at this time and was determined to the nearest .1 g by providing a set amount of food each day and weighing the remaining food 24 hours later, including the uneaten food in the bedding.

After 28 days of dietary regimen (i.e., on the 29th day) all rats were injected intraperitoneally with either 1 ml/500 g of saline or 50 μg/kg of LPS from *Escherichia coli* (serotype 0111:B4: Sigma, Castle Hill, NSW) in 1 ml/500 g saline. The LPS/saline challenge was performed approximately four hours after lights-on (ranging from 0800 hours to 1000 hours). All measures were continued for four days post-LPS.

Experiment 2 – Determination of serum levels of CORT, IL-6, and IL-10 after LPS

Thirty-nine male rats were used in Experiment 2 to determine serum levels of CORT, IL-6, and IL-10 at three time intervals after LPS administration. Rats were handled and exposed to the same dietary manipulations as in Experiment 1. There were two levels of diet (AL and CR50%) and three time points at which the rats were sacrificed (0, 2, and, 4 hours) after LPS (50 μg/kg) administration on the 29th day of CR.

Rats were rapidly decapitated with the aid of a guillotine at 0, 2, and 4 hours after LPS injection. Trunk blood was collected using plastic tubes for serum based enzyme-linked immunosorbent assays (ELISA). Tubes used to collect blood were left at room temperature for 15 minutes post-collection to ensure clotting. The blood was then centrifuged at 4 °C at

3000 rpm for 10 minutes and serum was transferred to cryovials and stored at -20 °C until required.

ELISA kits were used in accordance with the manufacturers' instructions, and samples were plated in duplicate for all assays. Serum was used to quantify CORT, with the intra- and inter-assay precision for the kit used being less than 7% and 9% respectively. The detection limit of this assay was 0.55 ng/mL (IDS, Boldon, United Kingdom). IL-6 was quantified using serum, with the intra- and inter-assay precision for the kit used being less than 9% and 10% respectively. The detection limit of this assay was 21 pg/mL (R&D Systems, MN). IL-10 was quantified using serum, with intra- and inter-assay precision for the kit used being less than 5% and 10% respectively. The detection limit for this assay was 10 pg/mL (R&D Systems, MN).

Optical density of the wells was measured at 450 nm with a Synergy HT Multi-detection Microplate reader (Bio-Tek Instruments Inc., Winooski, VT). Concentrations of the selected compounds were calculated using KC4 v 3.4 software (Bio-Tek Instruments).

Data Analysis

Analyses of sickness behaviour measures

All data collected over the 4-day baseline and 4-day testing period were averaged and means ± SEM were calculated for each group. For the T_b and body weight data a change from baseline measurement was used to determine differences between the groups due to the baseline differences between the groups on the day of injection. Data were then analysed using analysis of variance (ANOVA). To overcome violations of sphericity, the Greenhouse-Geisser statistic corrected degrees of freedom were reported. Where required, between-group simple main effects and within-group simple main effects were analysed using the Least Significant Difference method (LSD). When more than two comparisons were required, as in

the case of the T_b and locomotor activity data, a Bonferroni adjustment was used to protect for inflated type 1 error rate. Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$ as the criterion for significance.

Analyses of serum levels of CORT, IL-6, and IL-10

The effect of CR on serum levels of CORT, IL-6, and IL-10 after LPS-administration was determined by using a between groups multivariate analysis of variance (MANOVA) due to the high correlation between the three variables. Pillai's multivariate test statistic was used due to being robust in situations of unequal sample sizes and violation of assumptions (Tabachnick & Fidell, 1996). Univaritate ANOVAs were conducted for each variable for follow up analyses. A Bonferroni adjustment ($p < .017$) was used to assess the differences between groups on the univariate ANOVAs due to three comparisons being made and to protect for inflated type 1 error. Post-hoc tests were conducted where deemed appropriate using the LSD method. All data are presented as mean ± SEM. The correlation between CORT, IL-6, and IL-10 at each time point was evaluated statistically using Pearson product-moment correlation coefficient for each dietary group.

Results

Experiment 1 – Effect of CR on body weight and determination of sickness behaviour after 28 days of CR

Effect CR on body weight

The CR50% group lost weight, whereas the AL group gained weight. In the CR50% group it became evident that their weight loss plateaus at roughly day 21 of the CR period. On the initiation of the dietary regimens the AL animals weighed on average 354.8 g (± 10.9) and the CR animals weighed on average 345.8 g (± 9.5). On the last day of the CR period the

CR50% animals weighed on average 309.8 g (± 4.8) whilst the AL animals weighed on average 427.5 g (± 9.8).

Sickness behaviour measures

Core body temperature

LPS induced a fever in the AL rats whilst the CR50% animals displayed a profoundly attenuated fever profile (Figure 1). The T_b in AL animals peaked at 5 hours post-LPS at 1.1 °C (± 0.2) above baseline and the CR50% animals only recorded a 0.3 °C (± 0.1) increase in T_b from baseline at 3 hours post-LPS. The CR50% animals had a T_b profile similar to the two saline treated groups. ANOVA found a significant main effect for hour [$F(4.37,117.98 = 6.94$, $p < .001$, partial $\eta^2 = .21$], group [$F(3,27) = 14.42$, $p < .001$, partial $\eta^2 = .62$], and their interaction [$F(13.12,117.98) = 8.35$, $p < .001$, partial $\eta^2 = .48$]. Post hoc comparisons revealed a significant group and time effects from 2 hours until 6 hours post-LPS.

The AL group had a significantly higher T_b compared to both the AL saline and CR50% saline groups from 2 hours until 6 hours post-LPS ($p < .001$ for all). The CR50% animals demonstrated a lower T_b from 2 hours until 6 hours post-LPS compared to the AL animals (ranging from $p = .017$ to $p < .001$). Consequently, the CR50% animals were not different at any hour post-LPS compared to both saline groups. AL animals had a significantly higher T_b compared to baseline from 2 hours until 6 hours post-LPS (ranging from $p = .004$ to $p < .001$ for all). T_b in the CR50%, AL saline, and CR50% saline groups did not differ compared to baseline at any hour post-LPS/saline.

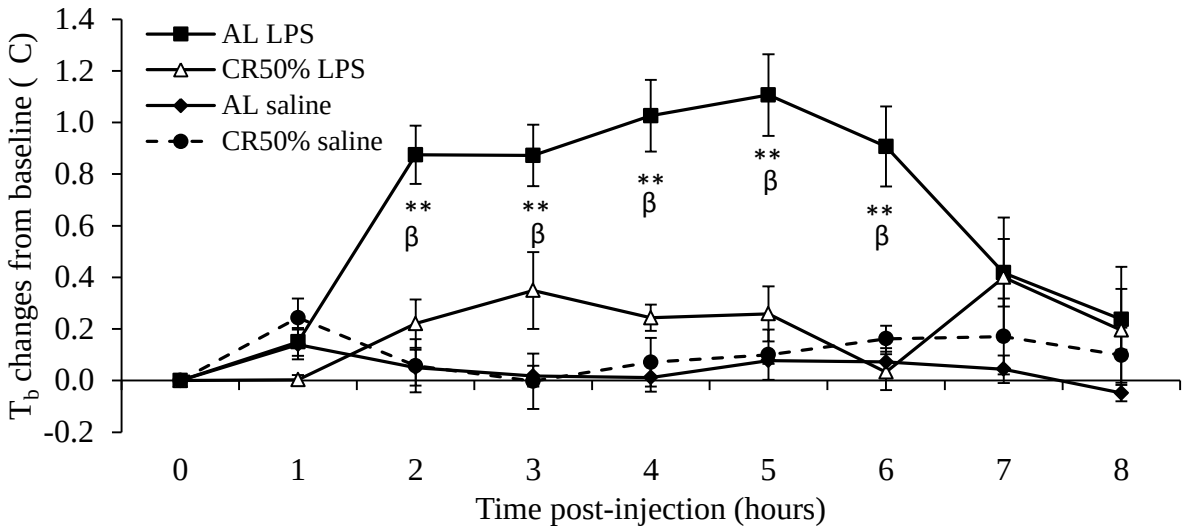

Figure 1. Mean T_b change from baseline (± SEM) for AL LPS (*n* = 14), AL saline (*n* = 8), CR50% LPS (*n* = 8), and CR50% saline (*n* = 8) animals for 8 hours post-injection – AL (LPS) and CR50% (LPS) data also presented in Figure #, Chapter 6. (**) denotes a significant difference from all other groups at $p < .01$ and (β) denotes a significant difference from baseline at $p < .01$.

Locomotor activity

For the 24 hour period post-LPS challenge the AL animals reduce their locomotor activity from baseline by 52% (-3.7 ± 0.5 counts/minute; Figure 2). The CR50% (-0.8 ± 0.2 counts/minute) animals reduced their locomotor activity by 20%. Both the AL saline and CR50% saline groups slightly increased their locomotor activity by 1% and 3% respectively (0.05 ± 0.1 counts/minute and 0.2 ± 0.2 counts/minute respectively).

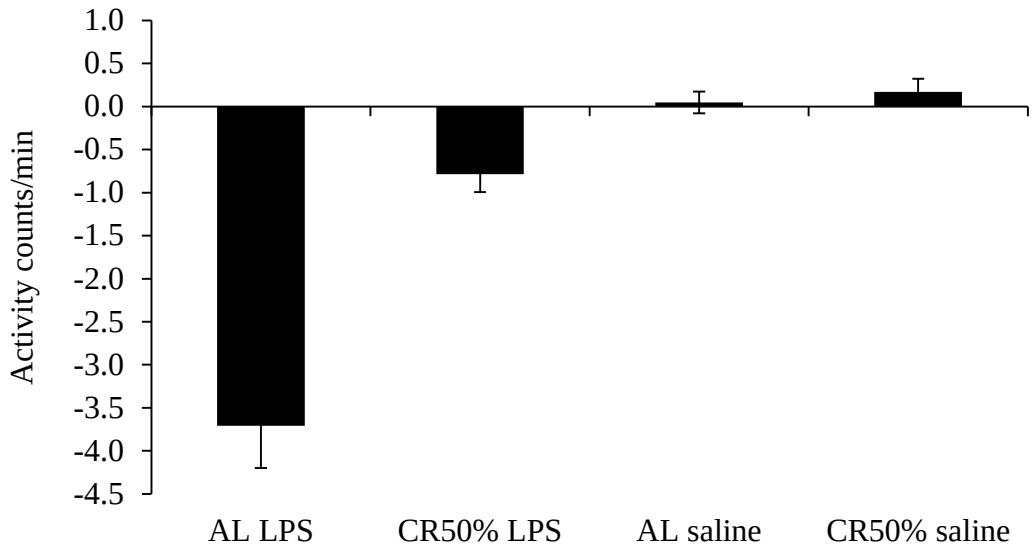

Figure 2. Mean (± SEM) 24 hour locomotor activity of AL LPS (*n* = 14), CR50% LPS (*n* = 8), AL saline (*n* = 8), and CR50% saline (*n* = 8) animals post-injection – AL (LPS) and CR50% (LPS) data also presented in Figure #, Chapter 6.

As seen in Figure 3, the AL animals have the largest decline in locomotor activity post-LPS, demonstrating this decline mainly during the dark phase. Aside from a reduction in locomotor activity during the 4-8-hour block post-LPS the CR50% group almost mirrors the activity pattern of the saline groups post-injection. Both saline groups remain at baseline levels throughout the 24 hour period post-injection. The ANOVA found a significant main effect for time [$F(2.98,68.53) = 24.61$, $p < .001$, partial $\eta^2 = .52$], group [$F(3,27) = 27.48$, $p < .001$, partial $\eta^2 = .78$], and their interaction [$F(8.94,68.53) = 9.33$, $p < .001$, partial $\eta^2 = .55$]. Post hoc comparisons revealed significant effects during five of the six 4-hour blocks post-LPS.

During the 0-4 hour block post-LPS the AL animals were significantly less active compared to all other groups (ranging from $p = .005$ to $p < .001$). During the 4-8 hour block the CR50% animals' activity declined near to the level of the AL group and both of these

groups were significantly different from both saline groups ($p < .001$ for all). During 8-12 hours post-LPS the AL group's activity declined further, differing from the other three groups ($p < .001$ for all). A similar pattern of activity remained during 12-16 and 16-20 hours post-LPS, with the AL group significantly less active compared to all other groups ($p < .001$ for all). AL animals were significantly less active compared to baseline from the 4-8 hour to the 16-20 hour blocks post-LPS (ranging from $p = .016$ to $p < .001$) The CR50% animals only differed from baseline during the 4-8 hours post-LPS ($p < .001$); the AL saline and CR50% saline groups did not differ from baseline at any point post-injection.

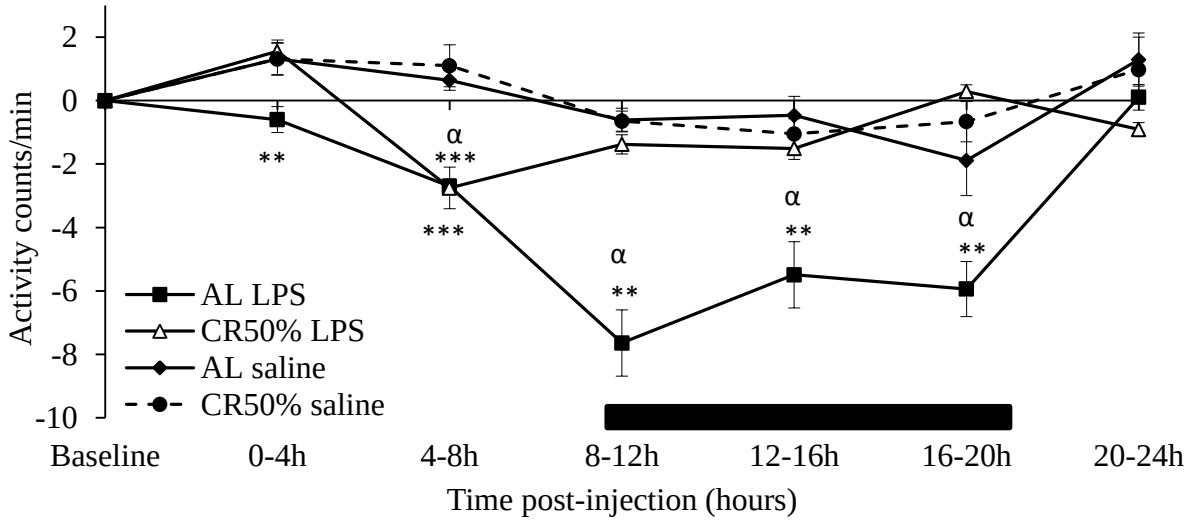

Figure 3. Mean activity change from baseline (± SEM) for AL LPS (n = 14), AL saline (n = 8), CR50% LPS (n = 8), and CR50% saline (n = 8) animals for 24 hours post-LPS, with the black box representing the dark phase – AL (LPS) and CR50% (LPS) data also presented in Figure #, Chapter 6. (**) denotes a significant difference from all other groups at $p < .01$, (***) denotes a significant difference from the CR50% saline and AL saline groups at $p < .001$, and (α) denotes a significant difference from baseline at $p < .05$.

Body weight

On day 1 post-injection the AL animals demonstrated the largest weight loss (-5.6% ± 0.8) compared to all other groups. The CR animals who received LPS only lost -1.1% (± 0.3) of their pre-injection body weight. The CR50% saline animals (-0.3% ± 0.1) lost the least amount of weight and the AL saline animals (0.2% ± 0.3) were the only group to gain weight (Table 1).

The ANOVA found a significant main effect for day [$F(2.33,53.64) = 24.51$, $p < .001$, partial $\eta^2 = .52$], group [$F(3,23) = 33.46$, $p < .001$, partial $\eta^2 = .81$], and their interaction [$F(6.99,53.63) = 18.15$, $p < .001$, partial $\eta^2 = .70$]. Post-hoc comparisons revealed significant treatment effects for all days post-LPS. The AL group lost significantly more weight compared to all groups on all days post-injection ($p < .001$ for all). AL LPS animals were significantly lighter compared to baseline on all days post-LPS (ranging from $p = .007$ to $p < .001$ for all). The CR50%, AL saline, and CR50% saline groups all did not differ significantly from baseline on any day post-injection.

Table 1. *Mean (± SEM) body weight changes (%) from baseline for AL LPS (n = 14), AL saline (n = 8), CR50% LPS (n = 8), and CR50% saline (n = 8) for four days post-LPS/saline injection – AL (LPS) and CR50% (LPS) data also presented in Figure #, Chapter 6.*

Group	Day 1 (%)	Day 2 (%)	Day 3 (%)	Day 4 (%)
AL LPS	-5.6 (0.8)***β	-5.2 (0.8)***β	-4.4 (0.7)***β	-3.3 (0.6)***β
AL saline	0.2 (0.3)	0.4 (0.3)	0.5 (0.3)	1.1 (0.4)
CR50% LPS	-1.1 (0.3)β	-0.9 (0.2)	-0.1 (0.2)	-0.2 (0.2)
CR50% saline	-0.3 (0.1)	-0.6 (0.2)	-0.7 (0.2)	0.1 (0.2)

Note: (***) denotes a significant difference from all other groups at $p < .001$ and (β) denotes a significant difference from baseline at $p < .01$.

Food intake

AL animals demonstrated a sharp reduction in food intake during the initial 24 hours following LPS injection (-66.8%) which was below that of both CR50% groups food intake, and did not recover to pre-LPS levels during the entire four day period post-LPS (Table 2). No other group reduced food intake following LPS/saline injection. The ANOVA found a significant main effect for day [$F(2.41,62.58) = 615.57$, $p < .001$, partial $\eta^2 = .96$], group [$F(3,26) = 25.07$, $p < .001$, partial $\eta^2 = .76$], and their interaction [$F(7.22,62.58) = 65.68$, $p < .001$, partial $\eta^2 = .88$].

Post-hoc comparisons revealed significant treatment effects for each day post-injection. On day one ($p < .001$), day two ($p < .001$), and day three ($p = .02$) the AL LPS group ate significantly less compared to the AL saline group. On day one post-LPS the AL group ate significantly less compared to the CR50% and CR50% saline groups ($p < .001$ for both); however, they ate more than the CR50% groups on day two post-LPS and remained this way for the remainder of the testing period (ranging from $p = .02$ to $p < .001$ for all). Within group simple main effect revealed that the AL animals food intake was significantly lower than baseline on all four days post-LPS (day one $p < .001$, day two $p = .002$, day three $p = .020$, and day four $p = .027$).

Table 2. *Mean absolute food intake (g) (± SEM) for AL LPS (n = 14), AL saline (n = 8), CR50% LPS (n = 8), and CR50% saline (n = 8) for four days post-LPS/saline injection.*

Group	Baseline (g)	Day 1 (g)	Day 2 (g)	Day 3 (g)	Day 4 (g)
AL LPS	25.9 (0.5)	8.7 (2.2)γ	15.3 (1.6)γ	20.3 (1.4)γ	22.3 (1.0)γ
AL saline	23.9 (0.8)	23 (0.7)***	24.4 (1.4)***	22.9 (1.2)**	22.2 (0.6)
CR50% LPS	12.7***	12.7***	12.7*	12.7***	12.7***

CR50% saline	12***	12***	12*	12***	12***

Note: (*) denotes a significant difference from the AL LPS group at $p < .05$, (**) denotes a significant difference from the AL LPS group at $p < .01$, (***) denotes a significant difference from the AL LPS group at $p < .001$, and (γ) denotes a significant difference from baseline at $p < .001$.

Experiment 2 - Serum concentration of CORT, IL-6, and IL-10 after LPS

In both the AL and CR50% groups CORT concentration increased after exposure to LPS (Figure 4); the largest increase was observed in the CR50% animals 2 hours post-LPS. Serum IL-6 concentration increased in both AL and CR50% animals after administration of LPS (Figure 5). Serum IL-10 concentration increased in both AL and CR50% animals post-LPS (Figure 6). Using Pillai's trace there was a significant effect of CR on CORT, IL-6, and IL-10 concentration, V = 1.08, $F(15, 36) = 3.35$, $p < .001$, partial $\eta^2 = .36$. Separate univariate ANOVAs revealed that there were significant treatment effects of CR on CORT [$F(5,30) = 5.06$, $p = .002$, partial $\eta^2 = .46$], IL-6 [$F(5,30) = 4.88$, $p = .002$, partial $\eta^2 = .45$], and IL-10 [$F(5,30) = 4.14$, $p = .006$, partial $\eta^2 = .41$].

Post hoc tests for serum CORT concentration

Post hoc tests revealed that the AL group demonstrated an increase in serum CORT concentration at 2 ($p = .013$) and 4 ($p = .021$) hours post-LPS, as did the CR50% animals (2 hours; $p = .004$, and 4 hours; $p = .049$). The CR50% animals demonstrated almost twice as much serum CORT concentration at 2 hours post-LPS compared to AL animals ($p = .020$).

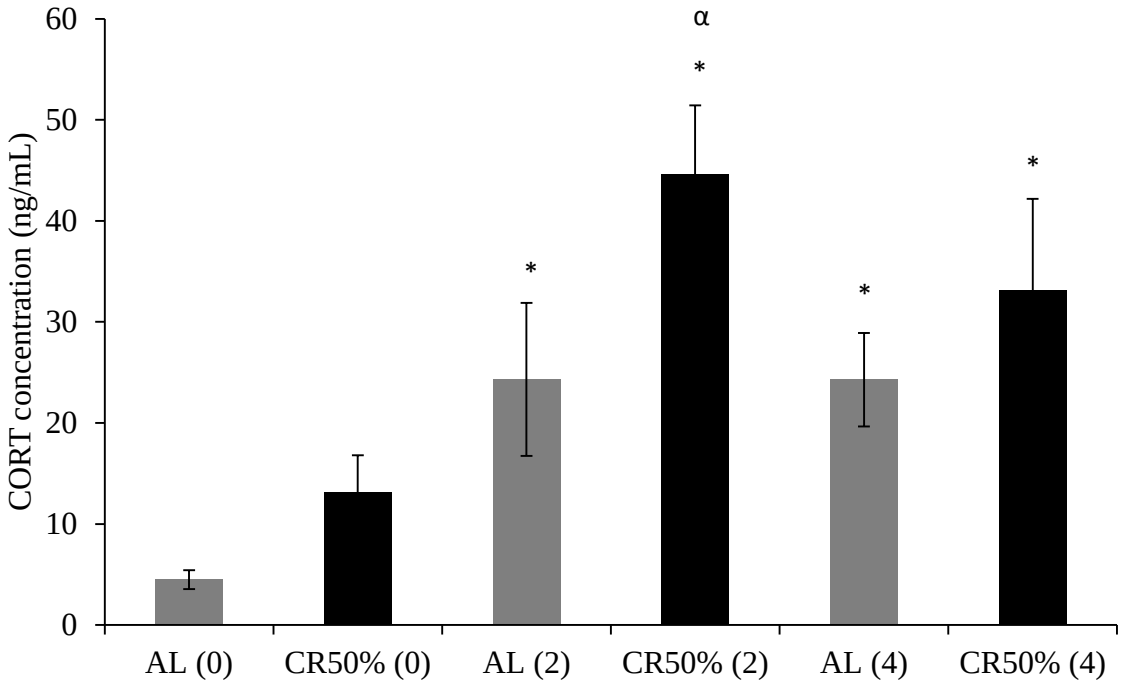

Figure 4. Mean (± SEM) CORT concentration (ng/mL) for AL (0), AL (2), AL (4), CR50% (0), CR50% (2), and CR50% (4) post-LPS injection (*n*'s ranging from 6 to 7 for each group), (*) denotes a significant difference from baseline within the same dietary regimen at $p < .05$, (α) denotes a significant difference from the AL group at the same time point at $p < .05$.

Post hoc tests for serum IL-6 concentration

IL-6 concentration was increased at 2 and 4 hours post-LPS compared to baseline in both the AL ($p < .001$ and $p = .003$ respectively) and a CR50% animals ($p = .004$ and $p = .049$ respectively). The AL animals demonstrated a larger increase in IL-6 at 2 hours post-LPS compared to the CR50% animals ($p = .034$). This trend continued at 4 hours post-LPS; however, it did not reach significance ($p = .052$).

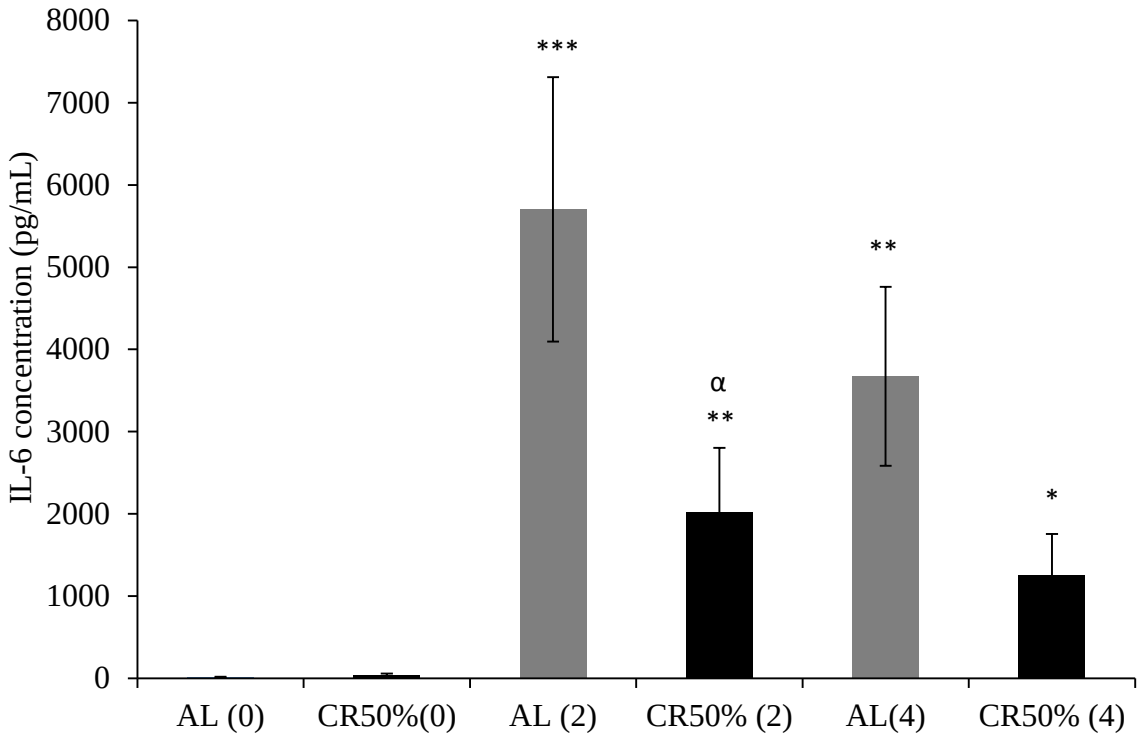

Figure 5. Mean (± SEM) IL-6 concentration (pg/mL) for AL (0), AL (2), AL (4), CR50% (0), CR50% (2), and CR50% (4) post-LPS injection (*n*'s ranging from 6 to 7 for each group). (*) denotes a significant difference from baseline within the same dietary regimen at $p < .05$, (**) denotes a significant difference from baseline within the same dietary regimen at $p < .01$, (***) denotes a significant difference from baseline within the same dietary regimen at $p < .001$, and (α) denotes a significant difference from the AL group at $p < .05$.

Post hoc tests for serum IL-10 concentration

IL-10 concentration was increased at 2 and 4 hours post-LPS compared to baseline in both AL ($p = .005$ and $p = .015$ respectively) and CR50% animals ($p = .044$ and $p = .008$ respectively). Although there was a slight trend towards the CR50% animals demonstrating a higher concentration of IL-10 at 4 hours post-LPS this difference was not significant.

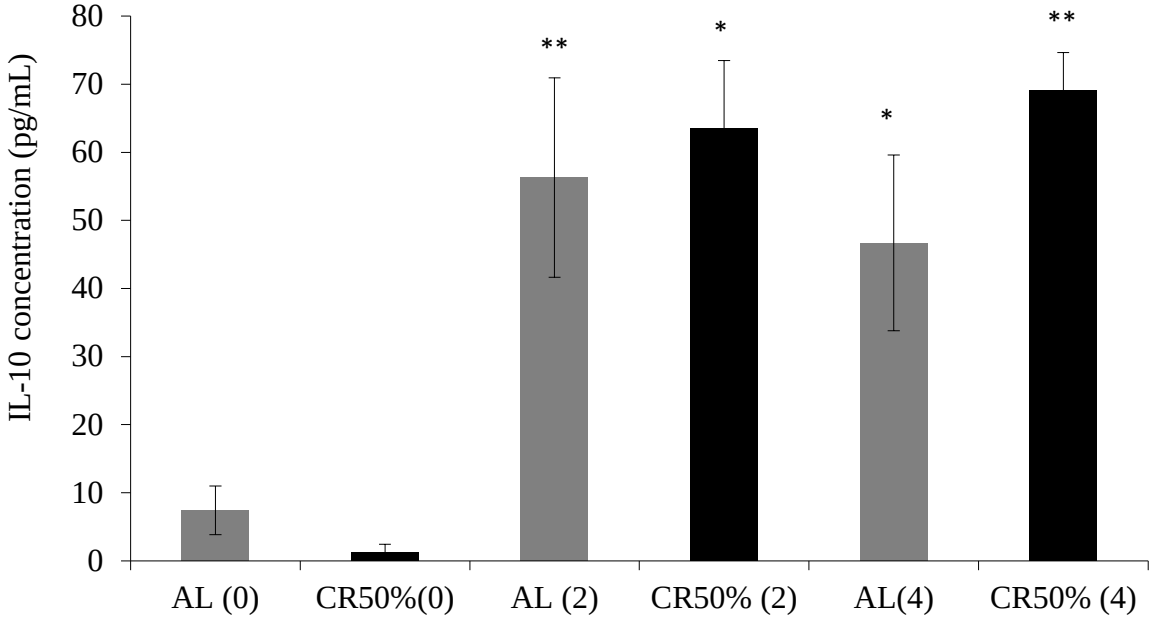

Figure 6. Mean (± SEM) IL-10 concentration (pg/mL) for AL (0), AL (2), AL (4), CR50% (0), CR50% (2), and CR50% (4) post-LPS injection (*n*'s ranging from 6 to 7 for each group). (*) denotes a significant difference from baseline within the same dietary regimen at $p < .05$ and (**) denotes a significant difference from baseline within the same dietary regimen at $p < .01$.

The relationship between CORT, IL-6, and IL-10 was investigated and it was determined that at two hours post-LPS the AL animals demonstrated a strong positive relationship between IL-6 and IL-10 ($r = .955$, $p = .003$). At four hours post-LPS there was also a strong positive relationship between CORT and IL-6 ($r = .804$, $p = .029$), CORT and IL-10 ($r = .891$, $p = .007$), and IL-6 and IL-10 ($r = .759$, $p = .048$) in the AL animals. The CR50% animals only demonstrated a significant correlation between variables at two hours post-LPS; CORT and IL-6 ($r = .809$, $p = .049$), CORT and IL-10 ($r = .880$, $p = .009$), and IL-6 and IL-10 ($r = .893$, $p = .017$).

Discussion

Effect of calorie restriction on sickness behaviour

This study is the first investigation to reveal that a 50% CR attenuates sickness behaviour post-LPS in rats. This study replicates the findings we have previously demonstrated in mice (MacDonald et al., 2011), which found that a 50% CR for 28 days attenuates sickness behaviour following LPS administration. The CR50% animals in the current study demonstrated a complete attenuation in the rise in T_b following LPS, an attenuation of the reduction in locomotor activity seen after LPS, attenuated weight loss, and no reduction in food intake normally seen post-LPS. Whilst the AL rats T_b increased by more than 1 °C following LPS, the CR50% group displayed a similar T_b profile to that of the animals who received saline injections; demonstrating a maximal increase of 0.3 °C at three hours post-LPS. This study also demonstrates that a dose-response in terms of the CR severity may exist due to previous literature demonstrating that a 20% CR in hamsters was not enough to alter T_b, circulating cortisol, or circulating IL-6 compared to controls post-LPS (Conn et al., 1995).

LPS-induced a decrease in total locomotor activity during the first 24 hours post-LPS was minimal in the CR50% animals compared to the large decline observed in the AL animals. The CR50% animals demonstrated a slight decline in activity 4-8 hours post-LPS; however, levels returned to baseline for the remainder of the 24 hour period post-LPS and resembled the locomotor activity of both saline treated groups. The AL animals' locomotor activity declined in the 4-8 hour block post-LPS and remained below baseline until 20-24 hours post-LPS.

LPS did not alter food intake in the CR50% group; it is noteworthy that the AL animals' food intake during the 24 hours post-LPS was lower than the CR50% groups' food intake. An increased drive to eat in food-deprived animals following leptin infusion or an

immune challenge has been demonstrated previously (Mrosovsky, Molony, Conn, & Kluger, 1989; Soos, Balasko, Jech-Mihalffy, Szekely, & Petervari, 2010). For example, rats CR by 33% demonstrated no reduction in food intake or a reduction in body weight during a seven day period of central leptin infusion (Soos et al., 2010). The authors stated that this was due to the extreme orexigenic tone of the CR animals. In the current study the AL animals ate 4 grams (or 27%) less than the CR50% animals post-LPS. One might speculate that the CR50% animals are not experiencing the anorexic effect produced by exposure to LPS and are not simply driven to eat their allocated food as an artefact of being on a restricted diet. However, if the orexigenic tone of the CR animals is enhanced, then it is possible that this overrides their need to suppress their food intake following LPS exposure.

Calorie restriction, sickness behaviour and peripheral targets

CR typically results in an increase in circulating CORT levels (Han et al., 1995; Heiderstadt et al., 2000; Holmes, French, & Seckl, 1997; Levay et al., 2010) and increased peripheral CORT levels after LPS has been consistently observed (Barnes, 1998; Besedovsky, Sorkin, Keller, & Muller, 1975; Campisi et al., 2003; Linthorst, Flachskamm, Muller-Preuss, Holsboer, & Reul, 1995). Although we did not observe a significant difference in CORT levels at baseline, there was a trend towards the CR50% animals demonstrating a higher CORT concentration at baseline. Both AL and CR50% animals demonstrated a rise in serum CORT levels two hours post-LPS administration; however, the CR50% animals demonstrated almost two-times the CORT concentration compared to the AL animals. CORT is well known to play a role in anti-inflammatory processes (Barnes, 1998). Therefore, the increased CORT levels seen in the CR50% animals may be one part of a multifaceted explanation for why CR50% animals demonstrate attenuated sickness behaviour. In previous investigations in CR mice we found that hypothalamic mRNA

expression of corticotropin-releasing hormone (CRH) was increased at baseline but reduced at 2 hours post-LPS compared to AL fed mice (MacDonald et al., 2011). Since CORT exerts a negative feedback role (Han et al., 1995; Levay et al., 2010) via activation of the glucocorticoid receptors in the hippocampus, hypothalamus, and the pituitary (Patel & Finch, 2002), it could be postulated that the increased CORT seen in these CR animals after LPS may be responsible for decreased hypothalamic levels of CRH. In our previous work we did not observe any change in glucocorticoid and mineralocorticoid receptor expression in the hypothalamus of CR mice post-LPS (MacDonald et al., 2011); however, we did not quantify receptors or investigate levels in the hippocampus nor did we determine levels of corticosteroid-binding globulin (CBG) after CR. It has been shown that after a short period (22 hours) of fasting CBG levels in birds was significantly reduced and subsequently free CORT was significantly increased (Lynn, Breuner, & Wingfield, 2003).

IL-6 release after LPS was attenuated in the CR animals, which was expected considering the animals CR for 28 days did not develop an increase in T_b. IL-6 is a major pro-inflammatory cytokine responsible for inducing a rise in T_b following exposure to LPS (Cartmell, Poole, Turnbull, Rothwell, & Luheshi, 2000; LeMay, Vander, & Kluger, 1990). Given the IL-6 levels in the CR animals were profoundly attenuated (only a third as high as the control animals at two and four hours post-LPS) it is possible that this is playing a major role in the CR animals attenuated fever. We have previously shown that the anti-inflammatory compound SOCS3, which is known to reduce the production of IL-6 (Bogdan et al., 1991), was significantly increased post-LPS in the hypothalamus of mice CR to 50% for 28 days (MacDonald et al., 2011). Accompanied with the results from the current study it appears that central increases in SOCS3 leads to reductions in peripheral IL-6, resulting in the attenuated fever and sickness behaviour seen in the CR animals post-LPS.

Further, others have demonstrated that peripheral IL-6 is crucial for the expression of mPGES-1 in the brain, with IL-6 treatment inducing mPGES-1 expression and a IL-6 anti-serum reversing the increase in mPGES-1 after LPS (Rummel, Matsumura, & Luheshi, 2011). In our previous work we demonstrated attenuated increases in mPGES-1 after LPS in the CR mice, which in conjunction with the results of this study indicate that reduced levels of peripheral IL-6 may lead to reduced levels of brain mPGES-1.

The profile of IL-10 concentration in the AL and CR50% animals was similar at both time points post-LPS; however there was a trend towards the CR50% animals demonstrating a heightened concentration of IL-10 at 4 hours post-LPS. Previously we have shown that hypothalamic mRNA levels of IL-10 in CR50% mice at 4 hours post-LPS are twice as high as that of AL animals (MacDonald et al., 2011); however, in the current study peripheral levels remain at levels similar to AL fed animals. It could be speculated that central IL-10 levels have more potent anti-inflammatory effects in the CR animals compared to the peripheral IL-10 levels. Along with CORT, IL-10 is known to exert anti-inflammatory effects (Bogdan et al., 1991; Leon, Kozak, Rudolph, & Kluger, 1999). In the AL animals at two and four hours post-LPS and in the CR animals at two hours post-LPS there were strong positive relationships between CORT, IL-6, and IL-10. These relationships are expected due to all of these three variables known to significantly alter during the inflammatory process (Barnes, 1998; Besedovsky et al., 1975; Bogdan et al., 1991; Cartmell et al., 2000; LeMay et al., 1990; Leon et al., 1999; Linthorst et al., 1995).

Although the increase in circulating IL-6 was attenuated in CR animals they were still significantly increased two hours post-LPS compared to baseline. It may be possible that the signal transducer, gp130, responsible for carrying the IL-6 molecule to the brain is not functioning properly. Further, it has been demonstrated that merely the activation of peripheral immune cells and secretion of cytokines is not enough to maintain a sustained

anorexic response post-immune challenge (Asarian & Langhans, 2010; Layé et al., 2000; Ogimoto, Harris, & Wisse, 2006). For example, it was seen in mice lacking the IL-1β signal transducer, MyD88, that peripheral exposure to LPS failed to induce an anorexic response (Wisse et al., 2007). These authors also found that LPS induced an increase in peripheral levels of IL-1β; however, the IL-1β message was unable to reach the brain and hypothalamic levels of IL-1β were significantly reduced in these mice compared to wild type mice (Wisse et al., 2007). It was recently found that MyD88, along with CD14 and Toll-like receptor 4 (all part of the receptor system for LPS) were down regulated in an obese human population undertaking a low calorie diet (Capel et al., 2009) and although MyD88 to the best of our knowledge has not been investigated after a period of CR in animals this may indicate that other receptors for LPS are also down regulated.

Conclusion

This study is the first to demonstrate that a 50% CR attenuates sickness behaviour post-LPS. Further, serum CORT was almost twice as elevated in CR50% animals than AL animals at two hours post-LPS. Elevated peripheral CORT levels would contribute to a heighted anti-inflammatory response in the CR50% animals and may play a role in the attenuated sickness behaviour seen in these animals. Additionally, circulating levels of IL-6 were significantly attenuated in the CR animals at two hours post-LPS indicating that this may be playing a major role in the attenuated fever seen in the CR animals. In contrast, IL-10 levels were similar in both AL and CR fed animals, suggesting that peripheral levels of IL-10 possibly do not impact upon the attenuated sickness behaviour.

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CHAPTER 6

DOSE-DEPENDENT EFFECT OF CALORIE RESTRICTION DURATION ON SICKNESS BEHAVIOUR IN RATS

Abstract

Previously our laboratory has demonstrated that 28 days of a 50% calorie restriction (CR) attenuates lipopolysaccharide (LPS)-induced sickness behaviour in mice and rats. This study examined the effect of differing durations of CR on the development of sickness behaviour (fever, anorexia, and behavioural depression). Male Sprague-Dawley rats fed ad libitum or restricted 50% (CR50%) in food intake for 14, 21, or 28 days were injected intraperitoneally on the 15th, 22nd, or 29^{th}day with 50 μg/kg of LPS. Changes in body temperature (T_b), locomotor activity, body weight, and food intake were determined. There was a dose-dependent response of sickness behaviour to LPS following the three different durations of CR. The rats CR for 28 days demonstrated the largest attenuation of sickness behaviour, which resulted in no development of fever, a limited reduction in locomotor activity, no sign of anorexia, and reduced cachexia following LPS. The rats CR for 14 and 21 days demonstrated a partial attenuation of sickness behaviour following LPS in a dose-dependent manner with the rats CR for 14 days demonstrating a larger increase in T_b, larger reduction in locomotor activity, and larger weight loss compared to the rats CR for 21 days. Rats CR for 14 and 21 days did not show any signs of anorexia. CR results in a suppression of sickness behaviour in a dose-dependent manner in relation to the duration of the CR period. These results provide an informative structure in which future studies can base their experiments on in terms of the optimal time frame in which rodents should be CR to elicit attenuations in sickness behaviour and/or alterations in immune function.

Calorie restriction (CR) has been widely studied in relation to prolonging lifespan (Austad, 1989; Conti et al., 2006; Han et al., 1995; Kubo, Day, & Good, 1984; Lin, Ford, Haigis, Liszt, & Guarente, 2004; McCay, Crowell, & Maynard, 1935; Weindruch, Walford, Fligiel, & Guthrie, 1986) and retardation the progression of certain diseases (Lane, Ingram, & Roth, 1999; Mascarucci et al., 2002; Maswood et al., 2004; Matsuzaki et al., 2000; Walford, Mock, Verdery, & MacCallum, 2002). However, there has been limited previous research that has investigated the effects of differing severities of CR on various outcome measures, for example oxygen consumption, gene expression, and cancer (Bevilacqua, Ramsey, Hagopian, Weindruch, & Harper, 2004; Cao, Dhahbi, Mote, & Spindler, 2001; Kumar, Roy, Tokumo, & Reddy, 1990). Further, there has been very limited research into how different durations of CR may impact on sickness behaviour. The effect differing durations of CR may have on investigatory outcomes is important to establish so that underlying mechanisms that play a role can be further clarified.

We have previously demonstrated that a 28 day 50% CR in mice (*Chapter 4*; MacDonald, Radler, Paolini, & Kent, 2011) and rats (*Chapter 5*) can fully attenuate lipopolysaccharide (LPS)-induced sickness behaviour. The CR mice and rats demonstrated no increase in T_b after an injection with LPS, a limited reduction in locomotor activity, reduced weight loss, and no reduction in food intake post-LPS (*Chapter 5*; MacDonald et al., 2011). Further, CR mice exhibit a central anti-inflammatory bias post-LPS, with levels of two key enzymes in the fever inducing pathway, cyclooxygenase 2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1), being lower in the CR mice compared to control mice. Further, levels of the anti-inflammatory cytokine interleukin-10 (IL-10) were increased, as were levels of suppressor of cytokine signalling 3 (SOCS3), which is induced by IL-10 (Cassatella et al., 1999) and inhibits production of IL-6 (Lang et al., 2003), post-LPS in the CR mice (MacDonald et al., 2011). In rats CR to 50% for 28 days we demonstrated that

increases in serum levels of corticosterone (CORT) post-LPS were enhanced compared to control rats (*Chapter 5*), possibly contributing to the attenuation of sickness behaviour seen in these animals due to the known anti-inflammatory role CORT plays (Barnes, 1998; Smoak & Cidlowski, 2004). Circulating levels of IL-6 were also significantly attenuated in the CR animals post-LPS, which is of great importance due to IL-6 being well known to be critically important during fever production (Cartmell, Poole, Turnbull, Rothwell, & Luheshi, 2000; LeMay, Vander, & Kluger, 1990). All of our previous investigations into sickness behaviour post-LPS have occurred after 28 days of CR. The aim of the current study is to determine if there is a dose-dependent effect of the duration of CR on the development of sickness behaviour post-LPS.

As mentioned above, there has been limited investigation of the impact of differing durations of CR, and although not directly related to sickness behaviour or immune function the following examples illustrate that the duration of CR plays an important role in investigatory outcomes. It was shown that body-mass adjusted oxygen consumption in rats was not significantly affected by a 40% CR for two weeks or two months; however, a six month period reduced oxygen consumption by 40% (Bevilacqua et al., 2004). The same study revealed that mitochondrial production of reactive oxygen species was reduced by 53% compared to controls after two weeks on a 40% CR, and increased to a 57% reduction at two months, and a 74% reduction at six months, indicating a dose-dependent response to the duration of CR (Bevilacqua et al., 2004). In contrast, it was found that when comparing a short and long term (precise length not specified) 44% CR, these CR durations were homologous in both direction and level of change in the expression of genes that change with age (Cao et al., 2001). Furthermore, the short term CR reproduced 100% of the same effects as long term CR on urinary protein and stress response gene expression and 67% of the effects on inflammatory response gene expression (Cao et al., 2001). These findings provide

mixed messages as to the precise difference between short versus long term CR. It may be that depending on the variables measured that there is a threshold that needs to be reached (in terms of the duration of CR) to see changes in variables. Thus, the timing of the CR period needs to be optimised for specific investigations.

Previously our laboratory has demonstrated that sickness behaviour after LPS attenuated by a 50% CR for 28 days in mice and rats. The current study aims to investigate the effect different durations of CR have on sickness behaviour after LPS. Due to our previous findings it is hypothesised that there will be a dose-dependent attenuation of sickness behaviour measures post-LPS. The rats CR to 50% for 28 days will demonstrate the largest attenuation in sickness behaviour measures, followed by the rats CR for 21 days, whereas the rats CR for 14 days will demonstrate the smallest attenuation of sickness behaviour.

Methods

Animals

Forty-one male Sprague-Dawley rats were procured from Monash SPF animal services (Clayton, Victoria, Australia) and allowed to acclimate to the facility for at least one week. During this period, standard rodent chow (Barastoc, Melbourne, Australia) and water were available ad libitum. At the beginning of experimentation the rats were aged between 9 and 12 weeks old. Rats were individually housed in polypropylene basin cages (30 × 50 × 15 cm) with sawdust and tissues provided as bedding. Rats were maintained at an ambient temperature of 26 ± 1 °C, which is within the thermoneutral zone for this species (Poole & Stephenson, 1977) on a 12:12 light/dark cycle (0500 – 1700 hours). Animal care and experimentation was performed in accordance with protocols approved by the La Trobe University Animal Ethics Committee.

Surgery

Following acclimation all of the rats were surgically implanted in the peritoneal cavity with a biotelemetry device (E-4000, Mini-mitter®, Bend, OR, USA: 23 × 8 mm, 1.6 g) under anaesthesia as previously described (Begg, Kent, McKinley, & Mathai, 2007). Rats were anaesthetised in an induction chamber using 4% isoflurane and 0.6 L/min oxygen flow and were maintained during surgery on 2.5% isoflurane and 0.4 L/min oxygen flow using a nose cone (refer to *Chapter 2* for more detail). Rats were allowed one to two weeks to recover before the initiation of the CR regimens.

Dietary regimens

Rats were divided into one of two CR regimens matched for weight, food intake, and age: ad lib (AL; n = 14) fed ad libitum (approximately 20-30 g per day); or CR50% (n = 27) rats received 50% of the amount consumed by the AL animals (approximately 10-15 g per day). The dietary composition of the control and CR50% diets has been published elsewhere (Levay, Govic, Penman, Paolini, & Kent, 2007; Levay, Tammer, Penman, Paolini, & Kent, 2010). The group sizes varied across dependent variables and precise numbers used for each variable are stated in the results section. The intake of the CR groups was determined weekly based on the average daily food intake of the control group for three consecutive days. Water was continuously provided to all groups. The dietary manipulation continued for 14, 21, or 28 days before, and four days after LPS administration. Food was provided daily, approximately one hour before the dark phase onset.

Determination of sickness behaviour after 14, 21, or 28 days of CR

Sickness behaviour was determined by continuous measurement of core body temperature (T_b), locomotor activity, body weight, and food intake for four days pre- and four days post-LPS challenge. T_b, locomotor activity, and body weight were also determined throughout the CR period.

T_b and locomotor activity were determined by placing each cage on a receiver with each individual biotelemetry device generating a continuous frequency signal proportional to the animal's T_b ($\pm 10^{-1}$ °C). The receiver sampled this frequency at 1-minute intervals and this sample was decoded by VitalView software (Mini Mitter Co., Inc., Bend, OR) and stored on a hard drive. Rats were weighed once a week approximately three hours after lights-on during dietary manipulation and daily at the same time for the four days pre- and post-LPS injection using top loading scales (± 0.1 g). Food consumption was also determined at this time and was determined to the nearest .1 g by providing a set amount of food each day and weighing the remaining food 24 hours later including the uneaten food in the bedding.

After 14, 21, or 28 days of dietary regimen (i.e., on the 15th, 21st, or 29th day) all rats were injected intraperitoneally with of 50 μg/kg of LPS from *Escherichia coli* (serotype 0111:B4: Sigma, Castle Hill, NSW) in 1 ml/500 g saline. The LPS challenge was performed approximately four hours after lights-on (ranging from 0800 hours to 1000 hours).

Data Analysis

All data collected over the 4-day baseline and 4-day testing period were averaged and means ± SEM were calculated for each group. Mean ± SEM change in T_b was presented as the first 8 hours post-LPS. Mean ± SEM change in locomotor activity was presented as the first 24 hours post-LPS as well as 4 hour block averages during the first 24 hours post-LPS. For the body weight and food intake data a change from baseline measurement was used to determine differences between the groups due to the baseline differences between the groups

on the day of injection. Data were then analysed using analysis of variance (ANOVA). To overcome violations of sphericity, the Greenhouse-Geisser statistic corrected degrees of freedom were reported. Where required, between-group simple main effects and within-group simple main effects were analysed using the Least Significant Difference method (LSD). Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$ as the criterion for significance.

Results

Determination of sickness behaviour after 14, 21, or 28 days of CR

Core body temperature

LPS induced a fever in the AL rats and to a lesser extent in the CR50% LPS (14d) animals (Figure 1). The AL animals T_b increased within 2 hours and was at its highest at 5 hours post-LPS at 1.1 °C (± 0.2) above baseline whereas a significant increase in the CR50% LPS (14d) animals T_b occurred only after 4 hours with a maximum of 0.7 °C (± 0.1) also at occurring 5 hours post-LPS. LPS induced a slight and non-significant rise in T_b in the CR50% (21d) animals to 0.4 °C (± 0.1) at 3 hours post-LPS and a further increase to 0.8 °C (± 0.2) above baseline at 8 hours post-LPS. The CR50% (28d) animals only recorded a 0.3 °C (± 0.1) increase in T_b from baseline at 3 hours post-LPS. Although not shown, the CR50% (14d) animals T_b mirrored that of the AL animals between 8 hours and 24 hours post-LPS (data not shown).

ANOVA found a significant main effect for hour [$F(2.76,110.50) = 3.70$, $p = .016$, partial $\eta^2 = .02$], group [$F(5,40) = 9.07$, $p < .001$, partial $\eta^2 = .53$], and their interaction [$F(13.81,110.50) = 3.50$, $p < .001$, partial $\eta^2 = .30$]. Post hoc comparisons revealed a significant group effect from 2 hours until 7 hours post-LPS.

The AL and CR50% (14d) animals displayed similar T_b profiles until 4 hours post-LPS, when the AL group had a significantly higher T_b compared to the CR50% (14d) group and remained higher than the CR50% (14d) group until 6 hours post-LPS (ranging from p = .021 to p < .001 for all). The CR50% (21d) animals T_b increase was lower compared to the AL animals only from 4 hours to 6 hours post-LPS (ranging from p = .003 to p < .001) and was similar at all other time points. The CR50% (28d) animals demonstrated a lower T_b at each hour post-LPS compared to the AL animals (ranging from p = .052 to p < .001).

AL animals had a significantly higher T_b compared to baseline from 2 hours until 6 hours post-LPS (ranging from p = .004 to p < .001). The CR50% (14d) animals T_b profile was slightly different, demonstrating an increased T_b from baseline from 4 hours until 8 hours post-LPS (ranging from p = .008 to p < .001). The CR50% (21d) animals T_b did not differ from baseline at any hour post-LPS. However, during the remainder of the 24 hours period the CR50% (21d) animals T_b did increase significantly compared to baseline (data not shown). The CR50% (28d) group T_b did not differ compared to baseline at any hour post-LPS.

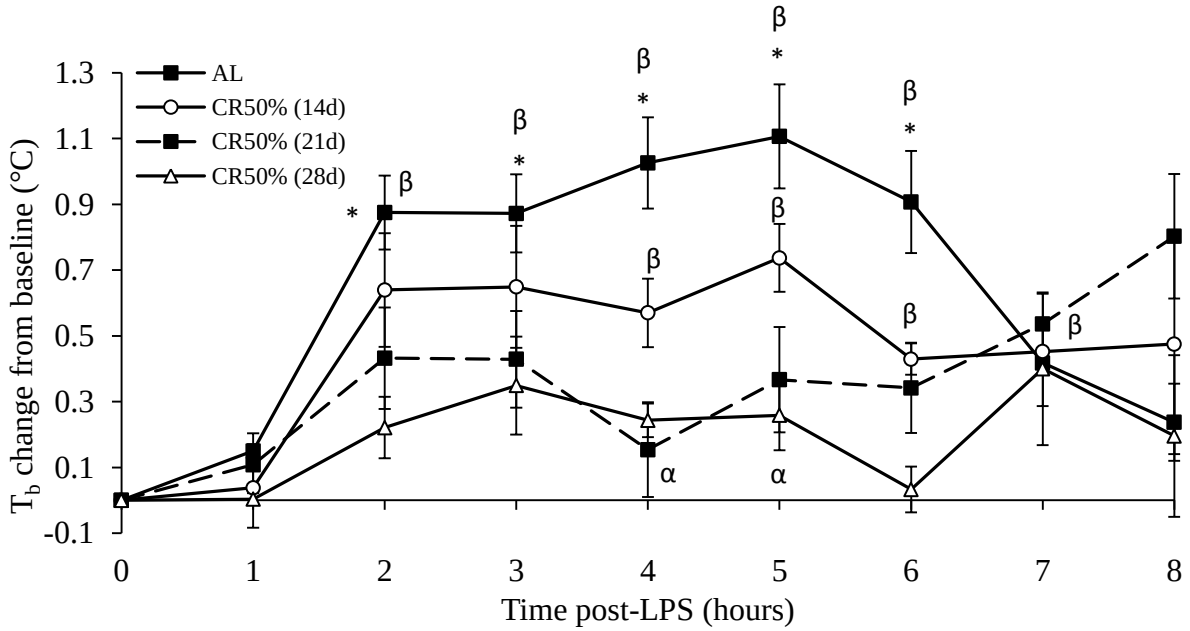

Figure 1. Mean T_b change from baseline (± SEM) for AL (n = 9), CR50% (14d; n = 11), CR50% (21d; n = 8), and CR50% (28d; n = 8) animals for 8 hours post-injection – AL and CR50% (28d) data also presented in Figure #, Chapter 5. (*) denotes a significant difference from the CR50% (28d) group at $p < .05$, (**) denotes a significant difference from the CR50% groups at $p < .01$, (α) denotes a significant difference from the CR50% (14d) group at $p < .05$, and (β) denotes a significant difference from baseline at $p < .01$.

Locomotor activity

For the entire 24 hours post-LPS challenge the AL animals reduce their locomotor activity from baseline by 52% (-3.7 ± 0.5 counts; Figure 2). The CR50% (14d; -2.6 ± 0.3 counts), CR50% (21d; -1.2 ± 0.2 counts), and CR50% (28d; -0.8 ± 0.2 counts) animals reduce their locomotor activity in a dose-dependent manner (39%, 27%, and 20% respectively). The ANOVA found a significant effect [$F(5,40) = 20.61$, $p < .001$] with the AL animals reducing their activity significantly compared to all CR50% groups ($p < .01$ for all) and compared to baseline ($p < .001$). The CR50% (14d) animals were significantly less active compared to the CR50% (21d) and CR50% (28d; $p < .01$ for both) and all three CR50% groups were less active compared to baseline (ranging from $p = .007$ to $p < .001$)

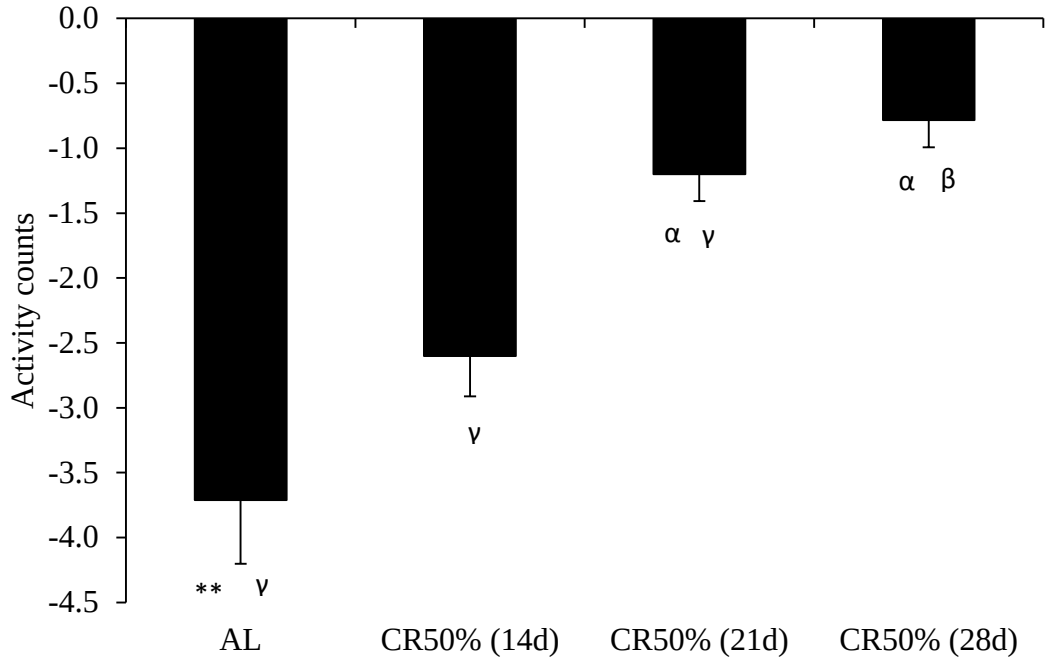

Figure 2. Mean (± SEM) 24 hour locomotor activity of AL (n = 9), CR50% (14d; n = 11), CR50% (21d; n = 8), and CR50% (28d; n = 8) animals post-injection – AL and CR50% (28d) data also presented in Figure #, Chapter 5. (**) denotes a significant difference from the CR50% groups at $p < .01$, (α) denotes a significant difference from the CR50% (14d) group at $p < .01$, (β) denotes a significant difference from baseline at $p < .01$, and (γ) denotes a significant difference from baseline at $p < .001$.

As seen in Figure 3, the AL animals have the largest decline in locomotor activity post-LPS, demonstraing this decline mainly during the dark phase. The CR50% (14d) animals also show a decline in activity; however, not to the same degree as the AL animals. Aside from a reduction in locomotor activity during the 4-8-hour block post-LPS the CR50% (21d) and CR50% (28d) groups remain near baseline levels post-injection.

The ANOVA found a significant main effect for hour [$F(3.40,136.01) = 30.41$, $p < .001$, partial $\eta^2 = .43$], group [$F(5,40) = 20.63$, $p < .001$, partial $\eta^2 = .72$], and their

interaction [$F(17.00,136.01) = 6.32$, $p < .001$, partial $\eta^2 = .44$]. Post hoc comparisons revealed a significant effect during five of the six 4-hour blocks post-LPS.

During the 0-4 hour block post-LPS the AL animals were significantly less active compared to the CR50% (21d) and CR50% (28d) groups ($p = .047$ and $p = .003$ respectively), and more active compared to the CR50% (14d) group ($p = .027$). Further, the CR50% (14d) group was significantly less active compared to the CR50% (21d) and CR50% (28d) groups ($p < .001$ for both). During the 4-8 hour block all of the CR50% LPS groups' activity declined near to the level of the AL group and all groups were not different from each other. During 8-12 hours post-LPS the AL group's activity declined again, differing from the other three groups ($p < .001$ for all). The CR50% (14d) animals were also still significantly less active compared to the CR50% (21d) and CR50% (28d) groups ($p = .043$ and $p = .011$ respectively). This pattern persisted during the 12-16 hours post-LPS; both the AL and CR50% (14d) groups were less active compared to the CR50% (21d) and CR50% (28d) groups (ranging from $p = .008$ to $p < .001$ for all). By 16-20 hours post-LPS only the AL group differed from all other groups ($p < .001$ for all).

AL and CR50% (14d) animals both differed from baseline at more than one time point post-LPS. AL animals were significantly less active compared to baseline during the 4-8 hour to the 16-20 hour blocks post-LPS (ranging from $p = .016$ to $p < .001$) and the CR50% (14d) animals were significantly less active compared to baseline during the 0-4 hour to the 12-16 hour block post-LPS (ranging from $p = .045$ to $p < .001$). The CR50% (21d) rats only differed from baseline during the 4-8 hour block post-LPS ($p = .028$). The CR50% (28d) group did not differ from baseline at any point post-injection.

During the 0-4 hour, 8-12 hour, and 12-16 hour blocks post-LPS the CR50% (14d) animals were significantly less active post-LPS compared to the CR50% (21d) and CR50%

(28d) groups (ranging from $p = .043$ to $p < .001$ for all). During the 4-8 hour and 16-20 hour blocks post-LPS all of the CR groups were similar to one another.

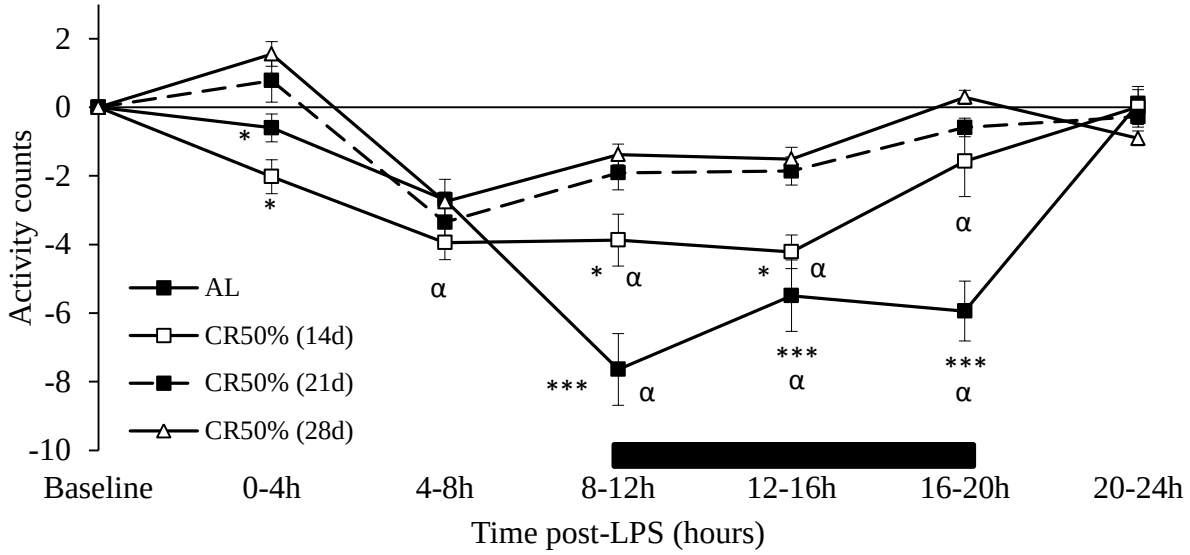

Figure 3. Mean activity change from baseline (± SEM) for AL ($n = 9$), CR50% (14d; $n = 11$), CR50% (21d; $n = 8$), and CR50% (28d; $n = 8$) animals, for 24 hours post-LPS, with the black box representing the dark phase – AL and CR50% (28d) data also presented in Figure #, Chapter 5. (*) denotes a significant difference from the other CR50% groups at $p < .05$, (***) denotes a significant difference from the CR50% groups at $p < .001$, and (α) denotes a significant difference from baseline at $p < .05$.

Body weight

On day 1 post-injection the AL animals demonstrated the largest weight loss (28.2 g ± 3.1 or -5.6% ± 0.8) compared to all other groups and did not return to their pre-LPS weight for the entire four days post-LPS (Table 1). The weight loss of the CR animals occurred in a dose-dependent manner, with the CR50% (14d) animals losing the most after the AL animals (-5.2 g ± 1.4 or -1.5% ± 0.4), then the CR50% (21d) animals (-4.5 g ± 0.4 or -1.4 % ± 0.1), followed by the CR50% (28d) animals (-3.4 g ± 0.9 or -1.1% ± 0.3). The CR50% (14d) and CR50% (21d) groups did not return to their pre-LPS weight before the end of the four day

period post-LPS; however, the CR50% (28d) group returned to their pre-LPS weight by day three post-LPS.

The ANOVA found a significant main effect for day [$F(1.04,33.29) = 4510.30$, $p < .001$, partial $\eta^2 = .99$] and the interaction [$F(3.12,33.29) = 33.84$, $p < .001$, partial $\eta^2 = .76$]. Post-hoc comparisons revealed significant treatment effects for all days post-LPS. The AL group lost significantly more weight compared to all groups on all days post-LPS ($p < .001$ for all). The only other difference was on day three post-LPS when the CR50% (14d) animals lost more weight compared to the CR50% (28d) animals ($p = .046$).

AL, CR50% (14d), and CR50% (21d) animals were significantly lighter compared to baseline on all days post-LPS ($p < .001$ for all). The CR50% (28d) only differed from baseline on day one and two post-LPS ($p < .001$ for both).

Table 1. *Mean (± SEM) body weight changes (g) from pre-LPS injection for AL (n = 13), CR50% (14d; n = 11), CR50% (21d; n = 8), and CR50% (28d; n = 8) for four days post-LPS injection – AL and CR50% (28d) data also presented in Figure #, Chapter 5.*

Group	Day 1 (g)	Day 2 (g)	Day 3 (g)	Day 4 (g)
AL	-28.2 (±3.1)***γ	-26.2 (±3.8)***γ	-18.7 (±3.4)*** γ	-14.2 (±3.1)*** γ
CR50% (14d)	-5.2 (±1.4) γ	-7.2 (±1.1)* γ	-5.5 (±1.2) γ	-5.2 (±1.2) γ
CR50% (21d)	-4.5 (±0.4) γ	-5.0 (±0.7) γ	-3.4 (±0.8) γ	-3.8 (±0.6) γ
CR50% (28d)	-3.4 (±0.9) γ	-2.8 (±0.8) γ	0.1 (±0.6)	-0.6 (±0.5)

Note: (*) denotes a significant difference from the CR50% (28d) group at $p < .05$, (***) denotes a significant difference from the three CR50% groups at $p < .001$, and (γ) denotes a significant difference from baseline at $p < .001$.

Food intake

The AL animals demonstrated a sharp reduction in food intake during the initial 24 hours following LPS injection (-64.5% ± 8.6) which was below that of all three CR50% groups food intake, and did not recover to pre-LPS food intake during the four days post-LPS (day two -37.1% ± 5.8, day three -21.8% ± 4.6, and day four -14.1% ± 3.2). All other groups did not display a reduction in food intake following LPS injection and continued to eat all the food provided to them. The ANOVA found a significant main effect for day [$F(1.89,60.57) = 120.95$, $p < .001$, partial $\eta^2 = .79$], group [$F(3,32) = 46.47$, $p < .001$, partial $\eta^2 = .81$], and their interaction [$F(5.68,60.57) = 44.50$, $p < .001$, partial $\eta^2 = .81$].

Post-hoc comparisons revealed significant treatment effects for each day post-injection. On all four days post-LPS the AL group ate significantly less compared to baseline compared to the CR50% groups ($p < .001$ for all); however, only ate less in absolute terms on day one post-LPS. Within group simple main effect revealed that the AL animals' food intake was significantly lower than baseline on all four days post-LPS ($p < .001$ for all). In contrast food intake did not differ from baseline on any day post-LPS for any of the CR50% groups.

Discussion

Dose-dependent effect of calorie restriction on sickness behaviour

This study is the first investigation to reveal that there is a dose-dependent effect of duration of a 50% CR on sickness behaviour after LPS with the longest duration, 28 days, eliciting the largest attenuation in sickness behaviour (see Table 2). This study also replicates the findings we have previously demonstrated in mice (MacDonald et al., 2011) and rats (*Chapter 5*), where a 50% CR for 28 days attenuated sickness behaviour following LPS administration. The CR50% (28d) animals in the current study demonstrated a full attenuation in the rise in T_b following LPS, an attenuation of the reduction in locomotor activity, attenuated weight loss, and no reduction in food intake normally seen after LPS.

Shorter durations of CR (14 and 21 days) resulted in a limited, but not a full attenuation of sickness measures. The CR50% (14d) animals demonstrated an elevation in T_b post-LPS; however, not to the extent of the AL animals. The CR50% (21d) animals also demonstrated a slight rise in T_b post-LPS; however the rise in T_b fell in between the CR50% (14d) and the CR50% (28d) animals indicating the dose-dependent response to the duration of the CR period. There was again a clear dose-dependent reduction in locomotor activity post-LPS. As a whole, locomotor activity post-LPS followed the same pattern as T_b, with the CR50% (14d) animals demonstrating the largest decline in activity of all the CR50% groups, and the CR50% (28d) animals demonstrating the least.

The AL animals lost the most amount of weight following LPS, not returning to baseline levels for the four days post-LPS. The CR50% (14d) and CR50% (21d) animals also lost a significant amount of weight and did not return to baseline levels for the full four days post-LPS. The CR50% (28d) animals were the only animals that received LPS and did not lose a significant amount of weight post-LPS and were almost at pre-LPS weight levels by day three post-LPS.

All CR50% groups did not lower their food intake following LPS. It is interesting to note that the AL animals' food intake on day one post-LPS is lower than all CR50% groups food intake. An increased drive to eat in food-deprived animals following an immune challenge has been demonstrated previously (Mrosovsky, Molony, Conn, & Kluger, 1989). For example, rats CR by 33% demonstrated neither reduction in food intake nor a reduction in body weight during a seven day period of central leptin infusion (Soos, Balasko, Jech-Mihalffy, Szekely, & Petervari, 2010). The authors stated that this was due to the extreme orexigenic tone of the CR animals. In the current study the AL animals ate on average 30% less than the CR50% animals. Consequently, it is tempting to speculate that the CR50% animals are not simply driven to eat their allocated food as an artefact of being on a restricted

diet. However, if the orexigenic tone of the CR animals is enhanced, then it is possible that this overrides their need to suppress their food intake following LPS exposure.

Another reason the CR animals may not reduce their food intake after LPS is due to the large increase in neuropeptide Y (NPY) that we have shown in mice CR to 50% CR for 28 days at baseline, and two and four hours post-LPS (MacDonald et al., 2011). NPY is well known to increase appetite (Brady, Smith, Gold, & Herkenham, 1990) and attenuate LPS-induced fever (Felies, von Horsten, Pabst, & Nave, 2004). Hypothalamic levels of NPY have been shown to increase after as little as 24 and 48 hours of food deprivation and after five days of a 50% CR (Gutman, Hacmon-Keren, Choshniak, & Kronfeld-Schor, 2008; Levin & Dunn-Meynell, 1997). Therefore, even the rats in the current study CR to 50% for 14 days possibly had higher levels of NPY which may be contributing to their slightly attenuated febrile response and no reduction in appetite. Further, a study that investigated various periods of food deprivation and CR periods on plasma leptin levels found that it was not until after 23 days of a 50% CR that leptin levels were significantly reduced (Gutman et al., 2008). Leptin is well known to reduce food intake (Campfield, Smith, Guisez, Devos, & Burn, 1995; Luheshi, Gardner, Rushforth, Loudon, & Rothwell, 1999); therefore, levels seen in previous research may indicate that after 14 and 21 days of CR in the current study leptin levels may not have begun to reduce which would make one question why our rats CR for 14 and 21 days did not reduce their food intake. However, these same authors also demonstrated that leptin infusion did not reduce food intake or body weight in the mice CR to 50%, indicating that leptin most likely cannot overcome the drive to eat in CR animals (Gutman et al., 2008).

The dose-dependent effect of CR duration has been shown to impact upon other variables, such as reactive oxygen species (Bevilacqua et al., 2004) and cancer (Kumar et al., 1990); however, others have demonstrated that the impact the length of CR has on certain gene expression is less clear (Cao et al., 2001). This has been the first study to demonstrate a

dose-dependent relationship exists between the duration of a 50% CR and the level of attenuation of sickness behaviour in rats.

In addition, it appears from the current study that some sickness behaviour related variables are more sensitive compared to others post-LPS in CR animals. For example, although the CR50% (28d) animals did not display an increase in T_b or reduction in locomotor activity they did demonstrate a significant reduction in body weight for the first two days post-LPS. Others have suggested that locomotor activity may be the most sensitive indicator of illness (Skinner, Mitchell, & Harden, 2009); however, in the current study this is not the case. Although the CR50% (21d) animals did not demonstrate a significant reduction in locomotor activity post-LPS, they did demonstrate a significant increase in T_b post-LPS and did not recover to their pre-LPS weight for the entire four days post-LPS. This indicates that depending on what variable is being investigated the threshold of CR that needs to be reached can differ (see Table 2).

Although peripheral and central targets were not measured at 14 and 21 days post-LPS in CR animals we could hypothesise that the effects we have seen after a 28 day CR would also demonstrate a dose-dependent effect in regards to the duration of the CR regimen. Namely, in rats CR to 50% for 28 days that peripheral CORT was increased and IL-6 was decreased post-LPS (*Chapter 5*). Given that it has previously been shown that CORT increases in a dose-dependent manner at increasing levels of CR severity (Levay et al., 2010) it may also be the case that CORT levels may also respond in a dose-dependent manner in regards to the duration of CR, with the longer period (28 days) eliciting the largest increase in peripheral CORT. For instance, shorter periods of food intake manipulation can alter CORT levels as after only 18 hours of food deprivation peripheral CORT levels have been shown to be higher than that of controls (McGhee, Jefferson, & Kimball, 2009). Further, mice CR to 50% for 28 days exhibit a central anti-inflammatory bias as demonstrated by attenuated

increases in COX-2, mPGES-1 and leptin receptor and increases in IL-10 and SOCS3 (MacDonald et al., 2011). Investigating peripheral and central targets at 14 and 21 days would be of benefit to further elucidate the mechanisms underlying the attenuation of sickness behaviour after CR and to see if there is a threshold of CR in relation to these variables.

As mentioned above in relation to CORT, the severity of the CR regimen plays a role in the outcome of investigations. We have also previously shown that sickness behaviour is attenuated in a dose-dependent manner, with a 25% CR resulting in a mild attenuation and a 50% CR resulting (similar to this chapter) in a full attenuation in mice (MacDonald et al., 2011). The 25% CR mice demonstrated a fever to the same magnitude as the control mice; however, it was shorter in duration, lasting only about five hours in comparison to more than eight hours for the control mice. The CR25% mice also demonstrated no reduction in food intake and limited weight loss (MacDonald et al., 2011). Therefore, these two investigations reveal that CR results in a dose-dependent attention in sickness behaviour in terms of both the duration and severity of the CR regimen.

Table 2. *The effect of different durations of CR on four measures of LPS-induced sickness behaviour in the rat.*

	T_b	Activity	Body weight	Food intake
AL	↑	↓	↓	↓
CR50% (14d)	↑	↓	↓	_
CR50% (21d)	↑	_	↓	_
CR50% (28d)	_	_	↓(partial)	_

Conclusion

The present study demonstrated that there is a dose-dependent effect of CR on sickness behaviour. Namely, the longer period of a 50% CR (28 days) was the most effective in attenuating sickness behaviour compared to the shorter periods of 14 and 21 days. These findings enable future research to use this template of methodology to base investigations concerning sickness behaviour and immune function after a period of CR. These results also add further weight to the argument that a relatively short period of CR (28 days) can attenuate sickness behaviour measures and that this phenomenon presents in a dose-dependent manner.

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CHAPTER 7

METABOLIC RATE IS REDUCED DUE TO CALORIE RESTRICTION IN RATS AND IS NOT ALTERED POST-LPS

Submitted for publication in combination with *Chapter 8* to Physiology & Behavior

Abstract

It has been widely reported that calorie restriction (CR) results in a decrease in metabolic processes. However, an examination of how CR affects metabolic rate post-lipopolysaccharide (LPS) has not been investigated. This study examined the effect of CR on sickness behaviour measures, energy expenditure (EE), and respiratory quotient (RQ) post-LPS. Male Sprague-Dawley rats fed ad libitum (AL) or restricted to 50% (CR50%) of the AL animals' food intake for 28 days were injected on the 28th day with saline (1 mL/500 g) and on the 29th day with 50 μg/kg of LPS. Core body temperature (T_b), locomotor activity, body weight, and food intake were determined for 24 hours post-LPS. Indirect calorimetry was used to determine total EE and RQ in AL and CR50% animals for 24 hours post-injection. The AL rats demonstrated an elevated T_b from 150 until 480 minutes post-LPS; however, the CR50% rats fever profile was dramatically attenuated, with the only significant increase occurring at 240 minutes post-LPS. The AL rats lost significantly more weight compared to the CR50% rats, although both groups lost weight compared to baseline. Only the AL rats reduced their food intake compared to baseline. Indirect calorimetry indicated that the CR50% demonstrated significantly reduced EE (-17.9 % ± 1.3) compared to the AL rats from 0 to 420 minutes post-saline. After LPS, the AL rats demonstrated an increase in EE at multiple time points between 90 and 420 minutes post-LPS, whereas the CR50% rats demonstrated no change compared to post-saline. The AL and CR50% rats demonstrated similar profiles of RQ after saline; however, after LPS the AL rats demonstrated a decrease in their RQ at 360, 450, and 480 minutes and for almost the entire dark phase post-LPS, whereas the CR50% rats demonstrated no difference compared to post-saline. The metabolic cost for rats to mount a fever during a period of low food availability may outweigh the benefits of producing a febrile response to a relatively small dose of LPS.

Calorie restriction (CR) has been consistently shown to reduce energy expenditure (EE) in animals and humans (Blanc et al., 2003; Duffy et al., 1989; Forsum, Hillman, & Nesheim, 1981; Gonzales-Pacheco, Buss, Koehler, Woodside, & Alpert, 1993; Lane et al., 1996; Leibel, Rosenbaum, & Hirsch, 1995; Martin et al., 2007; Ramsey, Roecker, Weindruch, & Kemnitz, 1997). For example, adult rats on a 40% CR for six weeks demonstrated a reduced EE (Gonzales-Pacheco et al., 1993), and in rhesus monkeys (aged eight to 14 years) on a 30% CR resting EE (during the dark phase) was reduced after 24 months of the CR regimen compared to controls (Ramsey et al., 1997). This was the first time point (when measured every six months) during their CR that the monkeys exhibited a reduced EE and their EE remained reduced until at least 30 months post-initiation of the CR regimens. However, when the CR monkeys were active (i.e., the light phase) their EE was similar to the controls (Ramsey et al., 1997). It has also been demonstrated in humans that a reduced calorie diet can lead to significant reductions in EE (Heilbronn et al., 2006; Martin et al., 2007). A six month 25% reduction in calories resulted in overweight, non-obese men and women to reducing their EE by almost 10% (Heilbronn et al., 2006).

Another example comes from rats that were CR to 30-40% of the control animals food intake for 17 days demonstrated reduced EE and also a reduction in another aspect of metabolic rate, respiratory quotient (RQ; measured via oxygen consumption) (Rothwell & Stock, 1982). RQ was reduced during the dark phase in the CR rats; however, the control and CR rats demonstrated similar RQ levels during the light phase. These same authors also observed a decline in brown adipose tissue (BAT) activity, which they suggested may account for the CR animals reduced EE (Rothwell & Stock, 1982). This study did not investigate core body temperature (T_b) which we have previously shown increases in CR animals in anticipation of meal delivery (MacDonald, Radler, Paolini, & Kent, 2011).

Therefore, even if BAT activity was reduced in the CR animals, they were still able to increase their T_b via other means.

An investigation in rats exposed to a 40% CR for 14 months found their RQ was markedly reduced compared to controls (Duffy et al., 1989). The CR rats demonstrated a reduced RQ during the light phase and a rapid increase in RQ surrounding the time they were fed, which was not seen in the control rats. This is in contrast to the findings presented above; a possible explanation of this comes from the time of day the rats were fed in each study. Rothwell and Stock (1982) fed their CR rats at the start of the light phase (their rest period) and Duffy et al. (1989) fed their CR rats five hours into the light phase. These differences in feeding times in respect to the relative light/dark periods may have entrained the CR rats' circadian rhythm of EE differentially. Duffy et al. (1989) suggested that this rapid increase of RQ in the CR rats was due to a rapid change in metabolic processes dependent on food availability which meant a shift from protein and fat metabolism to carbohydrate metabolism. Shortly after the CR rats finished eating their RQ gradually dropped as their carbohydrate stores depleted (Duffy et al., 1989).

Immune activation is well known to result in a marked increase in EE in a variety of animals and humans (Buchanan, Peloso, & Satinoff, 2003; Crawshaw & Stitt, 1975; Stitt, 1973; Stitt, Shimada, & Bernheim, 1985). For every 1 °C increase in core body temperature (T_b) metabolic rate increases by approximately 10% (Kluger, 1986). Rats injected intravenously with pyrogen prepared from human monocytes exhibited an increase in metabolic rate after 20 minutes, which was in accordance with the peak in rectal temperature increase (Stitt et al., 1985). The increase in EE post-LPS has been shown to vary from a limited increase in EE for only the first two hours post-LPS (Arsenijevic et al., 1998) to a sustained increase for at least the first four hours post-LPS (Arsenijevic et al., 2000). Even when fever is not present EE has been shown to be increased in chronic cases of

inflammation such as HIV infection (Hommes, Romijn, Endert, & Sauerwein, 1991; Melchior et al., 1991).

The impact of LPS on RQ has been less consistent; some investigations report that RQ does not change post-LPS (Harada et al., 2007), whilst others find that RQ is altered (increased in the first three hours and then decreased for the next seven hours) after LPS (Steiger et al., 1999). Rats injected with LPS demonstrated no difference in RQ post-LPS compared to rats injected with saline (Harada et al., 2007). On the other hand, heifers exposed to an infusion of LPS for 90 minutes demonstrated a limited increase in RQ for three to four hours post-LPS infusion; however, then demonstrated a decrease in RQ until at least 10 hours post-LPS (Steiger et al., 1999).

The effect of CR on EE and RQ has been investigated previously (Blanc et al., 2003; Duffy et al., 1989; Gonzales-Pacheco et al., 1993; Lane et al., 1996; Martin et al., 2007; Ramsey et al., 1997); however, to the best of our knowledge there has been no investigation of the effect of CR on EE and RQ after LPS exposure. The aim of the current study was to characterise the metabolic rate of the CR animals post-LPS via indirect calorimetry. It was hypothesised that the CR animals would display a reduced metabolic rate at baseline as indicated by reduced total EE and reduced RQ compared to control animals. Post-LPS it was hypothesised that the CR animals would demonstrate no change in the abovementioned variables due our previous investigations that have demonstrated an attenuated febrile response post-LPS (MacDonald et al., 2011); whilst the control animals would demonstrate a slight increase in EE following LPS injection.

Methods

Animals

Eighteen male Sprague-Dawley rats were procured from Monash SPF animal services (Clayton, Victoria, Australia) and allowed to acclimate to the facility for at least one week. During this period, standard rodent chow (Barastoc, Melbourne, Australia) and water were available ad libitum. At the beginning of experimentation the rats were aged between 12 and 14 weeks old. Rats were individually housed in polypropylene basin cages (30 × 50 × 15cm) with sawdust and tissues provided as bedding. Rats were maintained at an ambient temperature of 26 ± 1 °C, which is within the thermoneutral zone for this species (Poole & Stephenson, 1977) on a 12:12 light/dark cycle (0500-1700 hours). Animal care and experimentation was performed with approval from the La Trobe University Animal Ethics Committee.

Surgery

Following acclimation all of the rats were surgically implanted in the peritoneal cavity with a biotelemetry device (E-4000, Mini-mitter®, Bend, OR, USA: 23 × 8 mm, 1.6 g) under anaesthesia (Begg, Kent, McKinley, & Mathai, 2007). Rats were anaesthetised in an induction chamber using 4% isoflurane and 0.6 L/min oxygen flow and were maintained during surgery on 2.5% isoflurane and 0.4 L/min oxygen flow using a nose cone (refer to *Chapter 2* for more detail). These rats were allowed 10 days to recover before the initiation of the CR regimens.

Dietary regimens

Rats were divided into one of two CR regimens matched for weight, food intake, and age: ad lib (AL; n = 9) fed ad libitum (ranging from 24-30 g per day); and CR50% (n = 9) rats received 50% of the amount consumed by AL (ranging from 12-15 g per day). The dietary composition of the AL and CR50% diets has been published elsewhere (Levay,

Govic, Penman, Paolini, & Kent, 2007; Levay, Tammer, Penman, Paolini, & Kent, 2010). The intake of the CR groups was determined weekly based on the average daily food intake of the AL group for three consecutive days. Water was continuously provided to all groups. The dietary manipulation continued for 27 days before saline challenge and was continued until the end of experimentation. Food was provided daily, approximately one hour before the dark phase onset.

Measurement of T_b and locomotor activity

The rats had a biotelemetry device implanted to measure core T_b and locomotor activity. T_b and loco-motor activity were determined by placing each cage on a receiver with each individual biotelemetry device generating a continuous frequency signal proportional to the animal's T_b ($\pm 10^{-1}$ °C). The receiver sampled this frequency at 1-minute intervals and this sample was decoded by VitalView software (Mini Mitter Co., Inc., Bend, OR) and stored on a hard drive. The receiver for each cage was equipped with a matrix of antennas that were continuously signalled by the biotelemetry device. The receiver scanned the matrix in a sequential order to locate the position and orientation of the biotelemetry device, thus making it possible to detect the global locomotor activity of the rat. The receiver tallied the number of matrices crossed by the rat during 1-minute intervals and this information was recorded using VitalView software as described previously (Weiland, Voudouris, & Kent, 2004).

Indirect calorimetry

Calorimetric measurements were conducted on the day the rats were injected with saline and LPS using a custom built, four-cage, open-circuit calorimetry system. RQ and EE were measured via the calorimetry system (LabMaster; TSE Systems) as previously described (Jayasooriya et al., 2008). Both AL and CR50% rats were placed into the indirect

calorimetry system for a period of 72 hours; an acclimation stage (24 hours), the day they received a saline injection (24 hours), and the day they received a LPS injection (24 hours).

Procedure

Rats were weighed once a week approximately three hours after lights-on during dietary manipulation and daily at the same time on the day of saline and LPS injections using top loading scales. Food consumption was also determined at this time and was determined to the nearest .1 gram by providing a set amount of food each day and weighing the remaining food 24 hours later, including the uneaten food in the bedding. On day 27 of the CR period the animals were acclimated to the calorimetry chambers for a period of 24 hours. All rats received a saline injection on day 28 of the CR period (1 ml/500 g of saline). On day 29 all rats received an intraperitoneal injection of 50 μg/kg of LPS from *Escherichia coli* (serotype 0111:B4: Sigma, Castle Hill, NSW) in 1 ml/500g saline. Both saline and LPS challenges were performed approximately four hours after lights-on (ranging from 0800 hours to 0900 hours). All measures were continued for 24 hours post-saline and LPS.

Data analysis

All data are presented as means ± SEM for each group. The body weight data was collected once per week throughout the CR period. Body weight was also measured 24 hours prior and after both saline and LPS injections. Food intake data was collected over a 4-day baseline period prior to saline and LPS injections and averaged into one value for each animal. Food intake was also determined 24 hours after saline and LPS injections. Due to baseline differences both body weight and food intake post-saline/LPS values were presented as percentage change.

T_b, locomotor activity, EE, and RQ data collected during the day the rats received saline and LPS injections were averaged and means ± SEM were calculated for each group and presented as 30 minute averages. To determine the change in T_b and locomotor activity post-LPS the values of each variable on the day the rats received LPS were subtracted from the values of each variable on the day the rats received saline. Data were then analysed using 2 × 2 analysis of variance (ANOVA). To overcome violations of sphericity, the Greenhouse-Geisser statistic corrected degrees of freedom were reported. Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$ as the criterion for significance.

Results

Effect of CR on body weight

As expected the CR50% group lost weight, whereas the AL group demonstrated a slight weight gain (Figure 1). The CR rats lost weight steadily at the onset of CR; however, weight loss began to plateau between day 21 and day 28 of the CR period. On day 28 of the CR period the AL animals weighed 457.1 g (± 12.1) and the CR50% animals weighed 359.2 g (± 4.6).The ANOVA found a significant main effect for time [$F(1.93,30.90) = 15.11$, $p < .001$, partial $\eta^2 = .49$], group [$F(1,16) = 17.97$, $p < .001$, partial $\eta^2 = .53$], and their interaction [$F(1.93,30.90) = 243.06$, $p < .001$, partial $\eta^2 = .94$]. Upon allocation of the dietary regimens the AL and CR rats were not different in weight; however, they differed on each subsequent day of the dietary regimens (day 7 $p = .037$, day 14, 21, and 28 $p < .001$). The AL rats steadily put on weight compared to baseline from day 7 until day 28 ($p < .001$ for all) and the CR rats were consistently lighter compared to baseline from day 7 until day 28 of the CR regimen ($p < .001$ for all).

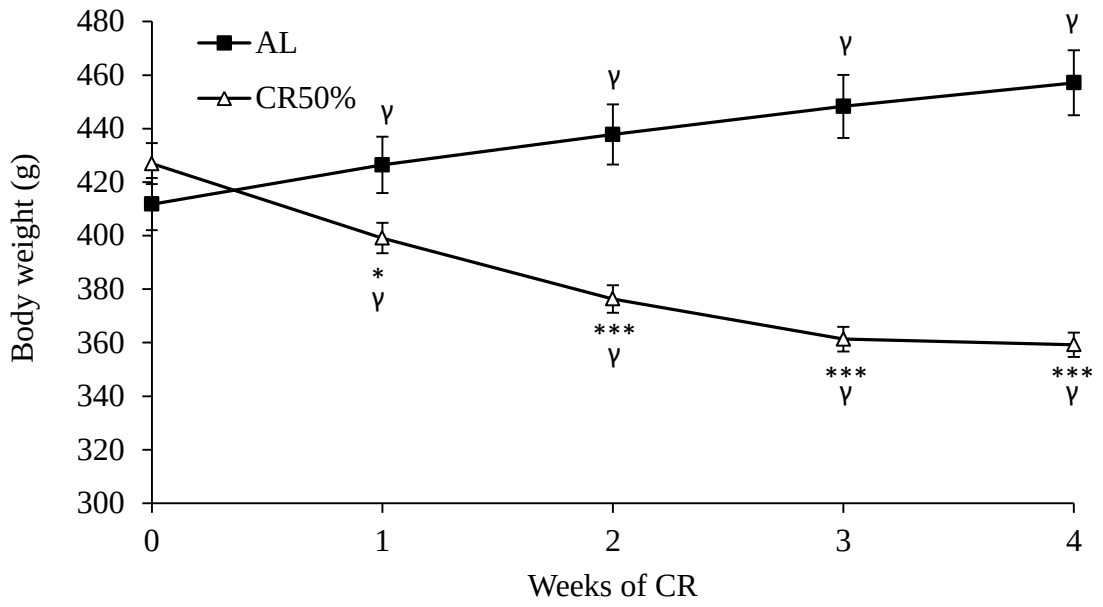

Figure 1.Mean (±SEM) body weight (g) of the AL (n = 9) and CR50% (n = 9) animals during the CR period. (*) denotes a significant difference from the AL group at $p < .05$, (***) denotes a significant difference from the AL group at $p < .001$, and (γ) denotes a significant difference from baseline at $p < .001$.

Sickness behaviour after LPS

Core T_b post-LPS

The AL rats demonstrated an increase in their T_b following LPS injection, peaking at 1.4 °C (± 0.2) 360 minutes post-LPS injection, whereas the CR50% rats only demonstrated a minimal increase of 0.5 °C (± 0.2) at 330 minutes post-LPS injection (Figure 2); the full 24 hours post-LPS can be seen in Appendix A. The ANOVA found a significant main effect for time [$F(3.31,39.76) = 17.46$, $p < .001$, partial $\eta^2 = .59$] and group [$F(1,12) = 10.65$, $p = .007$, partial $\eta^2 = .47$]. The AL rats demonstrated a higher T_b compared to the CR50% rats from 300 until 480 minutes post-LPS (ranging from $p = .035$ to $p = .008$). The AL animals T_b was lower compared to baseline at 30 and 60 minutes post-LPS ($p = .024$ and .036 respectively) and was elevated compared to baseline from 180 until 480 minutes post-LPS (ranging from p

= .003 to $p < .001$). The only time in which the CR50% rats T_b was significantly elevated compared to baseline was at 270 minutes post-LPS ($p = .045$).

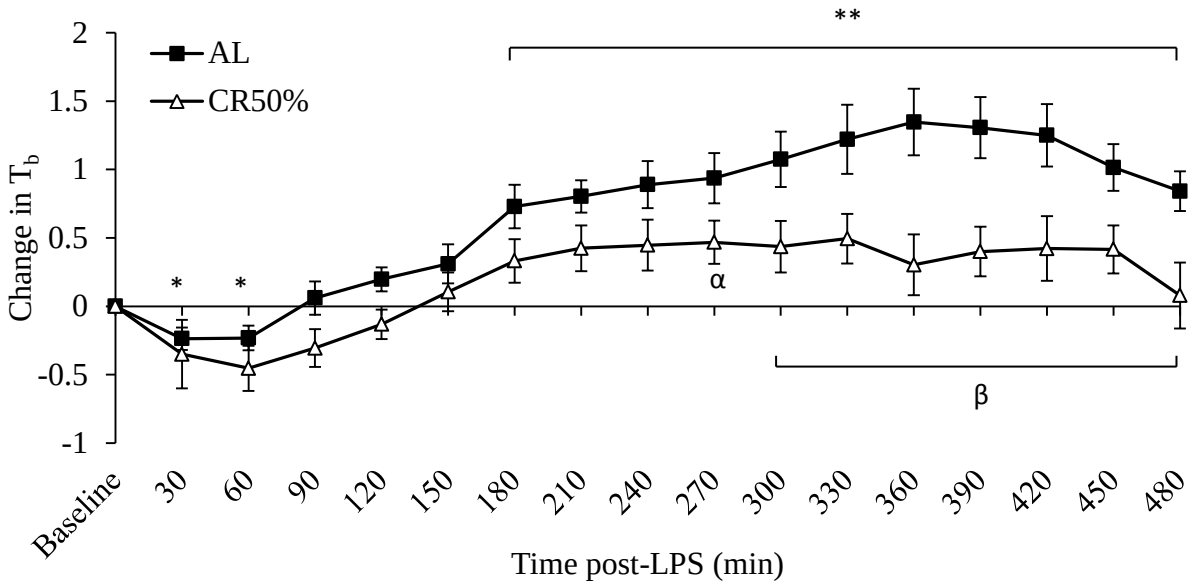

Figure 2. Change in T_b compared to baseline (the day of LPS subtracted from the day of saline) in AL ($n = 9$) and CR50% ($n = 9$) post-LPS. (*) denotes a significant difference from baseline in the AL group at $p < .05$, (**) denotes a significant difference from baseline in the AL group at $p < .01$, and (α) denotes a significant differences from baseline in the CR50% group at $p < .05$, (β) denotes a significant difference from the AL group at $p < .05$.

Body weight post-LPS

During the 24 hours post-LPS injection the AL rats lost -22.5 g (± 2.1 g) and the CR50% rats in comparison lost -5.8 g (± 1.0 g) body weight (Figure 3). The ANOVA found a significant main effect for time [$F(1,16) = 158.86$, $p < .001$, partial $\eta^2 = .91$], group [$F(1,16) = 40.24$, $p < .001$, partial $\eta^2 = .72$], and their interaction [$F(1,16) = 40.24$, $p < .001$, partial $\eta^2 = .72$]. Both the AL and CR50% group lost a significant amount of weight compared to their respective baselines ($p < .001$ for both); however, the AL group lost significantly more weight compared to the CR50% group ($p < .001$).

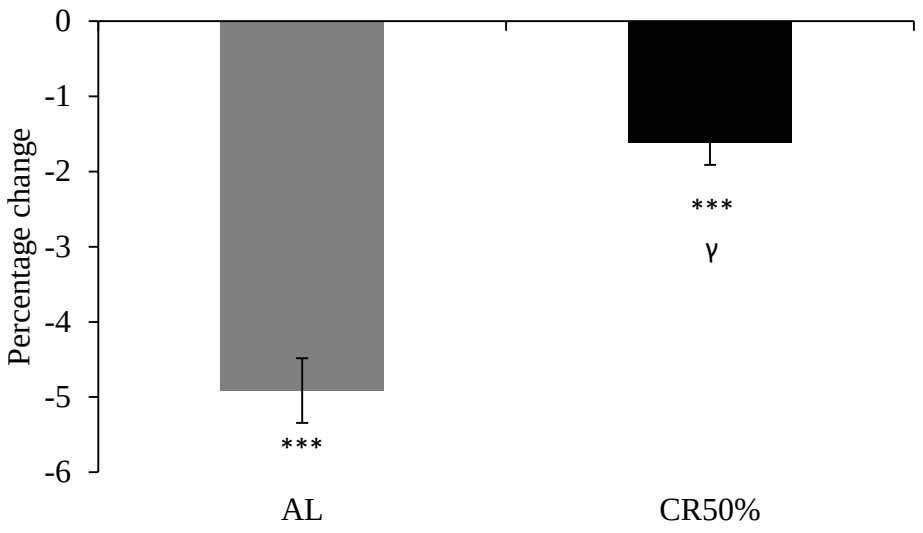

Figure 3. Percentage change in body weight compared to baseline (compared to the day prior to LPS injections) in AL (n = 9) and CR50% (n = 9) 24 hours post-LPS. (***) denotes a significant difference from baseline at $p < .001$ and (γ) denotes a significant difference from the AL group at $p < .001$.

Food intake post-LPS

The CR50% rats did not reduce their food intake post-LPS; however, the AL rats reduced their food intake in the 24 hours post-LPS to the level of the CR50% group (-49% ± 8%). The ANOVA found a significant main effect for time [$F(1,16) = 37.05$, $p < .001$, partial $\eta^2 = .70$], group [$F(1,16) = 37.05$, $p < .001$, partial $\eta^2 = .70$], and their interaction [$F(1,16) = 37.05$, $p < .001$, partial $\eta^2 = .70$]. The AL and CR50% groups differed significantly in their reduction of food intake following LPS ($p < .001$) and the AL group differed significantly from baseline ($p < .001$), whereas the CR50% group did not.

Locomotor activity post-LPS

The AL animals show a larger decrease in locomotor activity during the light phase post-LPS compared to the CR50% animals. During the dark phase both groups decrease their activity further, with the AL rats still demonstrating a larger decrease in activity compared to the CR50% rats (Figure 4). ANOVA only found a main effect for time [$F(1,7) = 6.50$, $p = .038$, partial $\eta^2 = .48$]. The AL rats were only marginally less active compared to the CR50% rats during the light phase post-LPS ($p = .055$). The AL rats were less active compared to the day before LPS during the light and dark phases ($p = .048$ and $p = .040$ respectively), whereas the CR rats were only marginally less active during the dark phase ($p = .055$).

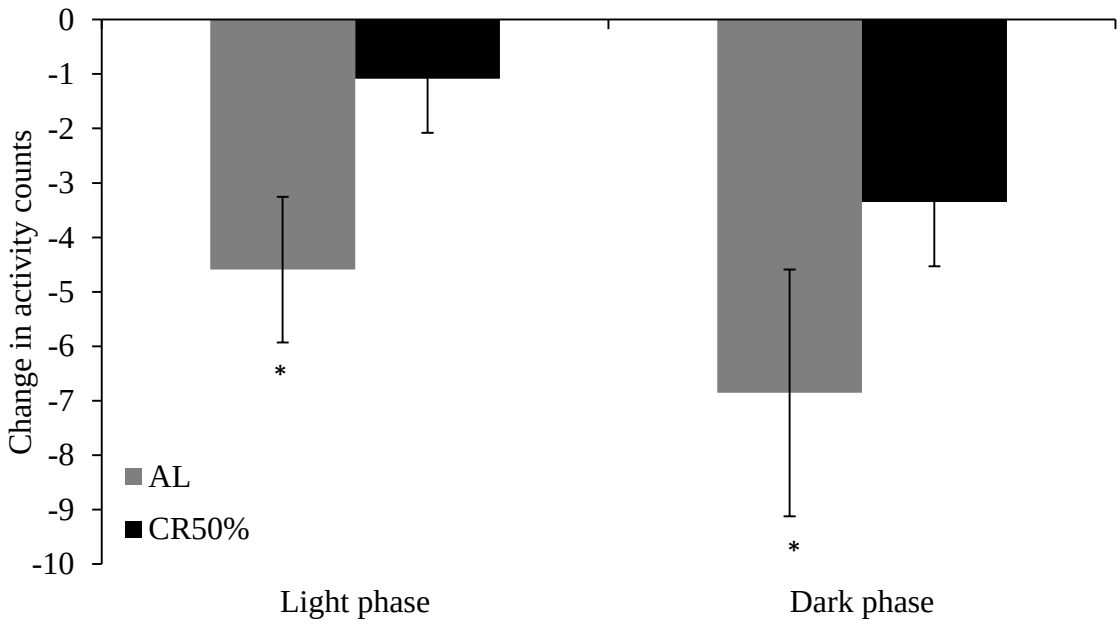

Figure 4. Change in locomotor activity counts in the light and dark phases post-LPS compared to the day before LPS (the day of LPS subtracted from the day of saline) in AL ($n = 9$) and CR50% ($n = 9$) rats. (*) denotes a significant difference from baseline at $p < .05$.

Indirect calorimetry

Energy expenditure post-saline and LPS

The CR rats demonstrated a reduced EE compared to the AL rats post-saline injection (Figure 5). Both groups demonstrate a slight increase immediately after the injection (30

minutes) and then reduce to a steady level for the next six hours. During the first six hours post-saline the CR50% rats demonstrate on average a 17.1% (± 3.1%) decrease in EE compared to the AL rats. Immediately prior to the dark phase (and feeding time) the CR50% rats demonstrate a marked increase in EE (at 450 minutes post-injection) then return to their reduced EE for almost the entirety of the dark phase (Appendix B). The ANOVA found a significant main effect for time [$F(6.25,99.97) = 21.56$, $p < .001$, partial $\eta^2 = .57$], group [$F(1,16) = 14.53$, $p = .002$, partial $\eta^2 = .48$], and their interaction [$F(6.25,99.97) = 5.11$, $p < .001$, partial $\eta^2 = .24$]. The AL rats demonstrated a significantly higher EE for all time points except for 450 and 480 minutes post-saline injection (ranging from $p = .025$ to $p < .001$).

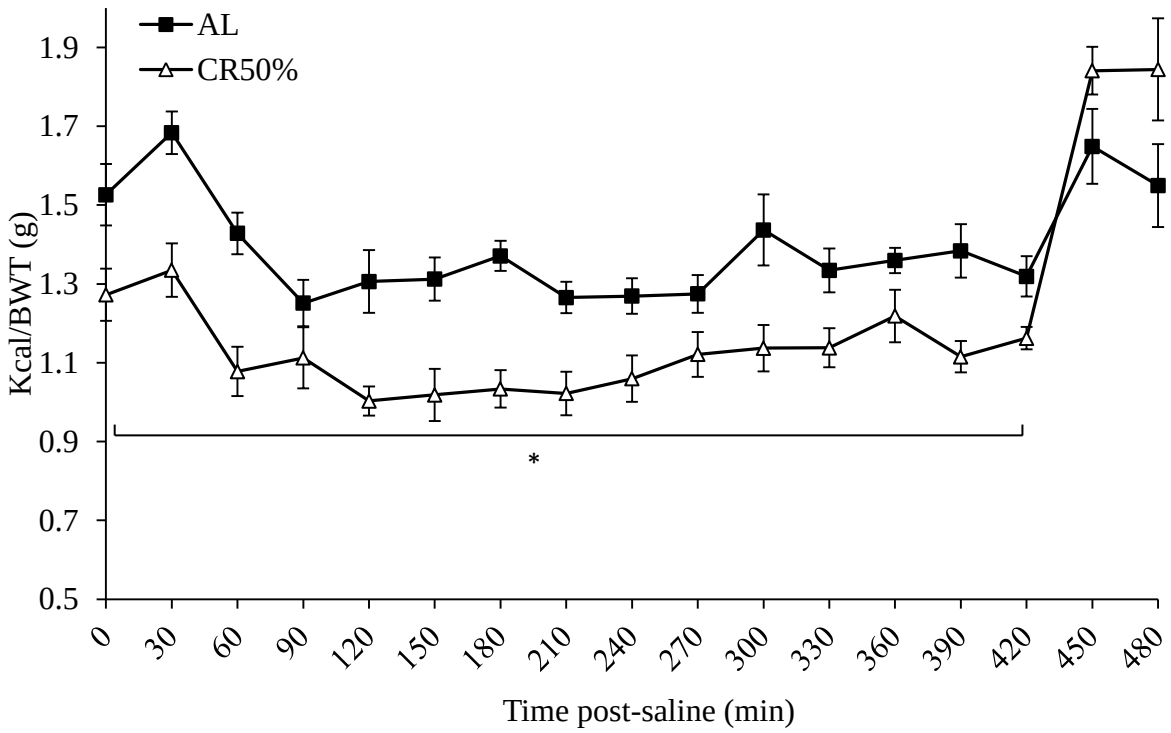

Figure 5. EE in AL ($n = 9$) and CR50% ($n = 9$) for 480 minutes post-saline injection. (*) denotes a significant difference from the AL group at $p < .05$.

A similar pattern to the day the rats received saline existed post-LPS (Figure 6). The increase in EE immediately after the injection is not as marked as it was post-saline injection and the AL rats do not demonstrate the increase in EE immediately prior to the dark phase as

they do on the day they received saline. The CR rats still demonstrate a reduced EE for the entire dark phase compared to the AL rats (Appendix C). The ANOVA found a significant main effect for time [$F(5.30,84.81) = 6.80$, $p < .001$, partial $\eta^2 = .30$], group [$F(1,16) = 53.02$, $p < .001$, partial $\eta^2 = .77$], and their interaction [$F(5.30,84.81) = 5.51$, $p < .001$, partial $\eta^2 = .26$]. The AL rats demonstrated a higher EE compared to the CR50% rats at every time point post-LPS except for 450 and 480 minutes post-LPS (ranging from $p = .005$ to $p < .001$), in the same pattern as the day the rats received saline.

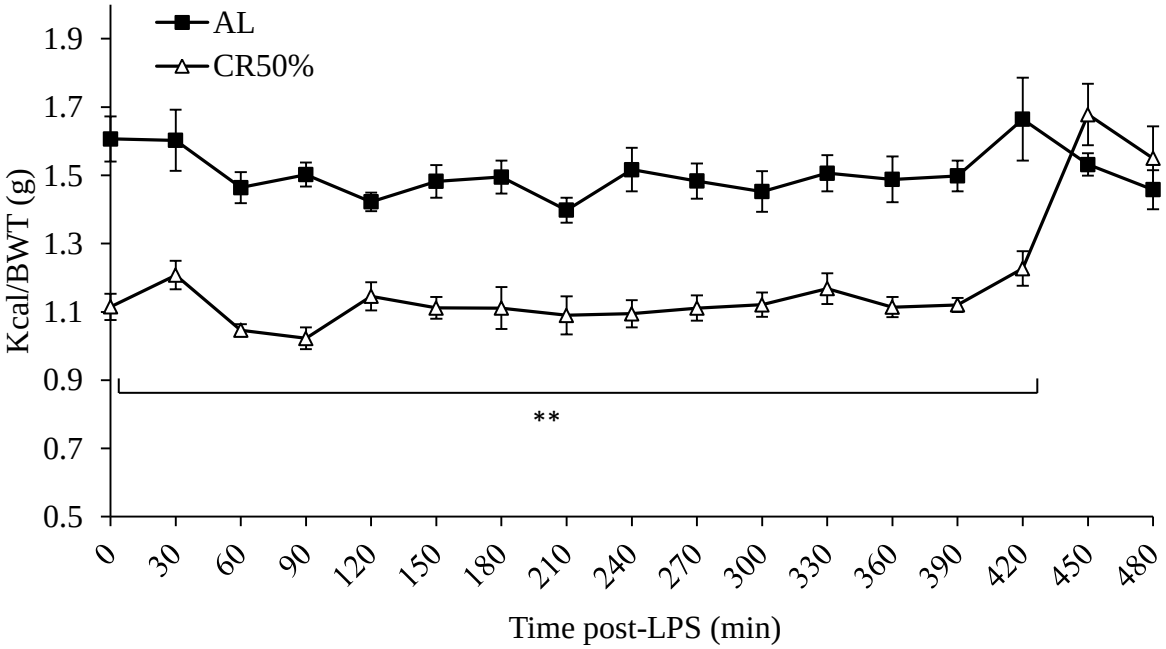

Figure 6. EE in AL ($n = 9$) and CR50% ($n = 9$) for 480 minutes post-LPS injection. (**) denotes a significant difference from the AL group at $p < .01$.

When comparing the AL rats on the day they received saline to the day they received LPS there are a number of differences (Figure 7). Apart from a slight increase in EE immediately after the injection on both days the EE of the rats post-LPS injection was higher than that of their EE post-saline injection for a number of time points. During the first six hours post-LPS the AL rats EE was on average 10.7% (± 2.2%) higher compared to their

post-saline EE. The AL rats post-LPS EE was also demonstrated to be lower at a number of time points during the dark phase post-LPS (Appendix D). The ANOVA found a significant main effect for time [$F(7.80,124.78) = 5.59$, $p < .001$, partial $\eta^2 = .26$] and the interaction [$F(7.80,124.78) = 3.06$, $p = .004$, partial $\eta^2 = .16$]. The AL rats demonstrated a higher EE at 90, 150, 210, 240, 270, 330, and 420 minutes post-LPS injection compared to post-saline injection (ranging from $p = .049$ to $p < .001$).

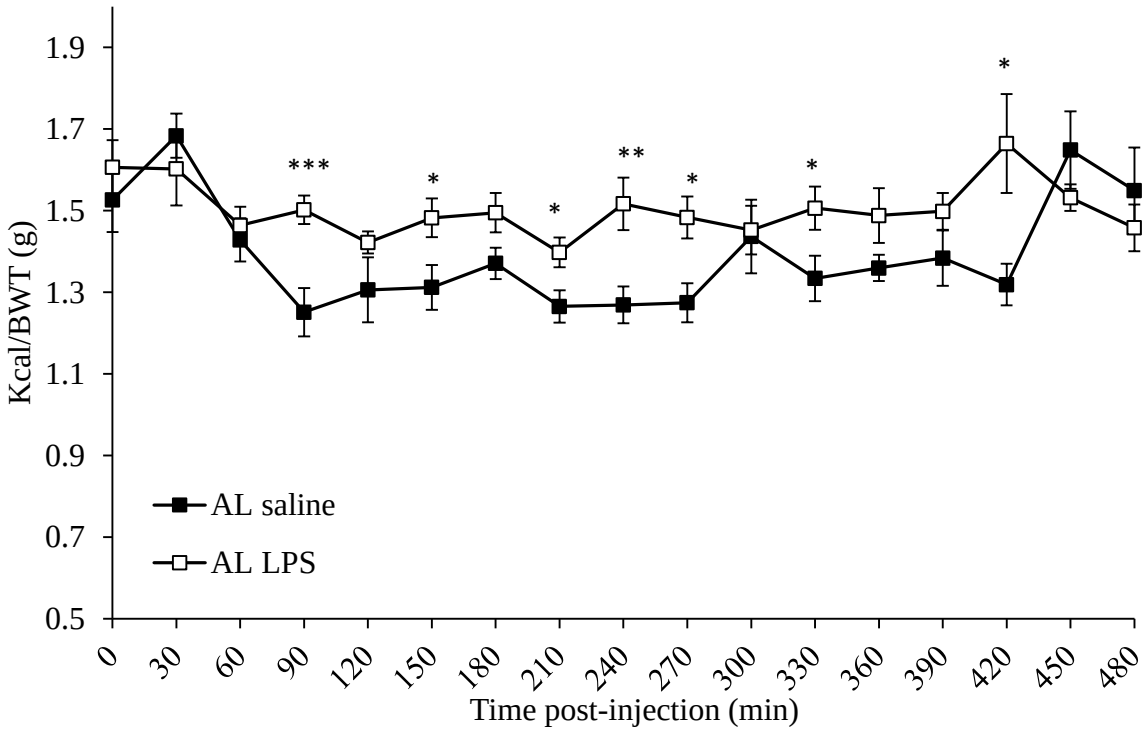

Figure 7. EE in AL ($n = 9$) animals for 480 minutes post-saline and LPS injections. (*) denotes a significant difference from the post-injection EE at $p < .05$, (**) denotes a significant difference from the post-injection EE at $p < .01$, and (***) denotes a significant difference from the post-injection EE at $p < .001$.

In contrast to the AL animals the CR50% animals demonstrate an almost identical pattern of EE after both saline and LPS injections (Figure 8). There is a slight peak in EE immediately post-injection and then also a dramatic increase in EE immediately prior to the

dark phase and around the time of food delivery. The pattern of EE during the remainder of the dark phase post-injection was also almost identical for the CR50% rats (Appendix E). The ANOVA only found a significant main effect for time [$F(4.75,75.94) = 25.89$, $p < .001$, partial $\eta^2 = .62$].

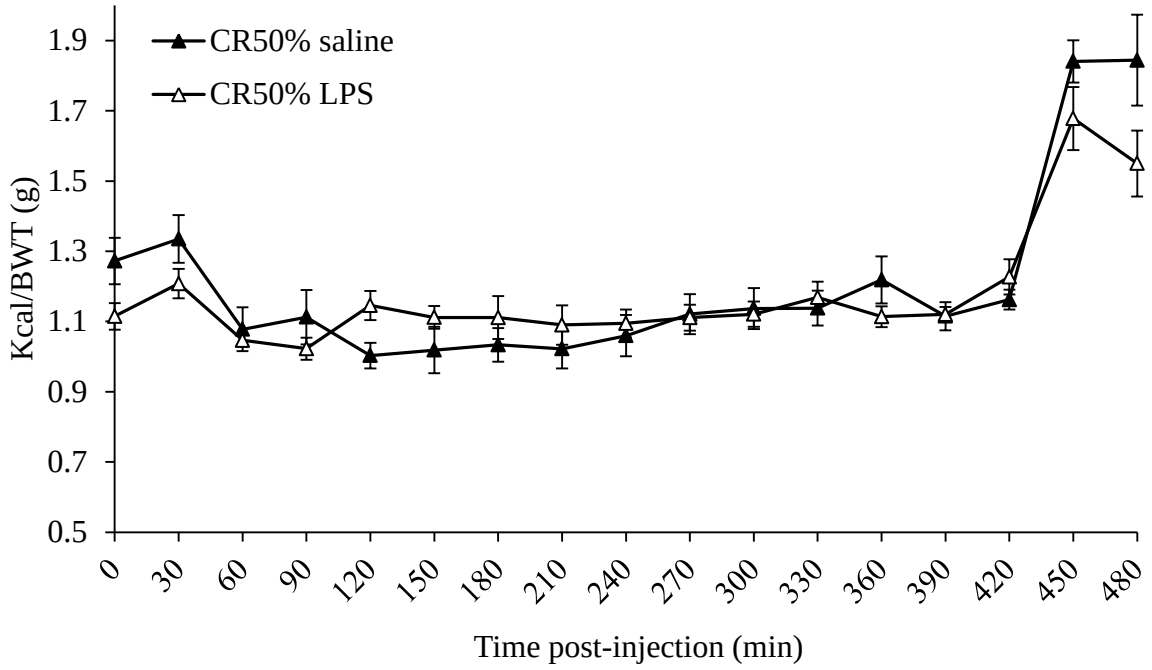

Figure 8. EE in CR50% (n = 9) animals for 480 minutes post-saline and LPS injections.

RQ post-saline and LPS in AL and CR50% rats

During the 8 hours post-saline injection the AL and CR50% rats demonstrated a similar pattern of RQ, with the ANOVA only finding an effect for time [$F(4.98,79.74) = 6.51$, $p < .001$, partial $\eta^2 = .29$]. It can be seen in Figure 9 that the AL and CR50% rats demonstrate relatively consistent RQ levels during the light phase, with the AL rats RQ gradually increasing in the second half of the light phase and the CR50% rats RQ stable until the presentation of food (which occurred immediately before the 480 minute time point). The CR50% rats demonstrated a reduced RQ compared to the AL rats during approximately half of the dark phase post-saline (Appendix F).

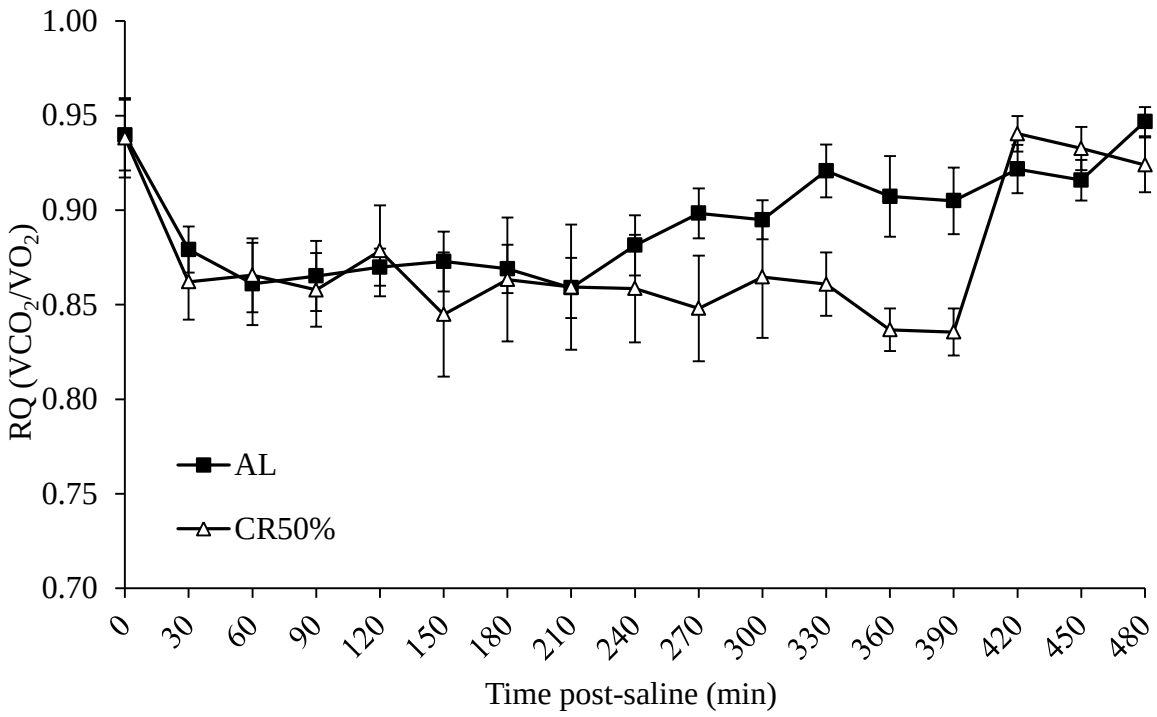

Figure 9. Half-hourly averages of RQ in AL (n = 9) and CR50% (n = 9) animals post-saline injection.

During the 8 hours post-LPS injection the AL and CR50% rats continue to demonstrate a similar pattern of RQ; however, whereas the CR50% rats again demonstrate an increase in RQ before the onset of the dark phase, the AL rats do not (Figure 10). The ANOVA found a significant main effect for time [$F(5.26,884.16) = 4.06$, $p = .002$, partial $\eta^2 = .20$] and the interaction with group [$F(1,16) = 4.15$, $p = .002$, partial $\eta^2 = .21$]. The AL groups RQ was higher compared to the CR50% groups RQ at the time of injection and at 300 minutes post-LPS ($p = .033$ and $p = .045$ respectively). This pattern was then reversed when the CR50% rats demonstrate a higher RQ at 450 and 480 minutes post-LPS ($p = .008$ and $p = .005$ respectively). During the first few hours of the dark phase post-LPS the CR50% appear to demonstrate slightly higher RQ levels compared to the AL rats, which then by half way through the dark phase the CR50% rats RQ levels drop down to the AL rats levels for the remainder of the dark phase (Appendix G).

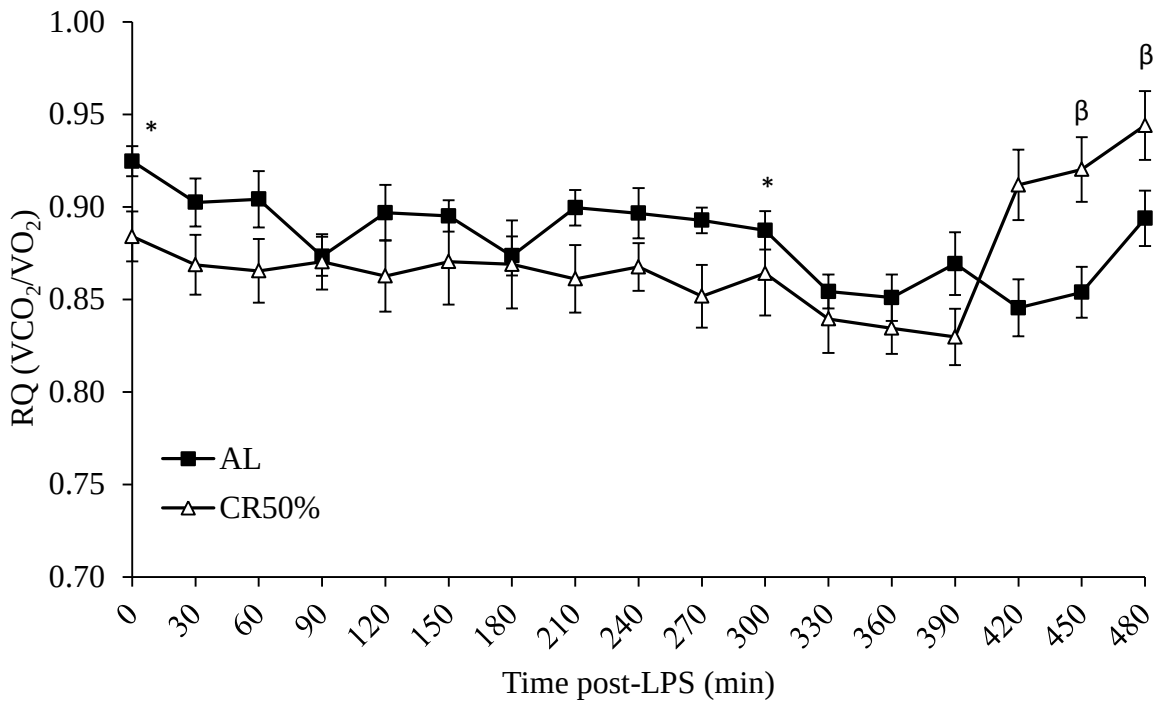

Figure 10. Half-hourly averages of RQ for in AL (n = 9) and CR50% (n = 9) animals post-LPS injection. (*) denotes a significant difference from the CR50% group at $p < .05$ and (β) denotes a significant difference from the AL group at $p < .01$.

When comparing the AL animals on the day they received saline to the day they received LPS it can be seen that when they received LPS their RQ reduced compared to when they received saline in the later period of the 8 hours post-LPS (Figure 11). The ANOVA found a significant main effect for time [$F(6.13,98.02) = 3.38$, $p = .004$, partial $\eta^2 = .18$] and the interaction with group [$F(3.13,98.02) = 6.08$, $p < .001$, partial $\eta^2 = .28$]. The AL animals saline RQ values were significantly higher than their LPS RQ at 360, 450, and 480 minutes post-injection (ranging from $p = .004$ to $p < .001$).This pattern continued into the dark phase, with the post-LPS RQ vales being lower than the post-saline values for the majority of the dark phase post-injection (Appendix H).

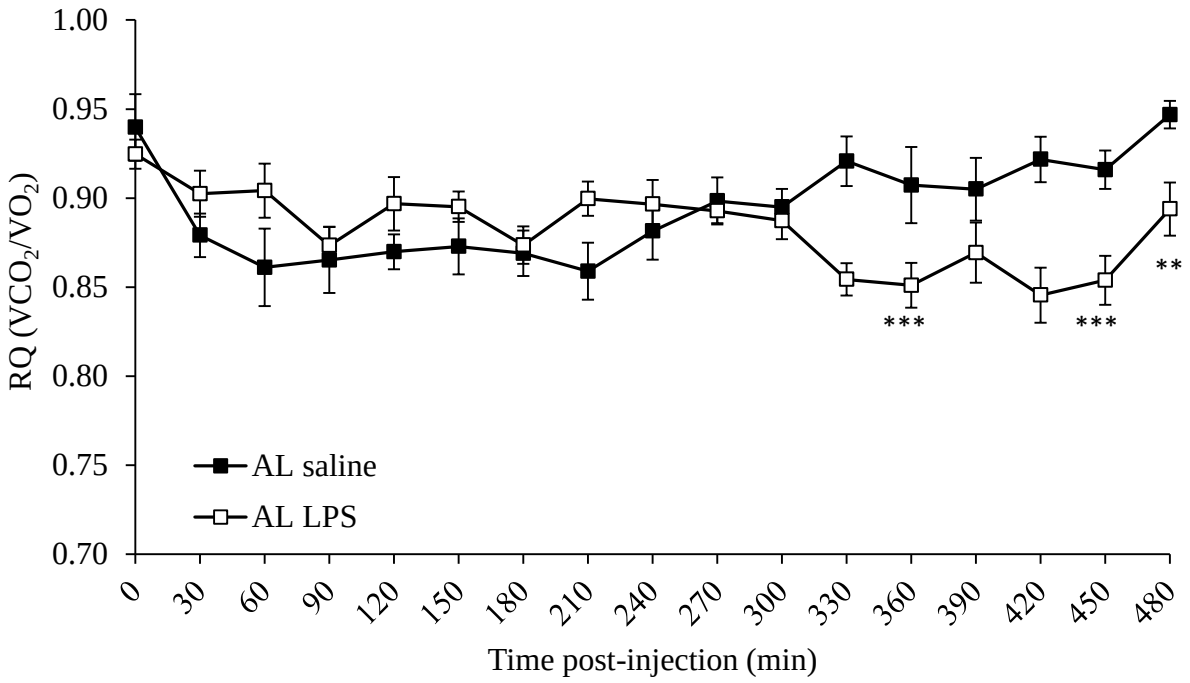

Figure 11. Half-hourly averages of RQ for AL (n = 9) animals post-saline and LPS injections. (**) denotes a significant difference from the AL LPS group at $p < .01$ and (***) denotes a significant difference from the AL LPS group at $p < .001$.

In comparison to the AL animals, the CR50% animals demonstrate almost identical RQ profiles post-saline and LPS (Figure 12). The ANOVA only found a significant main effect for time [$F(4.35,69.60) = 6.93$, $p < .001$, partial $\eta^2 = .30$]. This almost identical pattern continued throughout the dark phase post-injection (Appendix I).

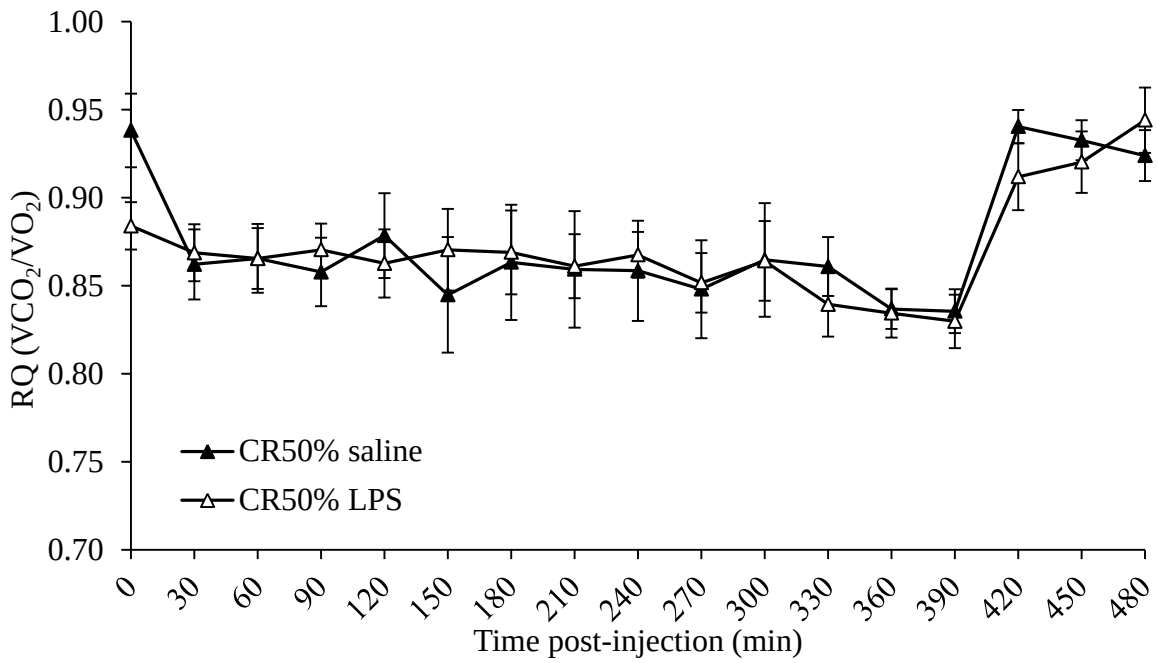

Figure 12. Half-hourly averages of RQ for CR50% (n = 9) animals post-saline and LPS injections.

The AL and CR50% rats demonstrate a similar pattern of RQ during the light and dark phases on the day they received saline, with both groups demonstrating higher RQ levels during the dark phase ($p < .001$ and $p = .032$ respectively; Figure 13). Although the AL and CR50% animals demonstrated equivalent RQ levels during the light phase, the AL animals demonstrated higher RQ levels during the dark phase ($p = .002$). LPS did not alter RQ levels in the light phase in the AL rats; however, RQ levels during the dark phase were significantly lower and a circadian difference was no longer present ($p = .003$). LPS did not alter RQ levels in the CR50% rats.

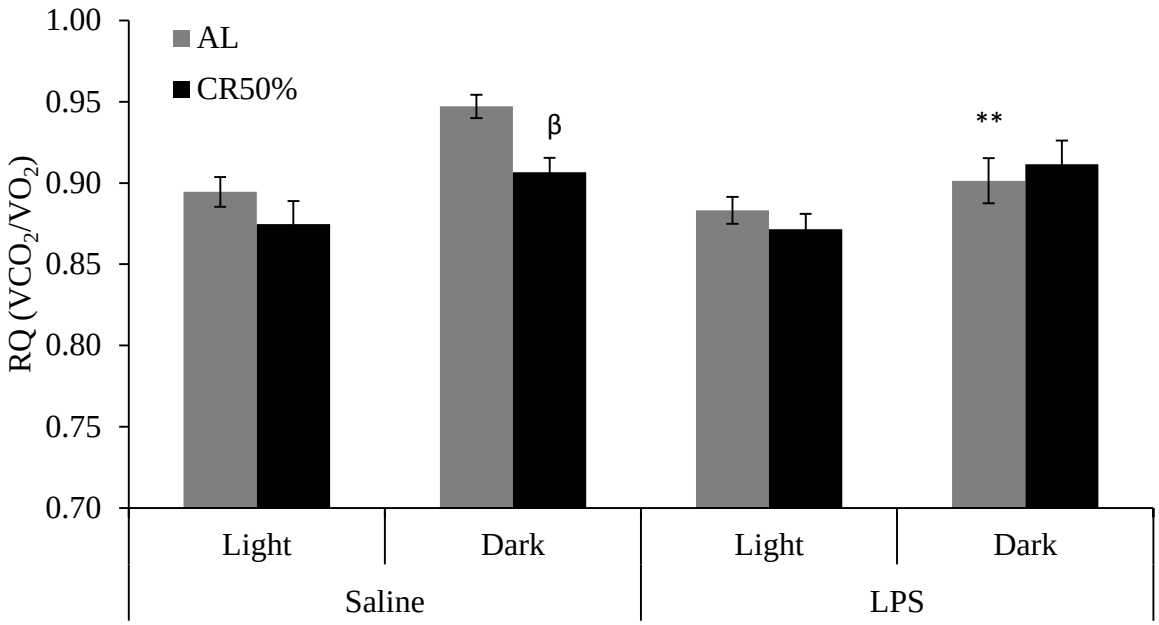

Figure 13. Light and dark averages of RQ for AL (n = 9) and CR50% (n = 9) animals post-saline and LPS. (**) denotes a significant difference from the same light/dark phase on the saline day at $p < .01$, and (β) denotes a significant difference from the AL group at $p < .01$.

Discussion

The CR50% rats demonstrated a significant attenuation of T_b increase, whilst the AL rats demonstrated a fever post-LPS. This attenuation in fever in the CR50% rats was also accompanied by a limited weight loss and no reduction in food intake post-LPS, consistent with previous research in our laboratory in rats (*Chapter 5*) and mice (MacDonald et al., 2011). The CR50% rats demonstrated reduced EE compared to the AL rats during the entirety of the time post-saline injection apart from a limited period surrounding the time when the CR50% rats were fed. LPS did not increase EE in CR50% rats in contrast to the AL rats increase seen at several time points in the eight hours post-LPS. RQ levels were relatively similar in the AL and CR50% rats post-saline; however, post-LPS the AL rats demonstrated a relatively similar RQ for the first few hours post-LPS and then there was a

decrease in their RQ levels leading into the dark phase. This was followed by a marked decrease in their RQ levels during the entirety of the dark phase post-LPS.

Effect of CR on EE and RQ

It has been shown previously that a period of CR can reduce EE and RQ (Blanc et al., 2003; Duffy et al., 1989; Forsum et al., 1981; Gonzales-Pacheco et al., 1993; Lane et al., 1996; Martin et al., 2007; Ramsey et al., 1997; Santos-Pinto, Luz, & Griggio, 2001). The current investigation supports these findings, with the rats CR50% rats demonstrating a reduced EE during almost the entire 24 hour period post-saline injection with the exception of three hours surrounding the CR animals food presentation. It has been shown previously that rats on only a 10% CR exhibited significant reductions in EE after one month of CR (Santos-Pinto et al., 2001). Further, it has been postulated that CR may not reduce total EE; however, when broken down into the active and resting phases it can be seen that CR results in a decrease in EE during the resting phase (Ramsey et al., 1997). As mentioned, the current study demonstrated a reduced EE for almost the entire 24 hour period; which may be an artefact of our relatively severe CR regimen. Others have used more mild CR regimens and this may account for the discrepancies between the results.

The CR rats also demonstrated similar RQ levels as the AL rats during the majority of the light and dark phases and then demonstrated a reduction in RQ levels for the second half of the dark phase on the day they received saline. This is in agreement with previous research that found RQ to be similar in CR (30-40%) and control rats during the resting phase (light) but decreased in CR rats during the active phase (dark) (Rothwell & Stock, 1982). However, it must be noted that the rats in the abovementioned study were fed their allotment of food at the beginning of their light phase, and the authors stated that this may have been why their RQ levels were similar to the control rats during this phase (Rothwell & Stock, 1982). In

relation to the drop in RQ levels in the CR rats during the dark phase the authors were unable to provide a solid explanation for why this may have occurred. The current study also demonstrated the rapid increase in the CR rats RQ levels immediately surrounding meal presentation, which has been seen in CR rats previously and is probably due to a rapid change in metabolic processes dependent on food availability (Duffy et al., 1989).

A reduction in EE, in conjunction with a reduced T_b, as a consequence of CR has been theorised as one of the major mechanisms in which CR extends the life-span. The reduction in EE and T_b in response to CR are thought to be due to the body activating an energy conservation mechanism (Lane et al., 1996). The mechanism by which reduced EE is thought to increase the life span by reducing oxidative stress (Barja 2004; Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; Shigenaga, Hagen, & Ames, 1994). Oxidants are produced as a by-product of aerobic metabolism, or increased EE (Shigenaga et al., 1994); therefore, it is thought that the lower the aerobic metabolism, the lower the oxidative damage.

Effect of CR on T_b, locomotor activity, EE, RQ and post-LPS

In agreement with previous research in this laboratory the CR rats demonstrated an attenuation of sickness behaviour, namely an attenuated febrile response, limited weight loss and reduction in locomotor activity compared to AL rats, and no reduction in food intake post-LPS (*Chapter 5*; MacDonald et al., 2011). This to our knowledge is the first study to also demonstrate that CR rats demonstrate no change in EE and RQ post-LPS.

As expected the AL rats demonstrated an increase in EE for the majority of the first 8 hours post-LPS (the light phase); however, during the dark phase their EE dropped to lower levels compared to before exposure to LPS. This is agreement with previous research demonstrating that EE was increased for up to four hours post-LPS in mice (housed at 29 °C) (Arsenijevic et al., 2000). Further, as mentioned earlier, rats injected intravenously with a

pyrogen demonstrated a fast increase in metabolic rate only 20 minutes after injection, which was in accordance with the peak in rectal temperature increase (Stitt et al., 1985). In the current study, the CR50% rats demonstrated an identical pattern of EE over the 24 hours period post-LPS compared to post-saline which helps to explain the lack of a sizable increase in T_b. In respect to RQ the AL rats demonstrated a similar RQ post-saline during the first 6 hours post-LPS; however, their RQ began to drop compared to post-saline just before the onset of the dark phase and remained lower during the majority of the dark phase post-LPS. Similar to their EE, the CR50% rats demonstrated no difference in their RQ post-LPS compared to post-saline. This replicates findings in control and restricted heifers (restricted to the amount the control animals ate post-LPS) as mentioned earlier. The control heifers demonstrated a slightly increased RQ immediately post LPS infusion, which turned into a reduced RQ for a sustained period for at least 10 hours post-LPS, whereas the restricted heifers demonstrated no change in their RQ profile post-LPS (Steiger et al., 1999).

The CR animals in the current study demonstrated no metabolic changes in response to LPS, whereas the AL rats did. This may indicate that the already reduced metabolic rate of the CR rats is preventing them from altering their metabolism any further, even in the presence of a bacterial compound such as LPS. The drop in EE in response to CR is thought to act as a protective mechanism to prevent the body from excessive depletion of energy stores (Poehlman & Horton, 1989). Therefore, post-LPS, when the body needs to use considerable amounts of energy to mount a febrile response [an increase in metabolic rate of approximately 10% is needed for every 1 °C rise in T_b (Kluger, 1986)], the CR animals may be reserving their energy stores. For example, in the current study the AL rats increased their metabolic rate by 10.7% in the first six hours post-LPS and given the ambient temperature of 26 °C the rats cannot just decrease heat loss, they need to increase thermogenesis in order to increase their T_b.

It is possible that the pyrogenic signalling pathways are disrupted and thus the CR animals brain does not receive a message to increase their T_b and subsequently increase metabolic rate post-LPS. Previously we have shown in 50% CR mice that hypothalamic levels of cyclooxygenase2 (COX-2) are attenuated at two hours post-LPS, microsomal prostaglandin E synthase-1 (mPGES-1) is also attenuated at two hours post-LPS, and suppressor of cytokine signalling 3 (SOCS3) is significantly increased at four hours post-LPS (MacDonald et al., 2011). Given that COX-2 [the rate-limiting step in PGE_2 production (Vane, Bakhle, & Blotting, 1998) and therefore the driver of fever production] and mPGES-1 [increased during inflammation and is down-regulated by glucocorticoids (Ivanov, Pero, Scheck, & Romanovsky, 2002; Kudo & Murakami, 2005)] are significantly attenuated in the CR animals suggests that pro-inflammatory signals are not reaching brain post-LPS. Further, SOCS3 is known to regulate the increase in the anti-inflammatory cytokine interleukin-10 (IL-10) and decrease the release of the major pro-inflammatory cytokine IL-6 (Bogdan, Vodovotz, & Nathan, 1991).We have also shown that peripheral levels of corticosterone (CORT) are significantly increased two hours post-LPS in 50% CR rats and that peripheral levels of IL-6 are significantly decreased at two hours post-LPS in CR rats (*Chapter 5*). CORT's anti-inflammatory properties are well known (Barnes, 1998), and IL-6 (as mentioned above) is the major pro-inflammatory cytokine (Cartmell, Poole, Turnbull, Rothwell, & Luheshi, 2000; LeMay, Vander, & Kluger, 1990). Therefore, given the changes in pro- and anti-inflammatory compounds post-LPS in the CR animals it is not surprising that they did not increase their metabolic rate in the current study as there possibly was no inflammatory message reaching the brain.

Mounting an immune response is a physiologically expensive process (Bonneaud et al., 2003; Demas, Chefer, Talan, & Nelson, 1997; Derting & Compton, 2003; Martin, Scheuerlein, & Wikelski, 2003; Svensson, Raberg, Koch, & Hasselquist, 1998), and there is

an ongoing process to balance the demand between low food availability, thermoregulatory demands, and immune processes. Further, it has been suggested that immunological process, like other physiological processes, should be optimised so that organisms can tolerate small infections if the energetic demands of mounting an immune response are too costly (Behnke, Barnard, & Wakelin, 1992). Trade-offs between immune function and thermoregulation and reproduction have been demonstrated (Bonneaud et al., 2003; Demas & Nelson, 1996); therefore, the CR animals in the current study have the competing demands of being exposed to a period of low food availability and also encountering an immune challenge. Further, the dose of LPS used in the current study was modest and it is possible that a larger dose of LPS might deliver a sufficient febrile signal to increase metabolism. Due to their already reduced EE, the CR rats physiologically favour thermoregulation over mounting an immune response (Behnke et al., 1992).

Conclusion

The current study demonstrated that a 50% CR in rats resulted in no change in EE and RQ post-LPS. Due to the CR rats EE already being significantly reduced compared to the AL rats it may be the case that the CR rats do not possess enough metabolic energy reserves to mount a febrile response and consequently the associated sickness behaviour. Trade-offs between mounting an immune response and other vital physiological processes during periods of low food availability has been demonstrated before. In this case the ability for the rats to thermoregulate during a period of low food availability may outweigh the benefits of mounting an immune response to a relatively small dose of LPS. Further, it is possible that the lack of pro-inflammatory cytokine message reaching the brain, as seen in our previous investigations, plays a large role in the CR animals not producing a febrile response and a subsequent increase in metabolic rate post-LPS.

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Appendix A

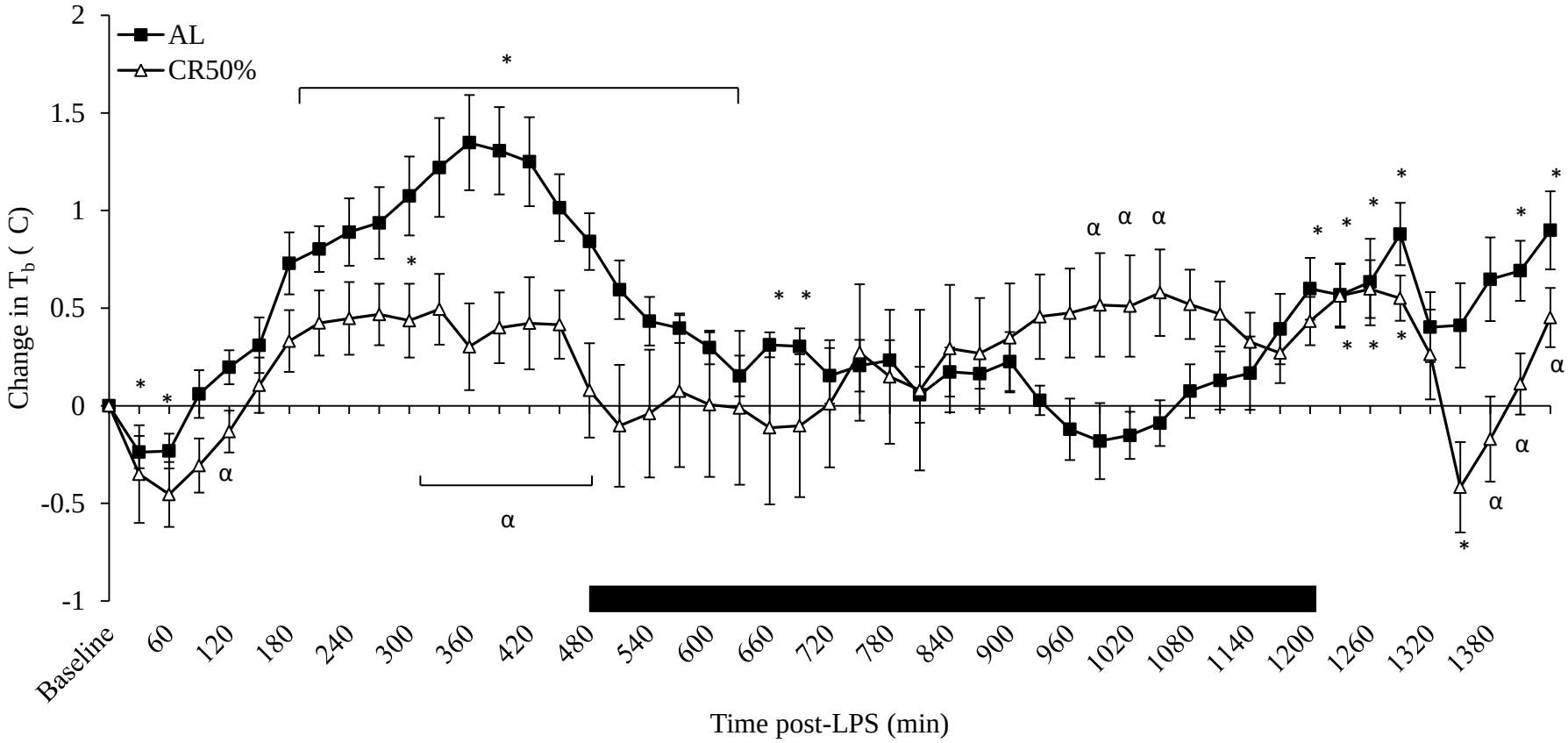

Figure A1. Change in T_b compared to baseline in AL (n = 9) and CR50% (n = 9) for 24 hours post-LPS, with the black box representing the dark phase. (*) denotes a significant difference from baseline in the AL and CR50% rats at $p < .05$, (α) denotes a significant difference from the AL group at $p < .05$.

Appendix B

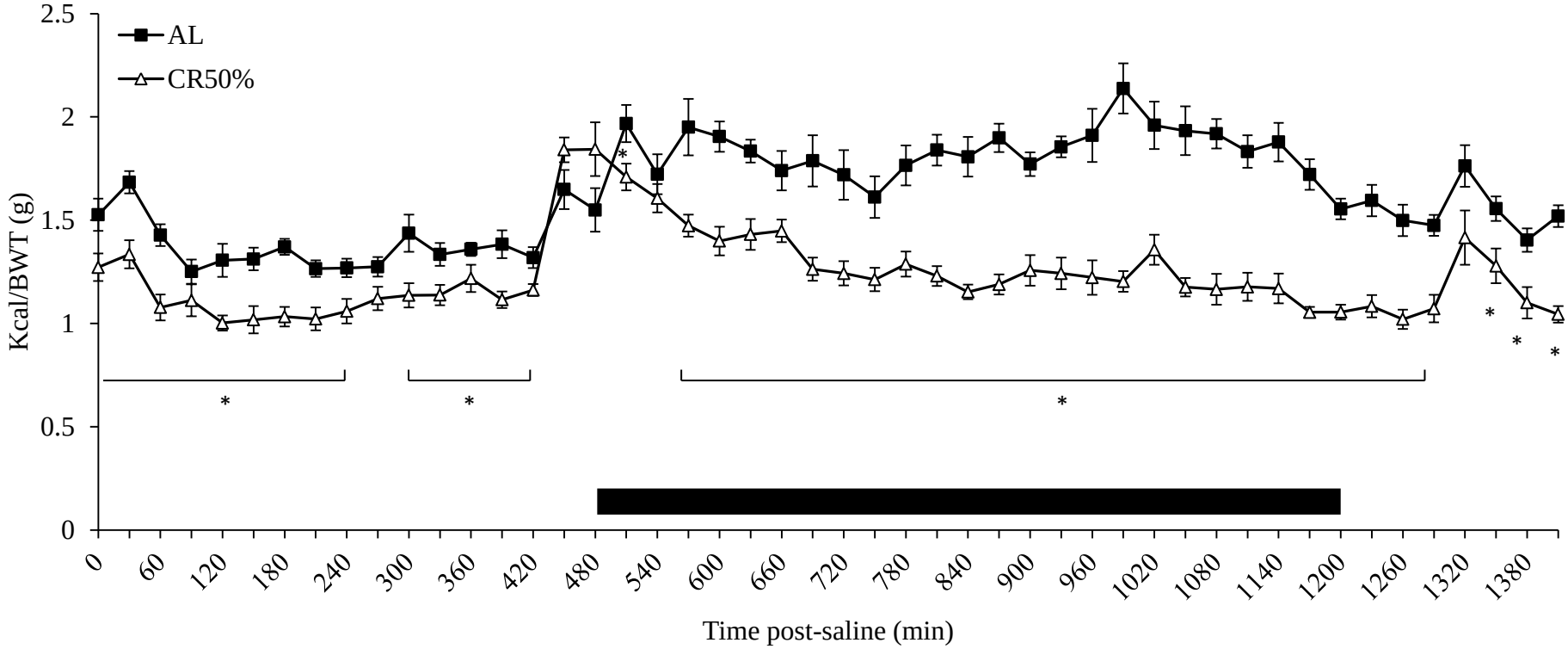

Figure B1.EE in AL (n = 9) and CR50% (n = 9) animals for 24 hours post-saline injection, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$.

Appendix C

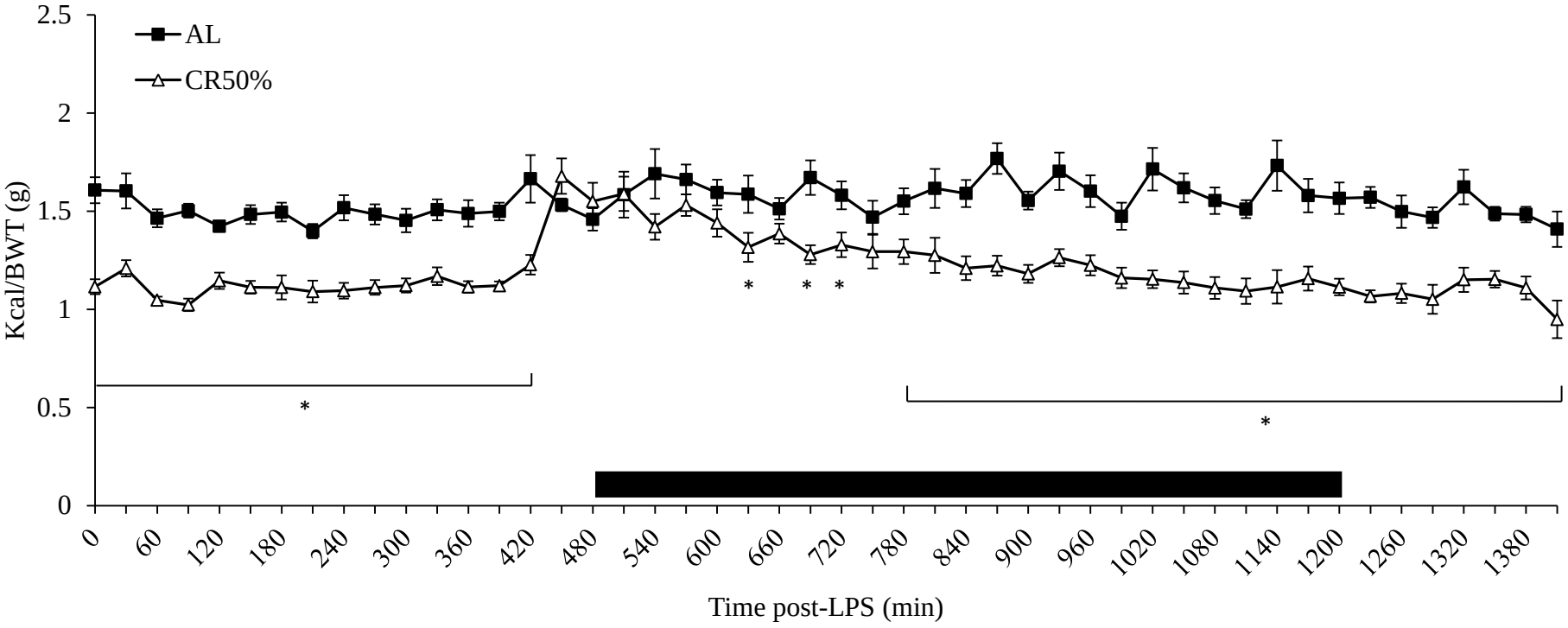

Figure C1.EE in AL (n = 9) and CR50% (n = 9) animals for 24 hours post-LPS injection, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$.

Appendix D

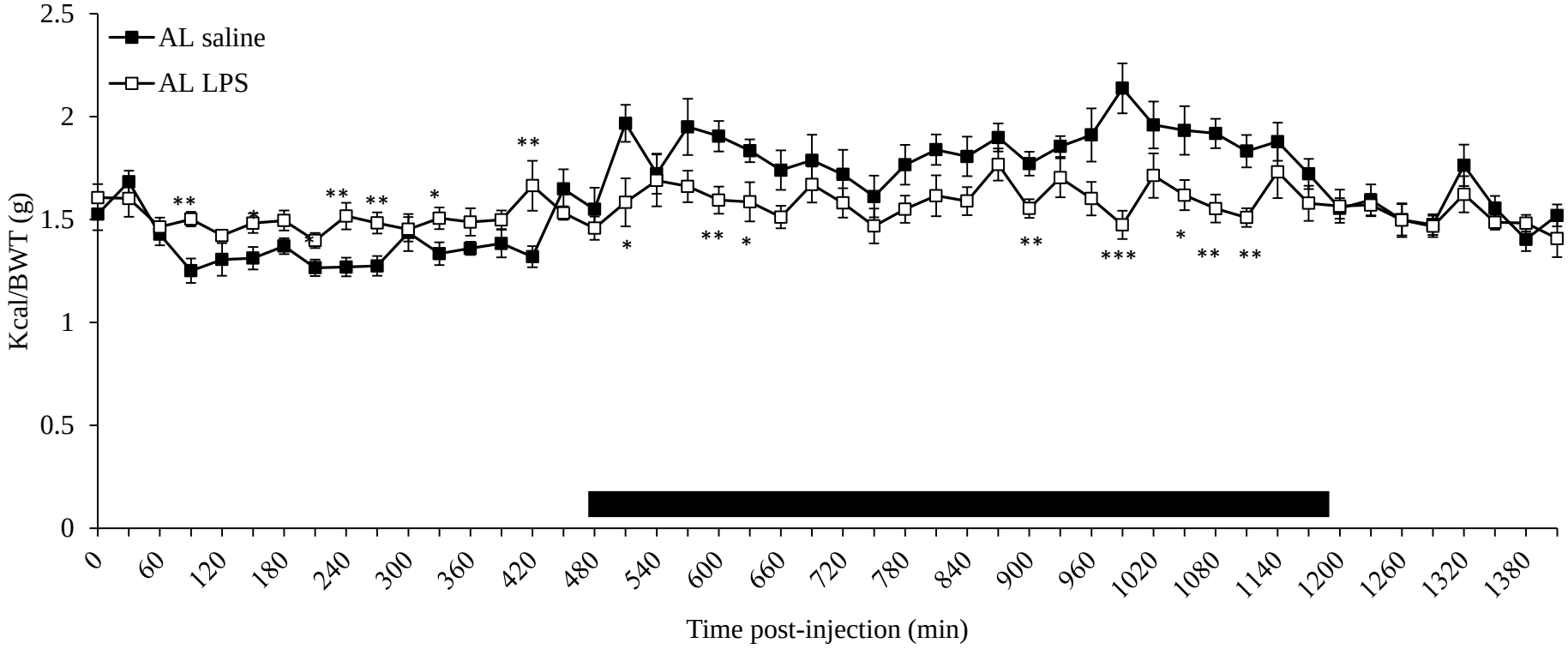

*Figure D1.*EE in AL (n = 9) animals for 24 hours post-saline and LPS injections, with the black box representing the dark phase. (*) denotes a significant difference from the AL saline group at $p < .05$, (**) denotes a significant difference from the AL saline group at $p < .01$, and (***) denotes a significant difference from the AL saline group at $p < .001$.

Appendix E

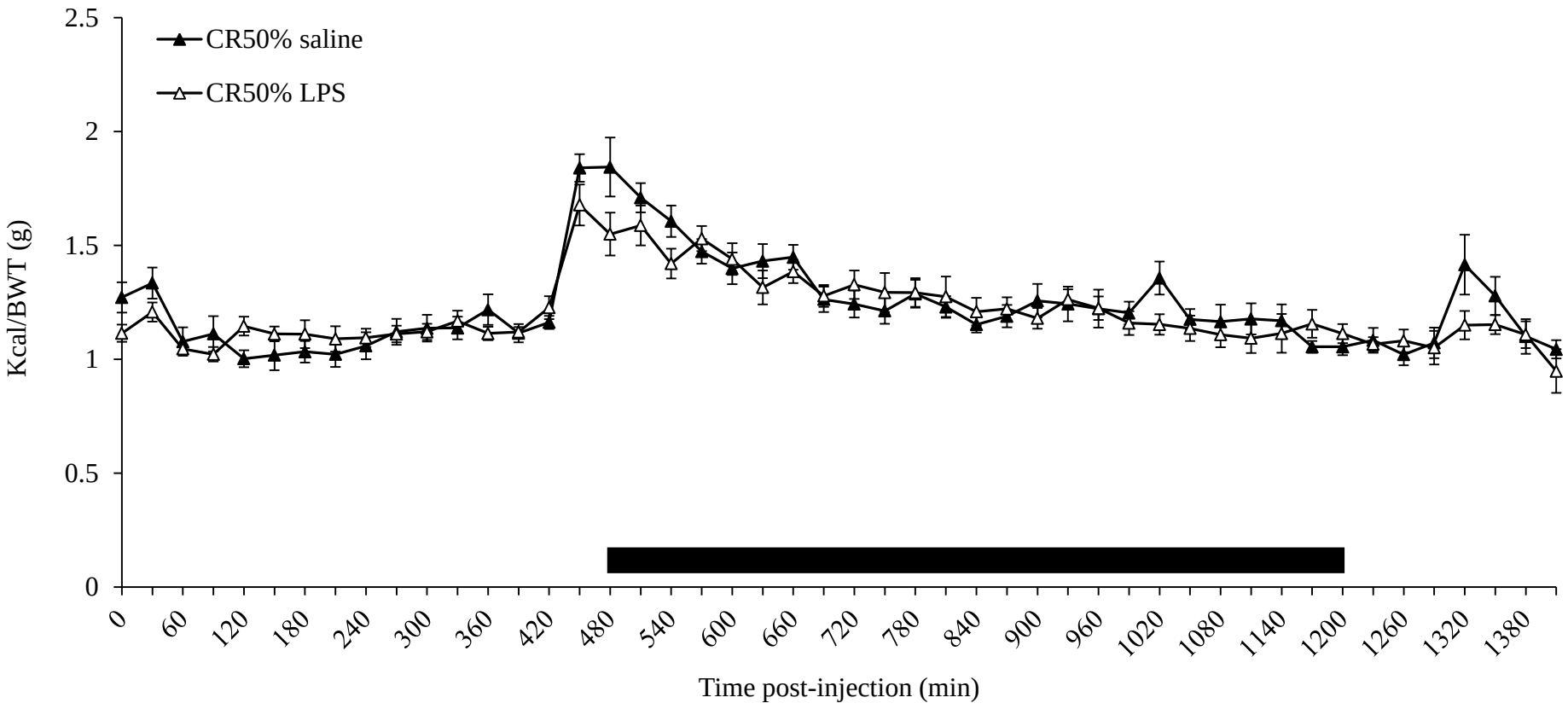

*Figure E1.*EE in CR50% (*n* = 9) animals for 24 hours post-saline and LPS injections, with the black box representing the dark phase.

Appendix F

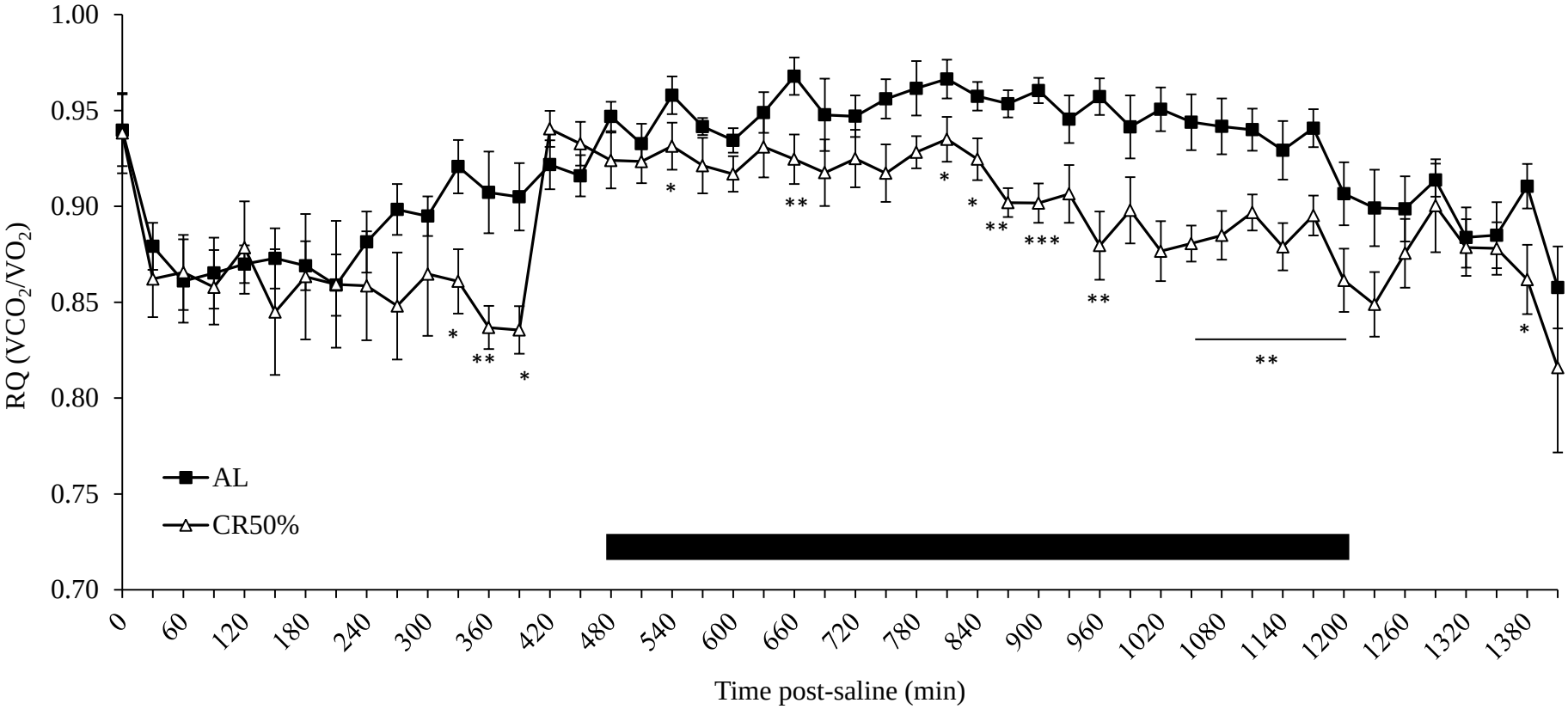

Figure F1. Half-hourly averages of RQ in AL (n = 9) and CR50% (n = 9) animals post-saline injection, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL saline group at $p < .01$, and (***) denotes a significant difference from the AL saline group at $p < .001$.

Appendix G

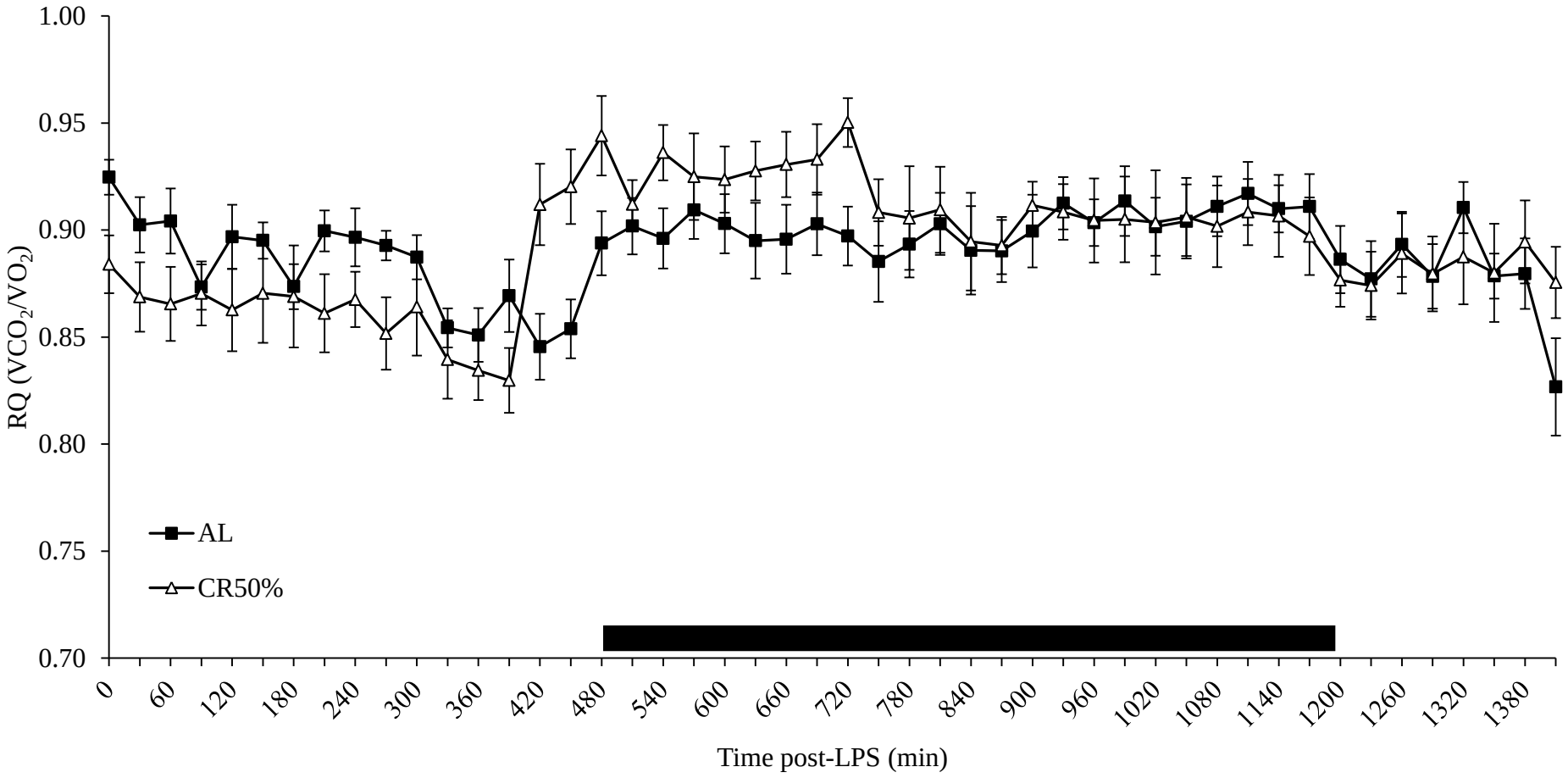

Figure G1. Half-hourly averages of RQ in AL (n = 9) and CR50% (n = 9) animals post-LPS injection, with the black box representing the dark phase.

Appendix H

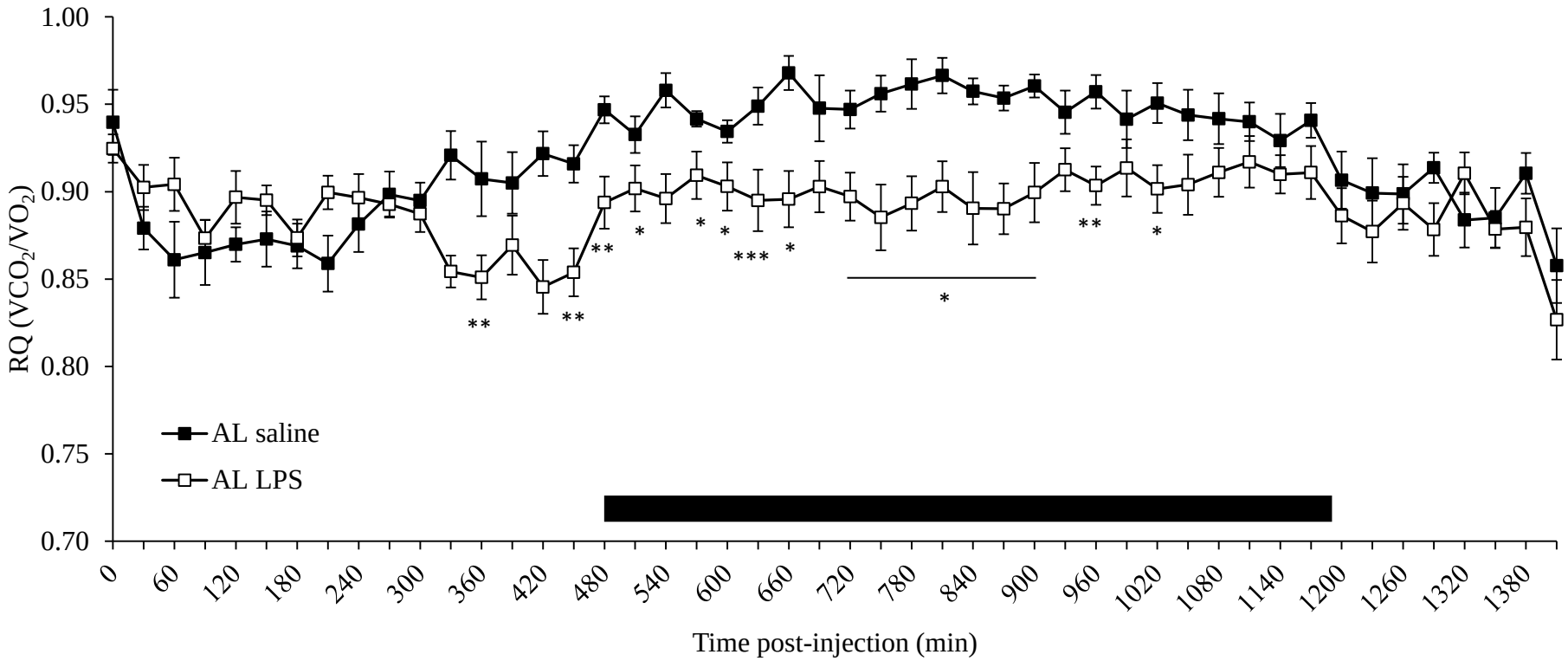

Figure H1. Half-hourly averages of RQ in AL (n = 9) animals post-saline and LPS injections, with the black box representing the dark phase. (*) denotes a significant difference from the AL saline group at $p < .05$, (**) denotes a significant difference from the AL saline group at $p < .01$, and (***) denotes a significant difference from the AL saline group at $p < .001$.

Appendix I

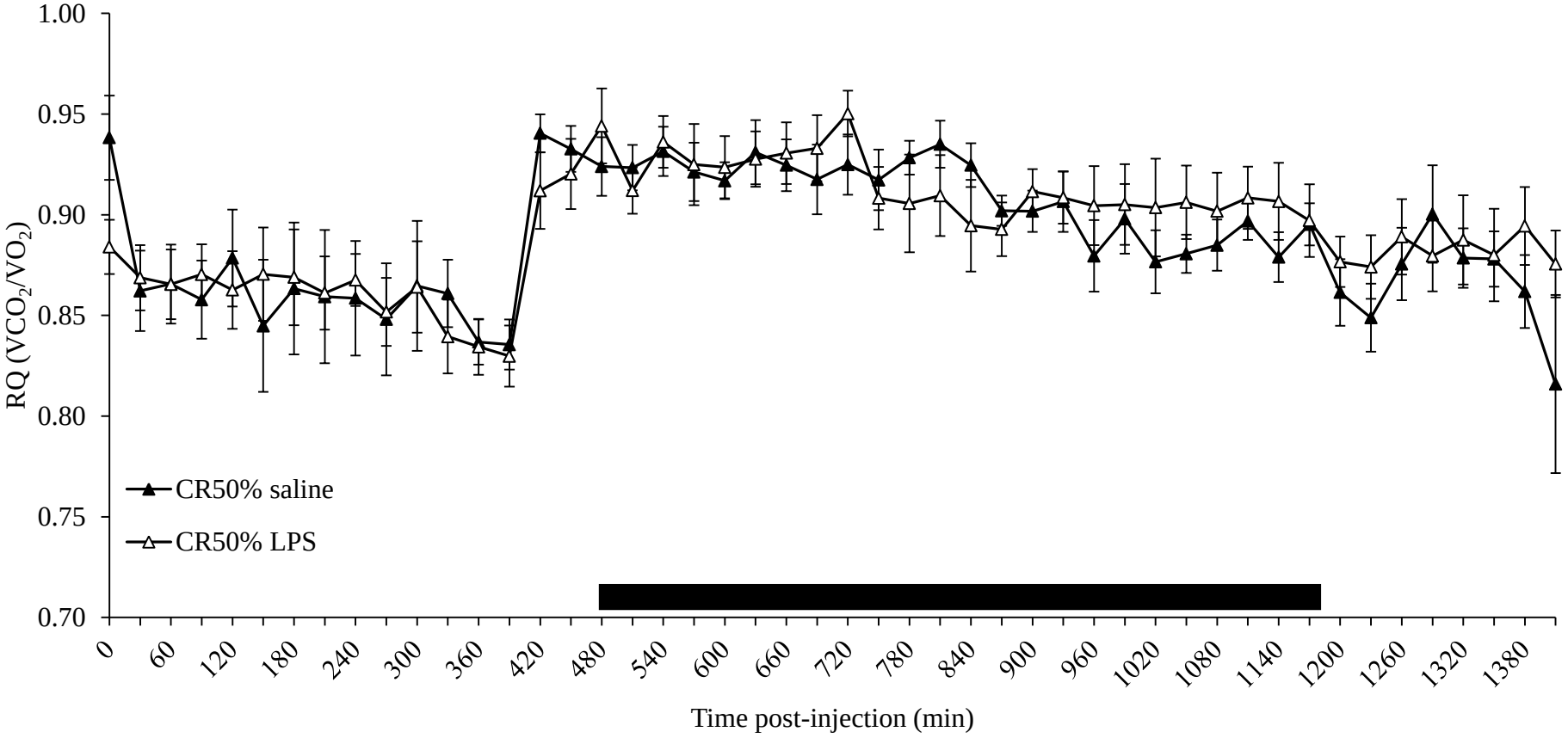

Figure I1. Half-hourly averages of RQ in CR50% (*n* = 9) animals post-saline and LPS injections, with the black box representing the dark phase.

CHAPTER 8

THE ABILITY TO SELECT A HIGHER AMBIENT TEMPERATURE ALLOWS CALORIE RESTRICTED RATS TO EXHIBIT FEVER POST-LPS

Submitted for publication in combination with *Chapter 7* to Physiology & Behavior

Abstract

Previous research has demonstrated that rodents injected with lipopolysaccharide (LPS) and fasted rats will self-select a warmer ambient temperature (T_a) compared to controls. However, an investigation of how calorie restriction (CR) may impact upon behavioural thermoregulation post-LPS has not been investigated. Therefore, this study aimed to examine the effect of CR on self-selected T_a post-LPS. Male Sprague-Dawley rats fed ad libitum (AL) or restricted to 50% (CR50%) of the AL animals' food intake for 30 days and were injected on the 28th day with saline (1 mL/500 g) and on the 29th day with 50 μg/kg of LPS. Core body temperature (T_b), self-selected T_a, body weight loss, and food intake were measured for 24 hours post-LPS. The CR50% rats chose a warmer T_a (28.1 °C ± 0.4) compared to the AL rats (23.7 °C ± 1.4) at baseline. Post-LPS the AL rats chose to sit at a warmer T_a from 30 minutes until 420 minutes post-LPS; however, the CR50% rats only selected a warmer T_a at 270 minutes post-LPS. The AL rats demonstrated a higher T_b compared to baseline at 120, 150, and from 240 until 480 minutes post-LPS. In contrast to previous findings in our laboratory the CR50% rats also demonstrated a higher T_b compared to baseline for most of the time between 270 and 420 minutes post-LPS. When allowed to select a warmer T_a the CR rats do so and thereby mount a febrile response, although delayed and shorter-lived. It is thought the CR rats are able to display a limited fever due to the possibility that the increased T_a allows for increased blood flow, and therefore, increased or enhanced ability for the cytokine message to reach the brain compared to a lower T_a.

Calorie restriction (CR) is well known to reduce core body temperature (T_b) in a number of species including humans (Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996). The reductions in core T_b have been linked to the life extension and anti-aging properties of CR via the reduction of oxidative damage (Barja, 2004; Duffy et al., 1989; Heilbronn et al., 2006; Shigenaga, Hagen, & Ames, 1994). A logical question is whether the decreased T_b alters preferred ambient temperature (T_a) selection. Limited research has investigated the impact of low food availability; however, it was shown in pigeons that low food availability impacted upon T_a preference, with pigeons exposed to a lower food intake selecting a higher T_a during their rest phase (dark phase) (Ostheim, 1992). Further, it was shown that rats fasted for three days selected a higher T_a compared to control rats and even though these fasted rats sat at a higher T_a they maintained their lower core T_b compared to the control rats (Sakurada et al., 2000). The authors concluded that the fasted rats do display an ability to regulate core T_b and that the selection of a higher T_a compliments physiological thermoregulation and aims to conserve heat loss and save energy in situations of low food availability (Sakurada et al., 2000).

The question of whether the hypothermia present in fasted and also CR animals is a regulated strategy exhibited by the animal or whether the animals thermogenic capability is compromised has been raised (Sakurada et al., 2000). These same authors found that rats fasted for a period of three days had no change in their threshold for non-evaporative heat loss; however, their threshold for the activation of thermogenesis had reduced (Sakurada et al., 2000). The authors concluded that this did not reflect an inability to activate thermogenesis, just a change in threshold for activation.

The effects of exposure to a pyrogen such as lipopolysaccharide (LPS) or prostaglandin E_1 (PGE_1) upon self-selected T_a has been investigated. Rodents allowed free access to food and exposed to LPS self-selected a higher T_a compared to animals injected

with saline (Akins, Thiessen, & Cocke, 1991; Briese, 1997; Florez-Duquet, Peloso, & Satinoff, 2001; Sugimoto, Shido, Sakurada, & Nagasaka, 1996). Further, the fever induced by an injection of PGE_1 into the pre-optic area of the hypothalamus in squirrel monkeys resulted in the monkeys selecting a higher T_a compared to pre-injection (Crawshaw & Stitt, 1975). These authors also examined the febrile response of these animals while they were injected at a T_a below or above their thermoneutral zone. They found that monkeys injected in an T_a lower than their thermoneutral zone exhibited a fever; however, it was produced solely by an increase in metabolic rate, whereas the fever in the monkeys at the higher T_a was produced mainly by vasoconstriction with less reliance on an increase in metabolic rate (Crawshaw & Stitt, 1975). At an T_a within thermoneutrality or slightly higher the main effector mechanism for fever is vasoconstriction, which is considered of low-energetic cost; however, in a cooler environment fever is mainly initiated by thermogenesis, which has a high energetic cost (Szekely & Szelenyi, 1979).

An investigation of newborn rabbit pups fasted for two days is also relevant. Fasting one to two day old rabbit pups for two days attenuated the expected rise in T_b after injection with a pyrogen (*Pseudomonas lipopolysaccharide*) (Kleitman & Satinoff, 1981). However, when the pups were placed into a thermal gradient at two hours post-injection they chose to sit at a warmer T_a compared to controls and subsequently were able to increase their T_b to febrile levels (Kleitman & Satinoff, 1981). Previously we have shown that CR rats housed at 26 °C exhibit an attenuation of LPS-induced fever and sickness behaviour; it is possible that the metabolic cost of increasing their T_b at this T_a is too costly as they need to conserve energy. Therefore, it would be of importance to investigate the role T_a selection may play post-LPS in CR animals.

Surprisingly, although the effect of food deprivation on self-selected T_a has been investigated (albeit in a limited capacity), the effect of LPS and other immune activators on

self-selected T_a have been investigated previously (Akins et al., 1991; Crawshaw & Stitt, 1975); however, the possible impact CR may have on self-selected T_a post-LPS has not. The aim of the current study was to investigate the impact of CR on LPS-induced selection of T_a. Due to past literature it was thought that the CR animals would choose a higher T_a at baseline (i.e., before exposure to LPS) and after exposure to LPS control and CR animals would increase their self-selected T_a. Due to the capability to self-select a higher T_a the CR animals may demonstrate an increase in T_b post-LPS.

Methods

Animals

Fourteen male Sprague-Dawley rats were procured from Monash SPF animal services (Clayton, Victoria, Australia) and allowed to acclimate to the facility for at least one week. During this period, standard rodent chow (Barastoc, Melbourne, Australia) and water were available ad libitum. At the beginning of experimentation the rats were aged between 12 to 14 weeks old. Rats were individually housed in polypropylene basin cages (30 × 50 × 15 cm) with sawdust and tissues provided as bedding. Rats were maintained at an T_a of 26 ± 1 °C, which is within the thermoneutral zone for this species (Poole & Stephenson, 1977) on a 12:12 light/dark cycle (0500 – 1700 hours). Animal care and experimentation was performed with approval from the La Trobe University Animal Ethics Committee.

Surgery

Following acclimation all of the rats were surgically implanted in the peritoneal cavity with a SubCue Datatlogger (SubCue Dataloggers, Calgary, Alberta, Canada: 15 mm × 5 mm, 4.2 g) under anaesthesia as described previously (Begg, Kent, McKinley, & Mathai, 2007).

Rats were anaesthetised in an induction chamber using 4% isoflurane and 0.6 L/min oxygen flow and were maintained during surgery on 2.5% isoflurane and 0.4 L/min oxygen flow using a nose cone (refer to *Chapter 2* for more detail). These rats were allowed one to two weeks to recover before the initiation of the CR regimens.

Dietary regimens

Rats were divided into one of two CR regimens matched for weight, food intake, and age: ad lib (AL; n = 7) fed ad libitum (on average 24-30 g per day); and CR50% (n = 7) rats received 50% of the amount consumed by AL (on average 12-15 g per day). The dietary composition of the AL and CR50% diets has been published elsewhere (Levay, Govic, Penman, Paolini, & Kent, 2007; Levay, Tammer, Penman, Paolini, & Kent, 2010). The intake of the CR groups was determined weekly based on the average daily food intake of the AL group for three consecutive days. Water was continuously provided to both groups. The dietary manipulation continued for 27 days before saline challenge and was maintained until the end of experimentation. Food was provided daily, approximately one hour before the dark phase onset.

Measurement of T_b

The rats were implanted with a SubCue Datalogger to measure core T_b. This device stores the animals core T_b at 5 minute intervals in a chronological sequence. Once the device was removed from the animal, data were immediately downloaded onto the hard drive of a computer equipped with SubCue software.

Measurement of T_a

The self-selected T_a of the rats was measured in a thermal gradient. The chamber (1500 mm × 100 mm × 100 mm) was constructed of a base of aluminium and fitted with a plastic floor. The chamber was fitted with a mesh lid to allow fresh air to enter the chamber. The chamber was cooled at the one end (5 °C) and heated at the other end (43 °C) by water; this established a thermal gradient approximately linear from 15 °C to 35 °C. The sides of the chamber were fitted with 24 infrared phototransistors; the photocell closest to the warm end of the chamber that was obscured by the rat recorded the position of the rat as previously described (Begg, Mathai, McKinley, Frappell, & Kent, 2008). A camera fitted above the thermal gradient also allowed for photos to be taken every 10 seconds in order to visually establish where each rat was within the thermal gradient. Food was available ad libitum for the AL rats and was distributed along the gradient and as per the CR regimen for the CR rats. Water was available at four sites, spaced evenly within the walls of the chamber. Rats were placed into the thermal gradient for a period of 72 hours; an acclimation stage (24 hours), the day they received a saline injection (24 hours), and the day they received a LPS injection (24 hours).

Procedure

Rats were weighed once a week approximately three hours after lights-on during dietary manipulation and daily at the same time on the day before and day of saline and LPS injections using top loading scales. Food consumption was also determined at this time and was determined to the nearest .1 gram by providing a set amount of food each day and weighing the remaining food 24 hours later, including the uneaten food in the bedding. On day 27 of the CR period the animals were acclimated to the thermal gradient for a period of 24 hours. All rats received a saline injection on day 28 of the CR period (1 ml/500 g of saline). On day 29 all rats were injected intraperitoneally with of 50 μg/kg of LPS from

Escherichia coli (serotype 0111:B4: Sigma, Castle Hill, NSW) in 1 ml/500 g of saline. Both saline and LPS challenges were performed approximately four hours after lights-on (ranging from 0800 hours to 0900 hours). T_b, self-selected T_a, body weight, and food intake measurements were continued for 24 hours post-LPS.

Data analysis

Analyses of body weight and food intake

The body weight data was collected once per week throughout the CR period and was averaged and means ± SEM were calculated for each group. Body weight was also measured 24 hours prior to and after saline and LPS injections. These values were averaged and means ± SEM were calculated for each group. Food intake data was collected over a 4-day baseline period prior to saline and LPS injections and averaged into one value for each animal. Food intake was also collected 24 hours after saline and LPS injections and means ± SEM were calculated for each group for post-injection values. The post-saline/LPS value was subtracted from the baseline value to achieve a change from baseline value for each animal. These values were then averaged and means ± SEM were presented. Data were then analysed using a 2 × 2 analysis of variance (ANOVA). To overcome violations of sphericity, the Greenhouse-Geisser statistic corrected degrees of freedom were reported. Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$ as the criterion for significance.

Analysis of T_b and T_a

T_b was presented as hourly averages for day 26 of the CR period to analyse the groups T_b before they were placed into the thermocline. T_b and T_a were collected during the day the rats received saline and LPS injections with an average of the four hours prior to each injection used as a baseline for each day (i.e., from 0500 until 0900). These values were

averaged and means ± SEM were calculated for each group. To determine the change in T_b post-LPS the values on the day the rats received LPS were subtracted from the values on the day the rats received saline and presented as 30 minute averages. Data were then analysed using 2 × 2 ANOVA. To overcome violations of sphericity, the Greenhouse-Geisser statistic corrected degrees of freedom were reported. Planned comparisons were conducted between 90 and 180 minutes post-LPS to assess the differences between the AL and CR groups T_b change post-LPS due to this being the critical window of fever development. Unless otherwise stated, all hypotheses tested used an alpha of $p < .05$ as the criterion for significance.

Results

Effect of CR on body weight

As expected the CR50% group lost weight, whereas the AL group demonstrated a slight weight gain (Figure 1). The CR rats lost weight steadily at the onset of CR; however, began to plateau between day 21 and day 28 of the CR period. On day 28 of the CR period the AL animals weighed on average 451.5 g (± 22.1) and the CR50% animals weighed on average 356.7 g (± 7.5). The ANOVA found a significant main effect for time [$F(1.97,21.68) = 6.33$, $p = .007$, partial $\eta^2 = .37$], group [$F(1,11) = 7.07$, $p = .022$, partial $\eta^2 = .39$], and their interaction [$F(1.97,21.68) = 75.40$, $p < .001$, partial $\eta^2 = .87$]. The AL and CR rats were similar weights upon allocation of the dietary regimens and began to differ from each other by day 14 of the CR period until day 28 (ranging from $p = .006$ to $p <.001$). The AL rats steadily put on weight compared to day 0 (ranging from $p = .002$ to $p < .001$), whereas the CR rats were consistently below their day 0 weight for the entire CR period ($p < .001$ for all).

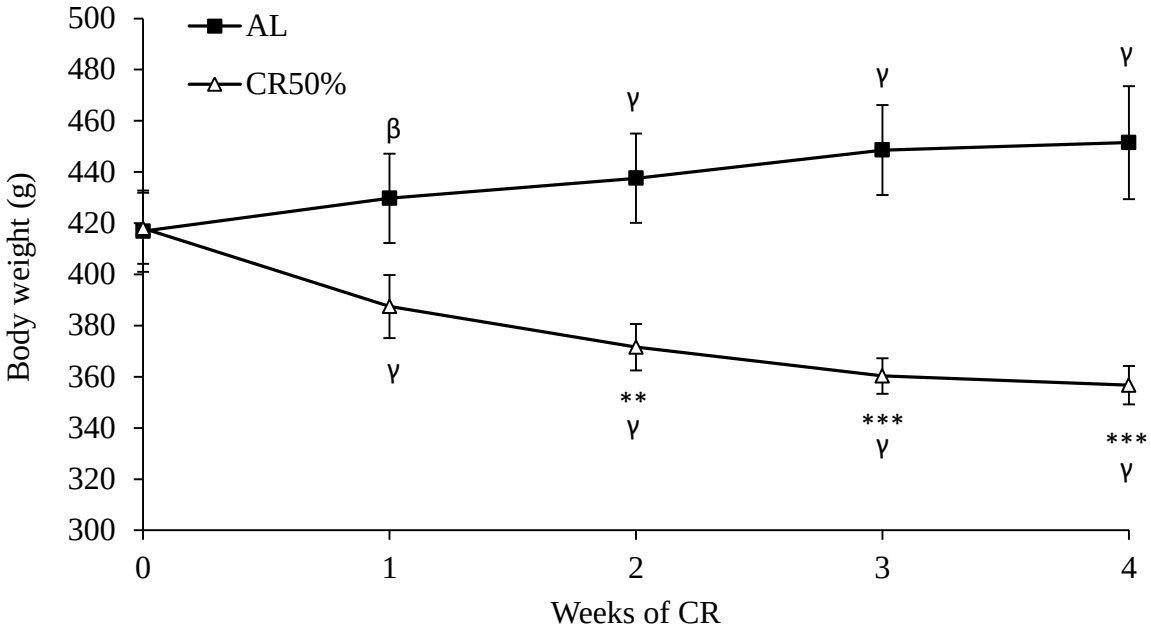

Figure 1. Mean (± SEM) body weight (g) of the AL (n = 7) and CR50% (n = 7) animals during the CR period. (**) denotes a significant difference from the AL group at $p < .01$, (***) denotes a significant difference from the AL group at $p < .001$ (β) denotes a significant difference from day 0 at $p < .01$, and (γ) denotes a significant difference from day 0 at $p < .001$.

Core T_b on day 26 of the CR period

The AL and CR50% rats demonstrated a similar pattern of T_b throughout the light phase on day 26 (the day before the animals were placed in the thermocline) of the CR period; however, the CR50% rats show an increase in T_b as it nears the time when food was provided (Figure 2). Then, during the dark phase the CR50% rats' T_b drops, whereas the AL rats' T_b remains elevated for the majority of the dark phase. The ANOVA found a significant main effect for time [$F(2.92,23.33) = 42.93$, $p < .001$, partial $\eta^2 = .84$] and the interaction between time and group [$F(2.92,23.33) = 14.05$, $p < .001$, partial $\eta^2 = .64$]. Post-hoc tests revealed that the CR50% rats demonstrated a higher T_b compared to the AL rats during the

hours of 1600 h to 1800 h (ranging from $p = .015$ to $p < .001$). From 2200 h until 0700 h the AL rats demonstrated a higher T_b compared to the CR50% rats (ranging from $p = .048$ to $p = .003$).

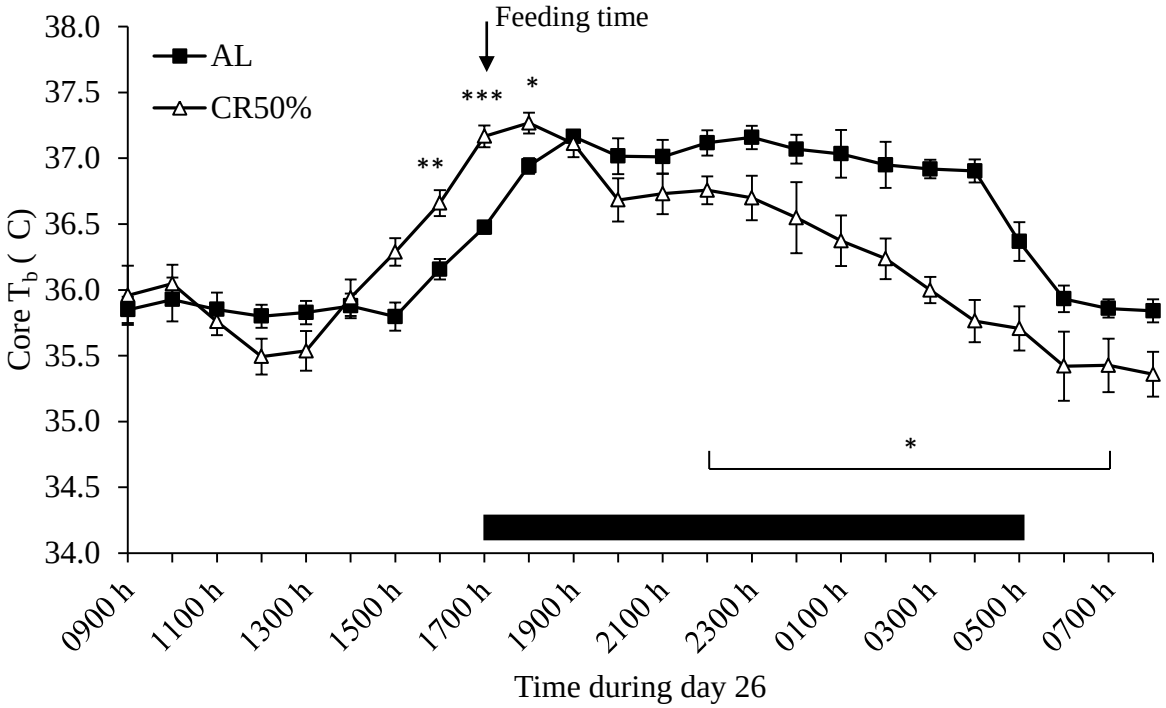

Figure 2. Mean (± SEM) T_b (°C) of the AL ($n = 7$) and CR50% ($n = 7$) animals on day 26 of the CR period, with the black box representing the dark phase. (*) denotes a significant difference from the AL group at $p < .05$, (**) denotes a significant difference from the AL group at $p < .01$, and (***) denotes a significant difference from the AL group at $p < .001$.

Sickness behaviour and chosen T_a after LPS in AL and CR50% rats

T_a post-LPS

T_a differed at baseline (an average of the four hours prior to LPS injections) between the AL (23.7 ± 1.4 °C) and CR50% (28.1 ± 0.4 °C) rats and while the CR50% rats remained at their baseline T_a for most of the 8-hour period post-LPS, the AL rats chose to sit at a higher T_a compared to baseline for 7 of the 8 hours post-LPS (Figure 3). The peak T_a in which the

AL rats selected was 28.3 °C (± 0.9) at 60 minutes post-LPS and the peak T_a for the CR50% rats was 29.7 °C (± 0.7) at 180 minutes post-LPS injection. The ANOVA found a significant main effect for time [$F(5.78,69.40) = 3.88$, $p = .002$, partial $\eta^2 = .26$] and the interaction [$F(5.78,69.40) = 2.57$, $p = .028$, partial $\eta^2 = .18$]. As mentioned above, the CR50% rats chose to sit at a higher T_a compared to the AL rats at baseline ($p = .012$). The CR50% rats remained at a warmer T_a compared to the AL rats at 120, 150, 180, and 270 minutes post-LPS injection (ranging from $p = .011$ to $p = .008$). The AL rats chose to remain at a higher T_a compared to their baseline from 30 minutes until 420 minutes post-LPS injection (ranging from $p = .049$ to $p = .013$). In comparison the CR50% rats only differed from their baseline T_a at 270 minutes ($p = .039$) and 480 minutes post-LPS ($p = .023$).

The relationship between the change in selected T_a and change in T_b was investigated and it was determined that in the CR animals the self-selected T_a at 60 minutes post-LPS was positively correlated with change in T_b at 150 (r = .972, p =.006), 180 (r = .988, p =.002), and 210 (r = .967, p =.007) minutes post-LPS. Further, the CR animals T_a at 150 minutes post-LPS was positively correlated with their change in T_b at 210 minutes post-LPS (r = .894, p =.040).

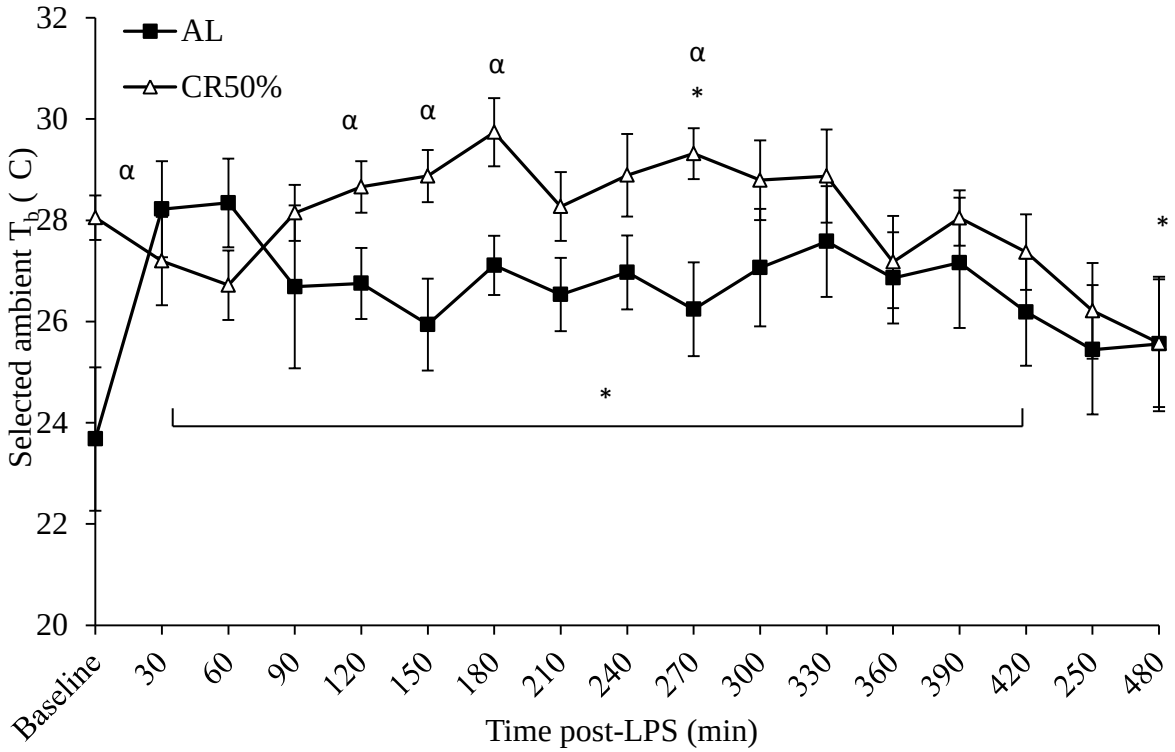

Figure 3. Selected T_a of AL (n = 7) and CR50% (n = 7) rats post-LPS. (*) denotes a significant difference from baseline (an average of the four hours pre-injection) in the AL rats at $p < .05$ and (α) denotes a significant difference from the AL group at $p < .05$.

Core T_b post-LPS

LPS induced a rise in T_b in both the AL and CR50% groups; with the CR50% group demonstrating a hypothermic response at 90 and 120 minutes post-LPS and demonstrating both a long delay before fever onset (approximately 5 hours) and shorted-lived fever (Figure 4). Both groups then followed a similar pattern of T_b for 24 hours post-LPS (data not shown). The peak of the fever in the AL rats (1.5 °C ± 0.2) occurred at 390 minutes post-LPS injection and the CR50% rats peak T_b increase (1.4 °C ± 0.4) occurred on average at 300 minutes post-LPS injection. The ANOVA only found a significant main effect for time [$F(4.09,32.77) = 12.69$, $p < .001$, partial $\eta^2 = .61$]. The AL group had a significantly higher T_b compared to baseline at 150 and 180 minutes post-LPS injection ($p = .031$ and $p = .037$

respectively) and from 270 until 480 minutes post-LPS (ranging from $p = .034$ to $p < .001$ for all). The CR50% animals T_b profile was slightly different, demonstrating a decrease in T_b at 90 and 120 minutes post-LPS ($p = .041$ and $p = .010$ respectively). Their T_b then started to increase and by 300 minutes post-LPS it was higher compared to baseline ($p = .039$) and remained higher than baseline at 330, 390, 420, and 450 minutes post-LPS (ranging from $p = .038$ to $p = .006$). Planned comparisons revealed that between 90 and 180 minutes post-LPS the AL and CR50% rats only differed in T_b at 90 [$F(1,8) = 13.92$, $p = .006$] and 120 minutes [$F(1,8) = 6.75$, $p = .032$].

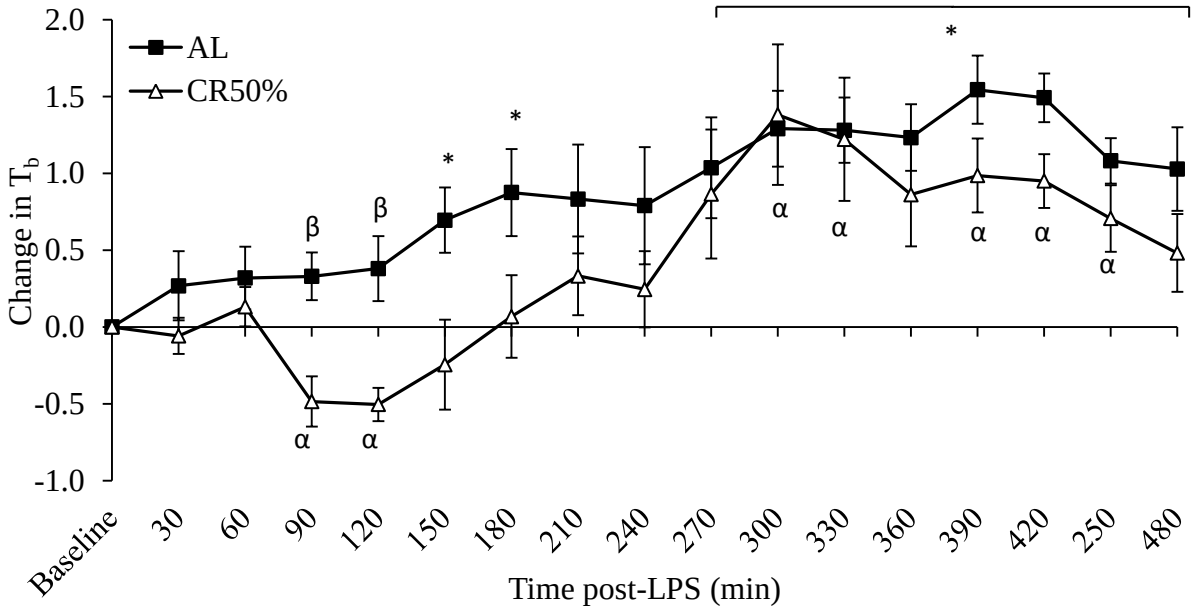

Figure 4. Change in T_b compared to baseline (the day of LPS subtracted from the day of saline) in AL ($n = 7$) and CR50% ($n = 7$) post-LPS. (*) denotes a significant difference from baseline in the AL group at $p < .05$, (α) denotes a significant difference from baseline in the CR50% group at $p < .05$, and (β) denotes a significant difference from the CR50% group at $p < .05$.

Body weight post-LPS

During the 24 hours post-LPS injection the AL rats lost a total of -16.2 g (± 1.8) body weight and the CR50% rats in comparison lost a total of -4.0 g (± 0.6) body weight (Figure 5). Due to baseline differences only percentage change was used for analysis. The ANOVA found a significant main effect for time (LPS day compared to the saline day) [$F(1,12) = 153.20$, $p < .001$, partial $\eta^2 = .93$], group [$F(1,12) = 42.60$, $p < .001$, partial $\eta^2 = .78$], and their interaction [$F(1,12) = 42.60$, $p < .001$, partial $\eta^2 = .78$]. The AL group lost significantly more weight compared to the CR50% group post-LPS ($p < .001$). The AL and CR50% groups were significantly lighter compared to their respective baselines ($p < .001$ for both).

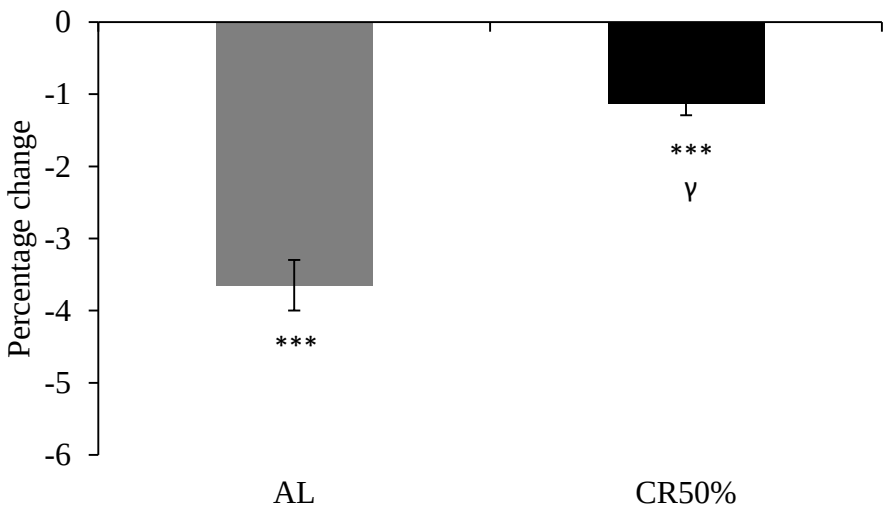

Figure 5. Percentage change in body weight compared to baseline (compared to the day prior to LPS injections) in AL ($n = 7$) and CR50% ($n = 7$) 24 hours post-LPS. (γ) denotes a significant difference from the AL group at $p < .001$ and (***) denotes a significant difference from baseline at $p < .001$.

Food intake post-LPS

During the 24 hours post-LPS injection the AL rats reduced their food intake by -47.0% (± 10.2) whereas the CR50% rats did not reduce their food intake. The ANOVA found

a significant main effect for time (LPS day compared to the saline day) [$F(1,11) = 48.06$, $p < .001$, partial $\eta^2 = .81$], group [$F(1,11) = 48.06$, $p < .001$, partial $\eta^2 = .81$], and their interaction [$F(1,11) = 48.06$, $p < .001$, partial $\eta^2 = .81$]. The AL group ate significantly less compared to baseline ($p < .001$), whereas the CR50% group did not differ from their pre-LPS food intake.

Discussion

CR resulted in a warmer self-selected T_a at baseline, with rats exposed to a 50% CR for 28 days preferring a T_a almost 4 °C higher than AL rats. Post-LPS the AL rats demonstrated a significant increase in self-selected T_a compared to baseline; however the CR50% rats only chose to sit a higher T_a at 270 minutes post-LPS. These CR rats then also developed a fever with the same peak; however, with a delayed onset and shorter duration compared to the AL rats. This is in disagreement with our previous findings that a 50% CR in rats (*Chapter 5*) and mice (MacDonald, Radler, Paolini, & Kent, 2011) elicits a full attenuation of fever and other sickness behaviour measures post-LPS. Even though the CR50% rats in the current study demonstrated a fever, they did not reduce their post-LPS food intake and lost significantly less body weight compared to the AL rats. Interestingly, even though the CR rats were able to choose a higher T_a compared to the AL rats during the baseline period on the day they received LPS (day 29), their core T_b remained significantly lower than the AL rats during the majority of the dark phase on day 26 of the CR regimen. These findings may suggest that the thermoneutral zone for these CR rats has shifted and that a higher T_a allowed the CR animals to develop a febrile response as it was no longer as metabolically as costly for the CR animals. In *Chapter 7* of this thesis the CR50% rats demonstrated significantly reduced energy expenditure (EE) compared to the AL rats; however, this reduced EE was not altered post-LPS. This demonstrates that the CR animals are possibly experiencing a trade-off between increasing their EE and mounting a febrile

response, or conserving their energy in order to aid thermoregulation Therefore, once the CR rats are able to select a warmer T_a the choice between thermoregulation and increasing EE to mount a febrile response may no longer fall with thermoregulation, but with mounting a febrile response.

Effect of CR on T_b and T_a

Consistently CR animals have been shown in this laboratory and others to exhibit a significantly reduced T_b (Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; MacDonald et al., 2011). Due to this reduced T_b it is not surprising that the CR rats in the current study self-selected a higher T_a compared to the AL rats. It was interesting that even though the CR rats were able to self-select a higher T_a they still demonstrated a significant reduction in core T_b, which has also been seen in fasted rats (Sakurada et al., 2000). However, the fasted rats T_b was reduced during the light phase, whereas in the current study the CR rats demonstrated a reduced T_b for the majority of the dark phase and a similar T_b to the AL rats during the light phase. A chronic change in dietary intake, such as CR may activate different mechanisms when reducing core T_b compared to fasting. For example, these two dietary manipulations have been shown to differentially impact upon hypothalamic neuropeptide Y expression (Bi, Robinson, & Moran, 2003; Brady, Smith, Gold, & Herkenham, 1990). It is also possible that only three days (including the acclimation day) in a higher T_a in the current study was insufficient to result in an increase in the CR animals core T_b and if they were housed at a higher T_a for the entire 28 days of the CR period they may show a similar core T_b to that of the AL rats.

Although not directly related to CR, an example that may be relevant comes from *ob/ob* mice, which have a reduced capacity for cold-induced thermogenesis. These mice, when housed at T_as within the thermoneutral zone of other mice (30 °C) display a lower core

T_b by almost 2 °C, which the authors speculated may be due to a reduced 'setting' for core T_b (Trayhurn & James, 1978). These mice were able to closely regulate their T_b, just at lower levels compared to lean mice (Trayhurn & James, 1978). It may be the same case for the CR rats, where their lower core T_b is at a reduced setting; however, they are still able to regulate and increase their T_b when needed as seen in the current study when the CR animals were fed. They exhibited a locomotor-induced increase in their T_b in anticipation of their feeding time.

Effect of CR on self-selected T_b and T_a post-LPS

Previous research has demonstrated that rodents exposed to LPS will select a warmer T_a by 2.4 °C compared to animals who receive saline (Akins et al., 1991; Briese, 1997; Florez-Duquet et al., 2001; Sugimoto et al., 1996). These authors have suggested that it is less physiologically expensive to behaviourally generate a fever than it would be to metabolically induce an increase in T_b. Similar findings have been seen in primates using PGE_1 as the inflammatory agent, with the primates self-selecting a higher T_a after PGE_1 exposure (Crawshaw & Stitt, 1975). Consistent with previous research the AL rats in the current study increased their self-selected T_a from 30 minutes post-LPS and remained at a warmer T_a (although this T_a was still below that of the CR rats) until 420 minutes post-LPS. However, in contrast, the CR rats (whose baseline T_a was already significantly higher than the AL rats) remained at their baseline T_a apart from two time points; at 270 minutes post-LPS they selected a higher T_a and at 480 minutes post-LPS they selected a lower T_a compared to baseline. On average the AL rats increase in T_a was 3.1 °C for the eight hours post-LPS; however the CR rats was only 0.1 °C.

The AL rats developed a fever; however, what was interesting was that the CR rats also developed a febrile response. This is in direct conflict with our previous work that

demonstrates that rats (*Chapter 5*) and mice (MacDonald et al., 2011; *Chapter 4*) display an attenuated febrile response, along with attenuated sickness behaviour measures. The lack of febrile response in our previous work was not simply due to reduced thermogenic capacity of the CR animals, as they demonstrated a sharp increase in T_b in conjunction with an increase in locomotor activity immediately prior to presentation of food. The thermogenic capacity of the animals in the current study can also been seen in Figure 2 as they demonstrated a sharp increase in T_b in the two hours prior to food presentation and the onset of the dark phase. Therefore, the CR animals were capable of increasing their T_b; however, in our previous studies they did not do so post-LPS, indicating that the attenuation was not due to reduced thermogenic capacity and was more likely due to a hypothalamic anti-inflammatory bias (MacDonald et al., 2011). Even though the CR rats in the current study developed a fever, albeit with a delayed onset and shorter duration, they did not show the same severity of sickness behaviour in other variables compared to the AL rats. They lost significantly less body weight post-LPS (although they did lose a significant amount compared to baseline) and did not reduce their food intake compared to baseline. The CR animals drive to eat may have competed with the physiological need to suppress their appetite post-LPS, which has been seen before in fasted rats exposed to infusion of interleukin-1 (IL-1) (Mrosovsky, Molony, Conn, & Kluger, 1989).

Evidence has shown that animals exposed to LPS at a T_a below their thermoneutral zone demonstrate blunted febrile responses (Boissé, Mouihate, Ellis, & Pittman, 2004; Buchanan, Peloso, & Santinoff, 2008; Marais, Maloney, & Gray, 2011; Peloso, Florez-Duquet, Buchanan, & Satinoff, 2003). Then, when housed in a T_a within their thermoneutral zone the febrile response is reinstated, and when housed at a higher T_a than their thermoneutral zone the febrile response is often enhanced; with higher increases in T_b recorded (Boissé et al., 2004; Buchanan et al., 2008; Marais et al., 2011). Further, it's been

described that phases two and three of the febrile response rely heavily upon thermoregulatory behaviour, and this means that the animals T_b becomes highly sensitive to the T_a (Romanovsky, 2004). It is possible that the CR animals demonstrate a shift in their thermoneutral zone and that the thermoneutral zone for AL fed rats may be too low for CR rats. The CR animals may need a higher T_a to minimize their metabolic effort to increase their T_b. In the current study, when the CR rats were able to choose a higher T_a (albeit only approximately 2 °C higher than the AL animals post-LPS) they were able to develop a delayed fever, possibly because it is closer to their new thermoneutral zone (after CR).

The ability of the CR animals to mount a febrile response at an T_a possibly below their thermoneutral zone may be another explanation for these findings. The impact that blood flow has on the distribution of LPS throughout the body has been highlighted elsewhere (Romanovsky et al., 2005). This distribution of LPS by blood is impacted upon by the T_a, such as directing a higher blood flow, and consequently inflammatory mediators, to the skin in conditions of warmer T_as (Ivanov, Patel, Kulchitsky, & Romanovsky, 2003). CR has been shown to lead to reduced blood pressure (Wright, Mc Murtry, & Wexler, 1981; Young, Mullen, & Landsberg, 1978), which may impact upon the ability of the CR animals' ability to transfer the cytokine message to the brain effectively. For example, it was seen in old rats that fever and transfer of IL-1β across the blood-brain-barrier following LPS exposure was attenuated at an T_a of 21 °C; however, was restored at 31 °C (Buchanan et al., 2008). One explanation offered by the authors was that the old rats did not develop a febrile response at the lower T_a due to reduced cerebral blood flow which would have led to less IL-1β travelling to the brain.

Investigations within our laboratory have shown that peripheral levels of IL-10 were similar in CR and AL rats at 2 and 4 hours post-LPS; however, the rise in IL-6 was significantly attenuated in the CR animals at 2 hours post-LPS. IL-6 is crucial in the

development of the febrile response (Chai, Gatti, Toniatti, Poli, & Bartfai, 1996) and thus the attenuated increase seen in the CR animals could be one of the key mediating factors for why they also display an attenuated fever post-LPS. These CR rats also demonstrated significantly increased peripheral corticosterone (CORT) levels post-LPS. Given that CORT has been shown to inhibit cytokine synthesis and release and plays a negative feedback role during inflammation (Barnes, 1998; Barnes, Adcock, Spedding, & Vanhoutte, 1993) and thus may be contributing to the cytokine message not reaching the brain. Further, we have observed reduced levels of central pro- and increased anti-inflammatory markers in conjunction with an attenuated fever in CR mice (MacDonald et al., 2011). Thus, it may be that the peripheral cytokine message does not reach the brain as effectively at an T_a within the thermoneutral zone of the respective control animals. Given the findings of the current study, the possibility that reduced peripheral blood flow may be impacting upon the passage of the cytokine message to the brain needs to be considered. Further investigations of peripheral and central levels of cytokines in CR animals housed at a higher T_a would be an imperative next step in further understanding the impact of CR post-LPS.

Interestingly, the CR rats in the current study did not demonstrate an increase in T_b until five hours post-LPS, which is past the critical stage for cytokine induced fever and sickness behaviour (Dantzer, 2001; Luheshi, 1998). In fact, at 90 and 120 minutes post-LPS the CR rats were slightly hypothermic. Therefore, we can only speculate to what is initiating the increase in T_b at five hours post-LPS in the CR rats, such as possibly the relationship between the CR animals self-selected T_a and subsequent increase in T_b. Between 90 and 180 minutes post-LPS there was an increase in the CR rats preferred T_a. This coincided with a subsequent increase in their T_b from 120 minutes post-LPS until their peak T_b increase at 300 minutes post-LPS.

Peripheral levels of tumour necrosis factor-α (TNF-α), IL-1, and IL-6 can be affected by the core T_b of the animal (Jiang et al., 1999). Immersion of anesthetised mice in a warm water bath post-LPS so that T_b peaked at 39.5 – 40 °C resulted in earlier and higher peaks of TNF-α and IL-6 levels compared to when the animals core T_b was within the basal T_b range post-LPS (36.5 – 37.5 °C) (Jiang et al., 1999). What this may mean for the current findings is that when the T_b of the animal is able to increase, so too are the cytokine levels that consequently help to produce fever. This may partially explain the findings of our previous work, when CR animals do not increase their T_b post-LPS there is a limited cytokine message sent to the brain (MacDonald et al., 2011). However, in the current study when the CR animals were able to self-select a higher T_a it was easier for them to increase their T_b post-LPS, and thus peripheral cytokine messages were better able to reach the hypothalamus. Further investigations of peripheral and central levels of cytokines in CR animals housed at a higher T_a would be an imperative next step in further understanding the impact of CR post-LPS.

Aside from the blood-brain-barrier, cytokines can also reach the brain via the vagus nerve (Hosoi, Okuma, Matsuda, & Nomura, 2005; Romanovsky, Simons, Szekely, & Kulchitsky, 1997; Watkins, Maier, & Goehler, 1995). Severing the vagus nerve can lead to an attenuated fever with low doses of LPS (Hansen & Kruger, 1997; Layé et al., 1995); however, with higher doses of LPS vagotomised animals still develop fevers (Romanovsky et al., 1997). T_a has been shown to play an important role in the development of the febrile response in vagotomised rats (Romanovsky et al., 1997). A high dose of LPS (1000 μg/kg) did not induce a fever in vagotomised rats exposed to at a slightly cooler T_a compared to thermoneutrality (25 °C) but did when rats were housed at 31 °C (Romanovsky et al., 1997). Although the current study used a markedly smaller dose of LPS (although the same strain) compared to the research cited above, the impact of the T_a may still be relevant (Hansen & Kruger, 1997; Layé et al., 1995).

In light of the evidence presented above it seems pertinent to determine accurately the thermoneutral zone of CR animals, especially if investigators are interested in changes in T_b and fever profiles. It has been shown that one of the most time and cost effective ways of determining the thermoneutral zone of an animal comes from measurements of skin and core T_b, with a strong negative correlation between the two the best indicator of thermoneutrality (Romanovsky, Ivanov, & Shimansky, 2002).

Conclusion

The findings of the current study show for the first time that CR animals able to self-select their preferred T_a select a warmer T_a and thereby increase T_b post-LPS. This is in disagreement with previous findings within this laboratory that demonstrate that CR rats and mice show an attenuation of fever and sickness behaviour measures at a T_a that is within an AL fed animals' thermoneutral zone. An imperative next step in investigating the febrile response of CR animals would be to determine CR animals' thermoneutral zone and then investigate circulating and central cytokine levels post-LPS in these CR animals at their new T_a. Further, investigations of peripheral blood flow in CR animals exposed to LPS would also be of benefit.

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CHAPTER 9

GENERAL DISCUSSION

Overview of findings

This thesis demonstrated that a period of calorie restriction (CR) resulted in an attenuated development of sickness behaviour (fever, anorexia, and cachexia) after exposure to lipopolysaccharide (LPS) in rats and mice. Further, it was demonstrated that CR resulted in a dose-dependent attenuation of sickness behaviour after LPS in terms of both severity (25% and 50% CR) in mice and duration (14, 21, and 28 days) in rats. Central pro-inflammatory markers were decreased and anti-inflammatory markers increased in mice CR to 50% for 28 days and then challenged with LPS. After administration of LPS circulating levels of corticosterone (CORT) were increased and circulating levels of interleukin 6 (IL-6) were attenuated in rats CR to 50% for 28 days. Further, 50% CR rats demonstrated no change in metabolic rate post-LPS compared to ad libitum (AL) fed rats. In contrast to the above findings 50% CR rats able to self-select their preferred ambient temperature (T_a) were able to increase their T_b which had a profoundly delayed onset and shorter duration compared to AL rats. This chapter will begin with a summary of each of the papers presented in this thesis, followed by a discussion of the key findings.

A number of anticipated and unexpected findings were obtained through the series of experiments presented here. These will be re-explored briefly and discussed in regard to the existing literature. Additionally, the methodological limitations and problems encountered within each study, and characteristic in all CR research, will be explored. Incorporated with the limitations of this research will be a discussion of future directions for this line of research.

Chapter 2: Dose-dependent effect of calorie restriction duration in rats on body weight, core body temperature, and locomotor activity

CR has been consistently shown to increase the mean and maximum life span in a wide range of species (Austad, 1989; Lin et al., 2004; McCay, Crowell, & Maynard, 1935; Weindruch, Walford, Fligiel, & Guthrie, 1986) and has been shown to illicit many health-promoting benefits (Halagappa et al., 2007; Maswood et al., 2004; Matsuzaki et al., 2000). CR reduces metabolic rate and T_b, which have been thought to play a significant role in the life-extension properties of CR (Blum et al., 1985; Duffy et al., 1989; Forsum, Hillman, & Nesheim, 1981; Lane et al., 1996).

This study aimed to examine the effect of three different durations (14, 21, and 28 days) of a 50% CR on body weight, T_b, and locomotor activity in male rats. There was a dose-dependent loss of body weight, reduction in core T_b, and alteration of locomotor activity seen in the CR rats; with the rats CR for the longest period of time, 28 days, demonstrating the largest change in all of the variables mentioned above. The locomotor activity of the CR rats was similar to the AL rats up until the dark phase of day 14. The CR rats then demonstrated a flattening of their circadian rhythm of locomotor activity, similar to their T_b. This was characterised by an increase in their light phase activity and a decrease in their dark phase activity. Interestingly, the thermogenic ability of the CR rats was intact, as evidenced by the locomotor activity induced increase in T_b (roughly 1 °C) prior to being fed during the CR period.

The duration of the CR regimen has been shown to have a significant impact upon the investigatory outcomes in some investigations (Hornick et al., 1998; Weithoff, 2007). Therefore, optimising the length of CR regimens employed by different research domains is important and not just for logistical reasons; depending on the investigatory aims, different durations of CR regimens can elicit markedly differing results. These results also support previous findings in which CR has been demonstrated to reduce core T_b (Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; Rikke et al., 2003), which has been implicated as

one of the mediators behind increased longevity in CR animals (Barja, 2004; Lane et al., 1996; Shigenaga, Hagen, & Ames, 1994). This reduced T_b is associated with reduced metabolic rate due to attempts to conserve energy (Blanc et al., 2003; Duffy et al., 1989; Gonzales-Pacheco et al., 1993; Heilbronn et al., 2006; Lane et al., 1996). Slowed metabolism has been shown to reduce the amount of cytotoxic agents released by macrophages and ultimately can lead to reduction in oxidative damage to cells (Chung et al., 2002). This has implications for the proposed longevity effects of CR as reduced core T_b, metabolic adaptation, and decreased oxidative stress are proposed biomarkers of longevity in rodent and monkey CR research (Lane et al., 1996).

Chapter 3: Dose-dependent effect of calorie restriction severity in mice on body weight, core body temperature, and locomotor activity

It has been demonstrated that different severities of CR have can elicit differing results (Kumar, Roy, Tokumo, & Reddy, 1990; Lane et al., 1996; Levay, Tammer, Penman, Paolini, & Kent, 2010; Magwere, Chapman, & Partridge, 2004). For example, rats CR to 90%, 80%, or 70% of AL rats' food intake demonstrated a dose-dependent decrease in colon cancer incidence (Kumar et al., 1990). In addition, rats CR to 12.5%, 25%, 37.5%, and 50% of the AL rats food intake demonstrated dose-dependent increases in CORT (Levay et al., 2010).

The aim of this study was to characterise the effect of two different severities of CR on body weight, core T_b, and locomotor activity. Male mice were fed AL or CR to 25% (CR25%) or 50% (CR50%) of AL animals' food intake for 28 days. There was a dose-dependent response of CR on body weight, with the CR50% mice losing the most weight, the AL mice putting on weight, and the CR25% mice falling in between both groups. Core T_b

reduced in a dose-dependent manner with the CR50% mice demonstrating the largest decline in light and dark phase T_b. The CR25% mice demonstrated an intermediate response, only consistently reducing their dark phase T_b compared to AL mice. Although neither group of the CR mice demonstrated an overall change in locomotor activity, both CR groups demonstrated an increase in their light phase activity. The CR50% mice were the only group to differ from the AL mice during the dark phase of the CR period, demonstrating a reduction in dark phase activity by day 28 of the CR period. Both CR groups demonstrated anticipatory increases in their T_b in the two hours surrounding food delivery, which was also reflected in an increase in locomotor activity during the same period on day 28 of the CR regimen.

These results support previous findings which have shown that CR reduces T_b (Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; Rikke et al., 2003). The larger magnitude of CR (50%) elicited the most dramatic changes in core body weight, T_b, and locomotor activity. Thethermogenic ability of both groups of CR mice was intact (similar to the rats in *Chapter 2*) as shown by the locomotor activity induced increase in T_b prior to being fed. The CR25% and CR50% mice demonstrated approximately a 2 to 2.5 °C increase in T_b in the two hours prior to the onset of the dark phase and food delivery. Due to their body size and surface-to-volume ratio, a period of CR for mice would understandably be more metabolically challenging compared to rats. Future investigations of CR in mice may want to consider the findings of the current study if they wish to take into consideration the impact of CR on T_b and locomotor activity.

Chapter 4: Calorie restriction attenuates LPS-induced sickness behaviour and shifts hypothalamic signalling pathways to an anti-inflammatory bias

The main aim of this Chapter was an investigation of possible central mediators of the attenuation of sickness behaviour. A further aim was to characterise the impact of differing magnitudes of CR on the sickness behaviour. Male mice were fed AL, restricted to 25%, or restricted to 50% of the AL animals in food intake for 28 days. After exposure to LPS on the 29th day the CR50% mice did not develop fevers, whereas the CR25% mice displayed a fever shorter in duration, but with the same peak as the AL fed mice. Both CR25% and CR50% mice showed no sign of anorexia, reduced cachexia, and limited reductions in locomotor activity after LPS administration in contrast to the large changes observed in the AL mice. Both CR groups ate all food presented to them whereas the AL mice reduced their food intake by 46% and both CR groups lost a minimal amount of weight in comparison to the AL mice that lost just under 5% on day one post-LPS. Although both CR groups demonstrated a slight reduction in locomotor activity post-LPS they were both more active compared to the AL group.

Further to this, it was found that a number of hypothalamic mRNA profiles were altered in the CR50% animals at baseline and post-LPS. Expression of neuropeptide Y (NPY) and corticotrophin-releasing hormone (CRH) were both increased by several fold in CR50% animals at baseline compared to AL mice. Increases in NPY in food deprived and CR animals have been seen previously (Bi, Robinson, & Moran, 2003; Brady, Smith, Gold, & Herkenham, 1990; Lewis et al., 1993; McShane, Wilson, & Wise, 1999). The CR50% mice demonstrated an attenuated increase in both cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) expression at 2 hours post-LPS, indicating that alterations in these compounds potentially play a major role in the attenuated fever seen in the CR50% animals. There was a significant increase in suppressor of cytokine signalling 3 (SOCS3) expression in the CR50% animals at 4 hours post-LPS, which was accompanied by an increase in IL-10 expression at the same time point.

Further, the normal increase in both leptin and pro-opiomelanocortin (POMC) expression were attenuated at 2 hours post-LPS in the CR animals, which has implications for both weight loss and food intake post-LPS considering leptin has been shown to exhibit pro-inflammatory effects (Harden, du Plessis, Poole, & Laburn, 2006; Luheshi et al., 1999; Sachot, Poole, & Luheshi, 2004) and increased POMC can lead to reduced food intake (Schwartz et al., 2000). Although not measured in this study, given the dose-response of T_b post-LPS in the CR25% mice it is tempting to speculate that there may also be a dose-dependent response of these mRNA compounds with regards to the severity of the CR regimen. However, it may not be as straightforward as this as some of the behavioural variables were not altered in a dose-dependent fashion, such as body weight and food intake post-LPS. Therefore further investigation of mRNA compounds after different CR severities needs to be considered. The present study was the first to reveal that CR results in a suppression of sickness behaviour responses, which may be due to CR leading to alterations in several central nervous, endocrine, and immune mechanisms.

Chapter 5: Calorie restriction attenuates LPS-induced sickness behaviour and increases circulating CORT, decreases circulating IL-6, and does not alter circulating IL-10 levels

We demonstrated previously (in *Chapter 4*) that a 50% CR for 28 days in mice led to a full attenuation of sickness behaviour and a central anti-inflammatory bias post-LPS. The aim of this study was to examine the effect of CR on the development of sickness behaviour in another species (rats) and to investigate potential peripheral mediators of the attenuated sickness behaviour. Male rats were fed AL or restricted to 50% of the AL animals in food intake for 28 days. On the 29th day the rats were injected with LPS or saline.

The CR rats demonstrated an attenuation of sickness behaviour, which resulted in no development of fever, a limited reduction in locomotor activity, no sign of anorexia, and reduced cachexia following LPS. Serum CORT levels were found to be significantly increased compared to baseline and the AL rats at 2 hours post-LPS in the CR50% animals. The increase in circulating levels of IL-6 was significantly attenuated in the CR rats compared to the AL rats at 2 hours post-LPS, whereas circulating levels of IL-10 were similar post-LPS in AL and CR50% rats. However, there was a trend towards the CR rats demonstrating higher levels of IL-10 at 2 hours post-LPS. The results from this study reinforce the notion that CR leads to an attenuated immune response and subsequently attenuated sickness behaviour and consolidates this phenomenon in another species. The increased peripheral levels of CORT and attenuated IL-6 levels post-LPS seen in the current study are likely additional contributors to the mechanism that result in the CR animals displaying attenuated fever and sickness behaviour post-LPS.

Chapter 6: Dose-dependent effect of calorie restriction duration on sickness behaviour in rats

Limited previous research has investigated the effects of differing durations of CR on various outcome measures, for example oxygen consumption, gene expression, and cancer are all examples of how different lengths of CR can impact on investigatory variables (Bevilacqua et al., 2004; Cao, Dhahbi, Mote, & Spindler, 2001). Given the findings from *Chapter 2* the aim of this study was to examine the effect of differing durations of CR on the development of sickness behaviour in rats. Male rats were fed AL or restricted to 50% in food intake for 14, 21, or 28 days. On the 15th, 22nd, or 29th day the rats were injected with LPS. There was a dose-dependent response of sickness behaviour to LPS following the three

different durations of CR. The rats CR for 28 days demonstrated the largest attenuation of sickness behaviour, which resulted in no development of fever, a limited reduction in locomotor activity, no sign of anorexia, and reduced cachexia following LPS. The rats CR for 14 and 21 days demonstrated a partial attenuation of sickness behaviour following LPS. Further, some sickness behaviour related variables appear to be more sensitive compared to others post-LPS in CR animals. Although the CR50% (28d) animals did not display an increase in T_b or reduction in locomotor activity they did demonstrate a significant reduction in body weight for the first two days post-LPS. The CR50% (21d) animals did not demonstrate a significant reduction in locomotor activity post-LPS; however, they did demonstrate a significant increase in T_b post-LPS and did not recover to their pre-LPS weight for four days post-LPS. This indicates that depending on what variable is being investigated the threshold of CR that needs to be reached can differ. These results also add further weight to the argument that a relatively short period of CR (28 days) can attenuate sickness behaviour measures and that this phenomenon presents in a dose-dependent manner.

Chapter 7: Metabolic rate is reduced after calorie restriction in rats and does not alter post-LPS

It has been widely reported that CR results in a decrease in metabolic processes in animals and humans (Blanc et al., 2003; Duffy et al., 1989; Forsum et al., 1981; Gonzales-Pacheco et al., 1993; Lane et al., 1996; Leibel, Rosenbaum, & Hirsch, 1995; Martin et al., 2007; Ramsey, Roecker, Weindruch, & Kemnitz, 1997). However, an investigation of how CR affects metabolic functions post-LPS has not been investigated. This study aimed to examine the effect of CR on sickness behaviour measures, energy expenditure (EE), and

respiratory quotient (RQ) post-LPS. Rats fed ad libitum or restricted to 50% of the AL animals' food intake for 28 days were injected on the 29th day with LPS.

The AL rats demonstrated an elevated T_b post-LPS; however, the CR50% rats fever profile was dramatically attenuated, only increasing slightly at one time point post-LPS. The AL rats lost significantly more weight compared to the CR50% rats, although both groups lost weight compared to their respective baselines. Only the AL rats reduced their food intake compared to baseline and both groups reduced their locomotor activity, although the AL rats were less active compared to the CR50% rats. Indirect calorimetry indicated that the CR50% rats demonstrated significantly reduced EE compared to the AL rats at baseline; however, the AL and CR50% rats demonstrated similar profiles of RQ at baseline. After LPS, the AL rats demonstrated an increase in EE compared to baseline, whereas the CR50% rats demonstrated no change in EE. After LPS the control rats demonstrated a decrease in their RQ for a limited period during the first eight hours (during the light phase) and then demonstrated a reduced RQ for almost the entirety of the dark phase post-LPS. In comparison the CR50% rats demonstrated no difference in RQ compared to baseline.

In *Chapter 4* and *Chapter 5* the CR animals demonstrate a shift towards an anti-inflammatory bias, both centrally and peripherally. In the CR rats in this study it may well be that the lack of increase in peripheral IL-6 and central COX-2 to drive fever is also resulting in the attenuated fever and subsequently sickness behaviour and EE. Further, it is possible that there is a trade-off between the metabolic effort to increase EE post-LPS and the metabolic effort to maintain important physiological processes such as thermoregulation, heart, and brain function. Mounting an immune response is a physiologically demanding process (Demas, Chefer, Talan, & Nelson, 1997; Martin, Scheuerlein, & Wikelski, 2003), and given the CR animals are experiencing a period of low food availability it may be more important for them to spend energy maintaining more important physiological processes

rather than mounting an immune response for a relatively small dose of LPS (Behnke, Barnard, & Wakelin, 1992). On the basis of these findings *Chapter 8* was designed to investigate whether allowing the CR rats to choose their preferred T_a would negate some of the metabolic costs of maintaining their T_b and therefore allow them to mount a febrile response.

Chapter 8: The ability to select a higher ambient temperature allows calorie restricted rats to exhibit fever post-LPS

Previous research has demonstrated that rodents injected with LPS self-select a higher T_a (Akins, Thiessen, & Cocke, 1991; Peloso, Florez-Duquet, Buchanan, & Satinoff, 2003), and that rats that have been fasted will also self-select a higher T_a compared to controls (Sakurada et al., 2000). However, an investigation of the combination of these two factors has not been conducted. Therefore, this study aimed to examine the effect of CR on self-selected T_a post-LPS. Rats fed AL or restricted to 50% of the AL animals' food intake for 28 days were injected on the 29th day with LPS.

The CR50% rats chose to sit at a warmer T_a compared to the AL rats at baseline. Post-LPS the AL rats chose to sit at higher T_a compared to baseline (although not as warm as the CR50% rats); however, the CR50% rats only differed from their baseline T_a at two time points post-LPS. The AL rats demonstrated a higher T_b compared to baseline post-LPS and in contrast to previous findings in our laboratory the CR50% rats also demonstrated a higher T_b compared to baseline post-LPS. However, the CR50% rats demonstrated a hypothermic response at 90 and 120 minutes post-LPS and did not reach their peak T_b until five hours post-LPS, well past the critical window for cytokine induced fever.

Given that the CR rats were able to demonstrate an increased T_b whilst they were situated at a warmer T_a (29 °C) compared to what they were traditionally housed at in our laboratory (26 °C) may indicate that their thermoneutral zone has shifted since being exposed to the CR regimen. It has been shown that animals exposed to LPS whilst housed below their thermoneutral zone demonstrated blunted febrile responses (Boissé, Mouihate, Ellis, & Pittman, 2004; Buchanan, Peloso, & Santinoff, 2008; Marais, Maloney, & Gray, 2011; Peloso, et al., 2003), which may indicate that the CR rats in the current thesis were essentially in an environment that was sub-neutral, even though 26 °C has been shown to be within the thermoneutral zone of control fed rats (Poole & Stephenson, 1977). These findings highlight the need to characterise the thermoneutral zone of CR animals before investigations involving T_b to ensure the CR animals are still in an environment that is thermoneutral.

Key Findings

Attenuation of sickness behaviour after calorie restriction and a potential mechanism

The studies detailed in this thesis were the first investigation of the impact of CR on the development of sickness behaviour in rodents. There has been limited investigation of food deprivation and sickness behaviour and cytokine responses after LPS (Inoue & Luheshi, 2010; Inoue, Somay, Poole, & Luheshi, 2008; Mrosovsky, Molony, Conn, & Kluger, 1989); however, there has not been an investigation of how a chronic manipulation in food intake would impact sickness behaviour. It has been demonstrated that food deprivation and CR impact upon differing central mechanisms (Bi et al., 2003; Johansson, et al., 2008) and thus the investigation of CR in relation to sickness behaviour was considered pertinent by this author.

This thesis was the first to demonstrate that CR rats and mice demonstrate an attenuated febrile response and limited changes in other sickness behaviour measures post-LPS challenge. Further, this thesis was the first to characterise the dose-dependent effects of CR on sickness behaviour development in relation the duration and severity of the CR regimen and to characterise a selection of potential peripheral and central mediators of the attenuation of sickness behaviour in CR rodents. Of interest, peripheral CORT levels in CR rats were elevated compared to AL animals at 2 hours post-LPS, increases in peripheral IL-6 levels were attenuated post-LPS in CR rats, and there was a limited, but delayed increase in COX-2 and mPGES-1 mRNA expression and substantial increases in both SOCS3 and the anti-inflammatory cytokine IL-10 levels post-LPS in CR mice.

Calorie restriction and changes in metabolic rate and ambient temperature preference post-LPS

As expected, and in agreement with previous research (Blanc et al., 2003; Duffy et al., 1989; Forsum et al., 1981; Gonzales-Pacheco et al., 1993; Lane et al., 1996; Martin et al., 2007; Ramsey et al., 1997), the CR rats demonstrated reduced EE compared to the AL rats. Lowered metabolic rate has been considered to play a role in the life-extending properties of CR, suggesting that reductions in metabolic rate can lead to reduced oxidative damage and ultimately extended life-span (Blum et al., 1985; Duffy et al., 1989; Lane et al., 1996)

Post-LPS the AL rats demonstrated an increase in EE for the first eight hours and then demonstrated a reduction in EE for the majority of 24 hour period post-LPS, as expected per previous research (Arsenijevic et al., 2000). However, the CR rats' EE remained the same as prior to LPS. For the CR rats this pattern was also seen in regards to their RQ levels; no different post-LPS compared to post-saline. The AL rats demonstrated similar RQ levels

during the first six hours post-LPS; however, then demonstrated a drop in RQ levels which was maintained for the majority of the dark phase post-LPS, which has also been demonstrated previously (Steiger et al., 1999).

In disagreement with the abovementioned studies within this thesis the CR rats that were able to self-select their preferred T_a demonstrated an increased T_b post-LPS although significantly delayed and shorted lived. It is possible that the ability to select a warmer T_a (possibly reflecting a new thermoneutral zone for CR animals) enabled the CR rats to metabolically afford to increase their T_b. It has been highlighted elsewhere that exposure to a bacterial compound at T_as below thermoneutral zone can inhibit the passage of peripheral cytokine messages to the brain and attenuate fever production (Boissé et al., 2004; Buchanan et al., 2008; Marais et al., 2011; Peloso et al., 2003).

Limitations and future directions

As with most research, the research presented in the current thesis is not without limitations that may have implications for the interpretation of the findings. Some of these were predictable; however, they were also unavoidable. Others arose during the process of data collection or analysis. Future studies aiming to investigate a similar area should be aware of these limitations and aim to rectify or amend them to increase the applicability of their findings.

Firstly, the method by which the animals in this thesis were CR may be a limitation. Similar to other CR studies we restricted calories by restricting the global amount of food the animal received. The majority of studies examining CR, whether it be longevity or physiological and behavioural outcomes, adopt the global method of restriction without providing nutritional supplements. However, we acknowledge that the observed effect of CR

may reflect reductions in specific components of food rather than just calories, which is certainly an inherent methodological flaw in all research investigating CR. In the current thesis the CR rats diet was deficient in fat and Vitamin B-12 and the CR mices diet was also deficient in fat, Vitamin B12, and Vitamin B6. The impact of the deficiency of one element of a rodents diet has been demonstrated before; for example, a diet free from protein can reduce the increase in IL-6 and TNF-α post-LPS (McCarter et al., 1998). We do not think that the results seen in this thesis would be due to any specific component of the animals' diet; however, since no investigation has been conducted we cannot be certain. Therefore, we are not aware what impact the small deficiencies in these dietary components would have on the findings reported in this thesis.

Another possible limitation of the current thesis was that the dose of LPS used would be considered moderate. On this basis the attenuated sickness behaviour seen in the CR animals may be an artefact of a small dose of LPS, not the physiology of the CR animal changing to attenuate the immune response. In explanation, the relatively small infection may be assessed by the CR animal as too small to mount an immune response given the high metabolic cost if they were to initiate an immune response. Further, the CR animals weighed significantly less compared to the control animals and therefore on the basis of a weight determined dose of LPS obviously received less LPS compared to the control animals. However, as discussed in *Chapter 4* the CR25% and CR50% mice were of a similar weight, and therefore would have received similar doses of LPS. The CR50% mice displayed a complete attenuation of LPS-induced fever, whilst the CR25% mice demonstrated a fever to the same peak as the AL mice, negating the impact of possible discrepancies in dose of LPS as a possible contributing factor as to why the CR50% animals did not develop sickness behaviour. Further, in *Chapter 6* the AL animals ate on average 30% less than the CR50% animals. However, even though we believe that the dose of LPS did not play a role in the

attenuated sickness behaviour in the CR animals it still would be of importance to investigate the impact of a higher dose of LPS. A larger dose of LPS may test the lack of anorexia in the CR animals. The AL animals decreased their food intake to roughly the same level as the CR animals food intake; therefore, possibly a larger dose of LPS would decrease feeding in the CR animals. Previously it has been shown that when the vagus nerve has been severed below the diaphragm there is an attenuation of fever and sickness behaviour in response to LPS (Hansen & Kruger, 1997; Layé et al., 1995). However, when exposed to large doses of LPS the vagotomised animals are able to demonstrate a fever (Romanovsky, Simons, Szekely, & Kulchitsky, 1997). This may indicate CR down-regulates fever and sickness behaviour in a proportional manner and that a larger doses of LPS may result in a fever; however, is still attenuated.

Another limitation of the research reported in this thesis was that the activity of brown fat thermogenesis (BAT) was not determined in the CR animals. BAT has been shown to be reduced in CR rats (Rothwell & Stock, 1982); and there have been suggestions of a correlation between the amount of brown fat and fever intensity, with reduced levels of brown fat being linked to reduced fever intensity (Cannon, Houstek, & Nedergaard, 1998). Future investigations need to determine the level of BAT in CR animals to elucidate this point more clearly and the potential role BAT may play.

Given the results of *Chapter 8* it would be imperative to investigate in a more in-depth manner the role T_a plays for the CR animal during an LPS challenge. In addition to this it would also be pertinent to investigate the thermoneutral zone of CR animals, as it appears that what is considered the thermoneutral zone for freely fed animals may not be the thermoneutral zone for CR animals. For example, if we housed CR rats at 29 °C would they develop a febrile response, sickness behaviour, and the associated increases in peripheral and central inflammatory signals. It has been shown that one of the most time and cost effective

ways of determining the thermoneutral zone of an animal comes from measurements of skin and core T_b, with a strong negative correlation between the two the best indicator of the thermoneutral zone (Romanovsky, Ivanov, & Shimansky, 2002).

Another relevant line of investigation given the findings of *Chapter 8* would be peripheral blood flow and subsequently peripheral cytokine messages reaching the brain in CR animals. As mentioned in this chapter a period of CR has been shown to reduce blood pressure (Wright, Mc Murtry, & Wexler, 1981; Young, Mullen, & Landsberg, 1978), which may impact upon peripheral cytokine messages reaching the brain post-LPS. Further, in relation to T_a it would also be important to investigate central and peripheral immune markers in CR animals at their preferred T_a or newly determined thermoneutral zone. Given *Chapter 8* indicated that T_a may play a crucial role in CR animals ability to mount an immune response and that there is possibly a trade-off between CR animals thermoregulation and mounting an immune response.

This thesis has been able to demonstrate that a period of reduced food intake can dramatically alter the immune response to a bacterial mimic. Further, the results of this thesis have been able to add to the wide body of existing literature in the areas of sickness behaviour, both peripheral and central immune responses, changes in metabolic rate during illness, and behavioural thermoregulation. In regards to the final point this thesis has also shown that manipulating food intake can potentially change the thermoneutral zone of the animal. This has potential consequences for all research investigating the impact of an altered food intake (including food deprivation) on various outcomes, especially in terms of T_b, metabolic rate, and immune function. It is critical that future research considers the impact of T_a when investigations involve manipulations of food intake.

Applying the findings of the current thesis to the human condition may not be viable; not many people would be willing or able to restrict their food intake by up to 50%, and nor

should they as this could impact detrimentally on their health. There has been limited research investigating the impact CR has on non-human primate immune function; however, early results suggest that CR retards immunosenescence in non-human primates and potentially improves immune function (Messaoudi et al., 2006; Nikolick-Zugich & Messaoudi, 2005). With this research in non-human primates it establishes that the positive impact of CR upon immune system functioning may also be attributed to humans. However, the aim of this research has been to investigate the mechanisms involved in food intake and its impact upon the immune system. Considering CR has been shown to reduce oxidative damage, one of the widely accepted indicators of aging (Barja 2004; Duffy et al., 1989; Heilbronn et al., 2006; Lane et al., 1996; Shigenaga et al., 1994), elucidating the full impact CR has on physiological functioning is important. Research has already begun into the impact the Sirt1 gene has on physiological functioning (Qin et al., 2006; Salminen, Kauppinen, Suuronen, & Kaarniranta, 2008); as it is this gene that researchers believe mimics the positive impacts of CR (Chen, Steele, Lindquist, & Guarente, 2005; Lamming, Wood, & Sinclair, 2004). On the other hand resveratrol, thought to activate Sirt1, has demonstrated mixed findings with some demonstrating increased life span (Howitz et al., 2003; Valenzano et al., 2006), others no increase in life span (Bass, Weinkove, Houthoofd, Gems, & Partrifge, 2007; Miller et al., 2011).

Non-steroidal anti-inflammatory drugs (NSAID) have analgesic, antipyretic, and anti-inflammatory effects and their primary mechanism of action is to block COX and therefore limit the inflammatory response (Hayllar & Bjarnason, 1995). However, there has been evidence to suggest that NSAID’s can detrimentally impact upon the gastrointestinal tract (Maiden et al., 2005). We have been able to demonstrate that CR can naturally limit the increases in COX-2 normally seen post-LPS, and although, as mentioned above, a 50% CR in humans would be untenable, the development of a mimic of the effects of CR may be a safer

option as opposed to NSAID in the treatment and management of inflammation. There has been limited investigation of the impact of CR on the other COX enzyme, COX-1; therefore, this would also be another avenue of future research, establishing the role CR may have in potentially altering levels of COX-1 as well.

Chronic inflammation has been cited as a characteristic of numerous diseases such as cancer (Coussens & Werb, 2002; Mantovani, Allavena, Sica, & Balkwill, 2008), obesity-related insulin resistance (Xu et al., 2003), polycystic ovarian syndrome (Kelly et al., 2001), inflammatory bowel disease (Itzkowitz & Yio, 2004), rheumatoid arthritis (McInnes & Schett, 2007), and Alzheimer's Disease (Sokolova et al., 2009) to name a few. The possibility of developing a mimetic of the potential anti-inflammatory properties of CR to help control the chronic inflammation seen in the abovementioned diseases and others may potentially be of great benefit to the medical field.

Conclusions

It has been well established that CR can exert positive effects upon the lifespan and certain age-related diseases (Austad, 1989; Bradbury et al., 2000; Chandrasekar, Nelson, Colston, & Freeman, 2001; Kubo, Day, & Good, 1984; Kubo, Gajjar, Johnson, & Good, 1992; Lane, Ingram, & Roth, 1999; Maswood et al., 2004; Matsuzaki et al., 2000; Sohal, Ferguson, Sohal, & Forster, 2009; Turturro et al., 1999; Walford, Mock, Verdery, & MacCallum, 2002). This thesis has demonstrated that a period of CR can attenuate sickness behaviour following LPS administration in mice and rats. It was demonstrated that hypothalamic mRNA levels of COX-2 and mPGES-1 were attenuated at 2 hours post-LPS and SOCS3 and IL-10 were enhanced at 4 hours post-LPS in the CR animals. There was also a significant increase in peripheral CORT and attenuation in the rise of peripheral IL-6 at two hours post-LPS in CR rats. These results indicated a switch from a pro-inflammatory bias to

an anti-inflammatory bias after LPS administration. Further, the CR animals displayed no change in metabolic rate post-LPS; however, when allowed to self-select their preferred T_a the CR rats were able to mount a limited increase in T_b. What remains to be elucidated is whether or not the response of the CR animals following LPS is adaptive and beneficial. It has been established that fever is adaptive (Hart, 1988), and therefore one may question why these CR animals do not display a fever. One possibility is that it may be too metabolically costly for them to develop a fever, and this outweighs the adaptive value of displaying a fever. Another possibility is that there is a trade-off between mounting an immune response and thermoregulatory processes. Irrespective of the reason as to why CR animals do not display sickness behaviour and the ‘normal’ patterns of pro- and anti-inflammatory compounds after LPS, this finding could be significant in investigating mechanisms important in chronic illness, and possible avenues of treatment for these diseases. An agent that could mimic the effect of CR on the immune response may be of potential benefit for the treatment and /or management of chronic inflammation.

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Appendix A

LA TROBE UNIVERSITY

HIGHER DEGREES COMMITTEE (RESEARCH)

ALTERNATIVE THESIS FORMAT GUIDELINES

La Trobe University
Higher Degrees Committee (Research)
Guidelines for preparing thesis by publication

1 While dissertations submitted for award of higher degrees often contain work by the candidate that is already published (or accepted for publication), it is also permissible for candidates to submit a higher-degree thesis that is in the form of a series of thematically-focused articles or chapters that are either published or accepted for publication by reputable journals or publishers. The presentation of the articles will take the same format as doctoral and masters by research theses, and will include full publication details for the published/accepted material.

2 Where the thesis submitted for a degree by published work includes jointly authored publications, the candidate is expected to have made a significant and leading contribution to such work. The candidate will provide at the time of submission a declaration for each article or chapter, stating the extent and nature of his or her contribution and justifying the inclusion of the material. In each case, a signed declaration from at least one co-author is to be provided, verifying the extent and nature of the candidate's contribution.

3 The presentation of a thesis as a collection of articles or chapters will include at least one substantial integrating chapter, or a separate introduction, general discussion and conclusion that in combination reveal the way the articles and chapters are thematically linked. This integrating material will not itself contain new or innovative research material. Where a dissertation contains a mixture of published and unpublished work which in combination represent a substantial and original contribution to knowledge, then it will be examined in the normal way, and will not be treated as a submission for a higher degree by published work.

4 The number of articles to be included will depend on the significance, originality and length of each and takes account of (a) the University's requirements for the degree, and (b) the amount of research normally expected to be undertaken for the degree in question. Discipline areas may set specific requirements, in addition to those described in these guidelines.

5 Normally most of the work submitted will have been completed during the period of Candidature. It is permissible to include work published prior to commencement of higher degree research candidature at La Trobe University when this fits with, and adds substantial content to, the research studies being assessed by the examiner(s).

6 A book published or definitely accepted for publication by a reputable publisher can also be submitted for examination for a Masters, PhD or professional doctorate, provided that guidelines 2 and 5 above apply.

7 The thesis will be examined in the normal way. In cases where the Chair of HDC(R) is persuaded that there has already been sufficient peer review of the contribution(s) a decision can be made to reduce the number of examiners to one examiner for a Master's degree and two examiners for a Doctorate.

Amended 12.5.2011

Appendix B

PUBLISHED MANUSCRIPT:

Calorie restriction attenuates LPS-induced sickness behavior and shifts hypothalamic signaling pathways to an anti-inflammatory bias

Appendix C

ETHICS APPROVAL DOCUMENTS