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Gestational hypothermia in mice is not proportional to foetal mass or affected by maternal thermal environment, but pups grow faster at temperatures below thermoneutrality

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ABSTRACT

During mammalian pregnancy, maternal thermal physiology and the associated thermoregulatory control systems undergo various changes. As the foetus grows, it produces an increasing amount of metabolic heat. That heat is dissipated either via feto-maternal exchange in the placental circulation (~85%), or via heat conductance pathways through the foetal skin, amniotic fluid and across the uterine wall (~15%). As gestation progresses, maternal core body temperature (T_{core}) decreases (gestational hypothermia). There are several theories concerning why gestational hypothermia occurs. The aim of this study was to confirm that gestational hypothermia occurs in mice. It was predicted that the amount of heat generated by foetal mass *in utero* would be proportional to the gestational decrease in maternal T_{core}.

It was also predicted that a change in maternal thermal balance by means of altered ambient temperature (T_a), may affect the extent of gestational hypothermia. Maternal intraperitoneal T_{core} was measured during gestation in mice assigned to two ambient temperature groups, thermoneutral (29°C, $T_{29,}$ n=8) and standard animal housing (22°C, $T_{22,}$ n=7). Thermoneutral and below thermoneutral environments were used to alter the maternal thermal balance by changing the thermal gradient between the animal and her surroundings. A cosinor analysis was applied to the temperature data to obtain the mesor, amplitude and acrophase of the fitted cosine wave at several defined stages of pregnancy. Food intake and maternal body mass were measured throughout pregnancy, and pups were weighed immediately after birth and during lactation.

The mesor of T_{core} decreased during gestation ($P<0.001$) by ~1°C from premating levels, between the middle of trimester 1, reaching a nadir one day prepartum and increasing significantly ($P = 0.001$) by 0.6°C on the day of birth and a further 0.4°C by day two of lactation ($P<0.001$). The decrease in maternal T_{core} was the same at the two housing temperatures. The extent of gestational hypothermia was not correlated with the foetal mass, the number of pups in the litter, or the proportion of maternal body mass made up by foetal mass at term. There was no difference in the litter mass or litter size at parturition between the treatment groups. However, pups grew faster in the T_{22} group than the T_{29} group during the first two days (by 0.2 g/day/pup, $P=0.003$), and from days four to six (by 0.1 g/day/pup, $P=0.03$) of lactation, but there was no difference in maternal body mass or maternal food intake between T_{22} and T_{29} at any stage of gestation or lactation.

The present study found that mice do exhibit gestational hypothermia as shown previously in rats, sheep, rabbits, dogs and goats. Gestational hypothermia is unaffected by housing temperature and is not correlated with the foetal parameters that influence feto-maternal heat exchange. This indicates that foetal temperature is not the regulated variable and some other mechanism or signalling pathway is causing the occurrence of gestational hypothermia. Pup growth during lactation appears to be more successful at sub thermoneutral temperatures, but is not associated with a difference in maternal mass or food intake between the two temperature groups. It is thought that maternal heat dissipation occurs at a faster rate at T_{22}, thus reducing the heat burden on those mothers, allowing them to process more food into milk.

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ANGII	angiotensin II
AT_1	angiotensin II type 1 receptor
AVP	arginine vasopressin
BMR	basal metabolic rate
BAT	brown adipose tissue
CNS	central nervous system
LCT	lower critical temperature
MHR	metabolic heat ratio
NA	noradrenaline
NST	nonshivering thermogenesis
SCN	suprachiasmatic nucleus
SNS	sympathetic nervous system
T_{core}	core body temperature
T_{22}	normal animal housing ambient temperature
T_{29}	thermoneutral temperature for mice
TNZ	thermoneutral zone
UCT	upper critical temperature
ΔT	change in maternal core body temperature between trimester 1 and the day before parturition

CHAPTER 1 – LITERATURE REVIEW

Preface

During gestation the growth of the foetus brings with it an increase in foetal metabolism and metabolic heat production. A range of physiological adaptations occur during gestation, one of which is a thermoregulatory adjustment in the mother. A gradual decrease in core body temperature (T_{core}) is observed in a host of mammals. The decrease in maternal T_{core} reaches its nadir during late pregnancy, coinciding with the approach of foetal mass to its maximum (Naccarato and Hunter, 1983; Kitrell and Satinoff, 1986; Laburn *et al*., 1992; Laburn *et al*., 1994; Fewell, 1995; Eliason and Fewell, 1997; Fewell and Tang, 1997; Faurie *et al*., 2001; Cairns *et al*., 2004). It is still uncertain why maternal T_{core} decreases, but it is hypothesized to be a regulated response (Eliason and Fewell, 1997; Cairns *et al*., 2004).

An important aspect of this study is the effect of ambient temperature (T_a) on the ability of an organism to reach a state of homeothermy. Heat dissipation to the environment is dependent on the temperature gradient that exists between the animal and its surroundings. In hotter environments, the gradient is smaller, which reduces the animal's ability to dissipate heat, and when heat loss cannot be achieved, T_{core} will increase. The opposite happens in colder environments, increasing the temperature gradient and the animal's ability to dissipate heat (Laburn *et al*., 2002). This review addresses the concept of mammalian thermal balance in detail and then contextualises the effect of T_a with the premise of gestational thermal physiology in mind. When an animal is pregnant, the mother serves as the "environment" for the

foetus, and these same rules of heat dissipation apply between mother and foetus (Gilbert *et al*., 1985).

The following literature review first sets the scene with a general overview of thermoregulation and thermal exchange of the mammalian organism with its environment. There then follows a review of the thermoregulatory literature, in which an attempt will be made to clarify the thermal physiology terminology, with particular reference to the terms that are relevant to this study. This review is important because there was some uncertainty surrounding what term to use to refer to the decrease in maternal T_{core} during gestation. It was necessary to determine what was normal with regards to thermoregulation and what potential thermoregulatory responses fell on either side of normal. From there it could be decided which term best suited the findings of our study. The term *gestational hypothermia* was settled on and the decision will be justified in the review.

Lastly, what is already known about gestational hypothermia is reviewed. The change to maternal T_{core} between the first trimester and the day before parturition is designated ΔT. The aim was to explore the effect of gestational hypothermia on the foetus and to unwrap the existing hypotheses that attempt to explain why maternal T_{core} decreases during gestation.

1.1 Thermoregulation

Temperature affects all biological, and therefore physiological, processes. Changes in temperature impact the rate and direction of almost all metabolic and biochemical processes within the body. Mammals have the capacity for both homeothermy, a

pattern of thermoregulation in which a relatively constant T_{core} in maintained, and endothermy, a process that facilitates the maintenance of homeothermy (Refinetti and Menaker, 1992; Schmidt-Nielsen, 1997). The range of T_{core} that classifies a state of homeothermy varies between species, but an example of the homeothermic range of a C57B/6 mouse is 2-4°C around an average T_{core} of 35.8°C (Gordon, 2012a).

Benzinger (1959) hypothesized that there are two critical sites involved in the regulation of T_{core}. Both are located in the preoptic area of the hypothalamus in the brain, and allow T_{core} to be regulated within a few tenths of a degree Celsius, even in a diverse range of ambient environments (Satinoff, 1978; Refinetti and Menaker, 1992; Jessen, 2000). The site in the brain that regulates autonomic responses to temperature change is in the anterior hypothalamus, and is triggered by activity in thermosensitive neurons (warm- and cold-sensitive neurons). The site in the brain that regulates metabolic rate is in the posterior hypothalamus (Kobayashi, 1989; Jessen, 2000). Homeothermic animals utilize a combination of mechanisms for thermoregulation, which are controlled via the autonomic nervous system (redistribution of blood by cutaneous vasoconstriction/vasodilation, nonshivering thermogenesis in brown adipose tissue [BAT], piloerection, sweating), as well as behavioural thermoregulation (*e.g.* saliva spreading, panting, huddling and temperature seeking behaviour [thermotaxis]) and shivering in skeletal muscle to maintain their T_{core} within such narrow limits (Gordon, 1993; Jessen, 2000; Gordon, 2012a).

Thermoregulatory behaviours have their effective limits when the animal is exposed to severe conditions which may lead to large fluctuations in peripheral temperatures (*i.e.* skin and oral temperatures) but do not necessarily cause changes in T_{core} (Jessen, 2000). Any fluctuations outside the species-specific homeothermic range of T_{core}

suggest physiological abnormality or pathological condition. In some cases this can also be indicative of a state of torpor.

1.1.1 Thermal exchange

Metabolic heat reaches the surface of the skin of an organism and is dissipated to the environment by four principal pathways, namely convection, conduction, radiation and evaporation. When T_a is below T_{core} and in the absence of a radiation source, metabolic heat is the only source of heat, and the four dissipation pathways are the sources of heat loss. If the metabolic heat production is greater than can be dissipated by means of radiation, convection, conduction and evaporation, then heat will be stored in the body, leading to an increase in T_{core}.

Newton's Law of Cooling states that the rate of change temperature of an object is proportional to the difference between the temperature of the object and the T_a (Strunk, 1971). The law loosely applies to the conditions of thermal exchange in an animal because the heat loss is proportional to the temperature difference between the animal and its surroundings, but animals are not passive bodies and are constantly generating heat. An animal's body mass, surface area-to-volume ratio, skin temperature, insulation thickness, metabolic rate, peripheral circulation, heat capacity, and the physical properties of the environment (barometric pressure, T_a, air movement around the body and humidity) all influence the rate of heat exchange. In turn these other factors will determine the extent of the four principal pathways of thermal exchange (Strunk, 1971; Pharo Gagge and Gonzalez, 2011).

Sensible heat loss occurs initially via conduction through the insulation layer (fat or fur) and then via convection and radiation to the environmental medium (Scholander *et al*.,

1950; Hammel, 1955; Schmidt-Nielsen, 1997; Jessen, 2000; Pharo Gagge and Gonzalez, 2011).

Convective heat loss depends on a temperature gradient and involves the mass movement of the medium that is transferring thermal energy. Convective heat loss can be natural (when a warmer body is placed in a cooler environment) or forced (when the medium around the body is moving, drawing heat away from the body) (Schmidt-Nielsen, 1997; Jessen, 2000).

Conductive heat transfer entails the transfer of heat energy from one site at a high temperature to another site of a lower temperature. Thermal conductance is the rate of heat flow between a body and its environment per ^{o}C of temperature difference (Schmidt-Nielsen, 1997; Jessen, 2000,). The heat flow from a body to its environment is also found to be influenced by the body mass of an organism, with an increase in body mass resulting in an allometric decrease in the thermal conductance, *i.e.* heat flow from a body to its environment is inversely proportional to its body mass (Hayssen and Lacy, 1985).

Thermal radiation is electromagnetic radiation and is the energy emitted to the ambient environment by the movement of photons at infra-red wavelengths. Evaporative heat loss occurs as a result of the vaporization of liquid on a surface of the body (the skin or the airways), and is dependent on the water vapour pressure difference between the liquid on the body surface and the surrounding environment (Strunk, 1971; Jessen, 2000; Pharo Gagge and Gonzalez, 2011).

Thermal exchange also occurs within the mammalian body and of the four pathways, conduction occurs between areas of different temperatures, but convection is the most efficient way to move heat within the body. For example, thermal energy is

transferred by convection through the mass movement of the blood from one area of the body to another, be it internally between organs or to the external environment by peripheral blood flow to the skin.

1.1.2 Nomenclature of thermoregulation

In thermal physiology there is ambiguity surrounding the nomenclature for describing changes in T_{core}, and whether these thermal changes reflect a regulated or a forced physiological response from a state of normothermy. Normothermia is the state of normal T_{core}, within a species-specific range when an animal is in a resting state in its TNZ (Thermal-Commission, 2001). The TNZ is the range of T_a at which metabolic heat production is balanced with dry heat loss (Jessen, 2000; Schmidt-Nielsen, 1997). Gordon (1983) proposed that T_{core} can be changed by one of two principal mechanisms, namely a change in set-point temperature, which results in a regulated shift of $T_{core,}$ or an excessive environmental or endogenous heat load that overwhelms the thermoregulatory mechanisms, causing a forced shift in T_{core}. He discussed in great detail the controversy, confusion and inconsistency that has arisen through a lack of definitive nomenclature in neuropharmacology and other disciplines that discuss thermal changes. For the purpose of this thesis, a review of the thermal physiological nomenclature surrounding thermal responses is necessary. The review will attempt to provide specific attention to variables relevant to the experimental study at hand.

This review refers to the International Union of Physiological Sciences Glossary of Terms for Thermal Physiology (The Glossary) for the base definitions. However, there have been different versions of the glossary published over the years between which thermal terminology definitions differ slightly. I would like to highlight the evolution of

some of these terms. The glossary was first published in 1973 by J. Bligh and K. G. Johnson, and has since been revised twice by the Commission for Thermal Physiology of the International Union of Physiological Sciences. It was republished in 1987 in *Pflügers Archiv,* and for a third time most recently in 2001 in *The Japanese Journal of Physiology*.

1.1.3 Set-point temperature

The first definition issue is the conception of set-point. There are countless arguments for and against this theoretical concept. In engineering terminology, the term set-point is defined as the value of the input into a control system at which the output is zero (Mitchell *et al*., 1970). The concept of a set-point in thermoregulation was originally proposed by J. D. Hardy in 1953, and was refined and officially published in his later collaborative works (Hammel *et al*., 1963; Hardy, 1965). Hammel *et al*. (1963) proposed that the central controller for all thermoregulatory responses is located in the preoptic nucleus of the anterior hypothalamus. Some thermoregulatory responses, they proposed, may be initiated earlier or at different body temperatures than others, manifesting as a variation in the threshold for different responses (Hammel *et al*., 1963; Satinoff, 1978).

By manipulating the hypothalamic temperatures of dogs and monkeys using hypothalamic heat clamps, Hammel *et al*. (1963) provided an example of the way the system operates by showing that when an animal is exposed to a cold environment, the decrease in skin temperature stimulates an increase in the rate of firing of cold thermal receptors, elevating the set-point temperature to a value above hypothalamic

temperature. This indicates that set-point temperature is not situated in one specific place, like the hypothalamus for example, but rather is a combination of all thermosensitive neurons. Hardy *et al*. (1964) and Wit and Wang (1968) found that there are neurons that are specifically sensitive to changes in temperature, with approximately 10-20% of hypothalamic neurones responding specifically to thermal changes of the skin, body core and other areas of the brain.

There are a few variations but most set-point definitions agree that set-point temperature is an abstract reference signal, to which the inputs of hypothalamic T_{core} are compared, resulting in the appropriate corrective thermoregulatory behavioural and autonomic responses (Hammel *et al*., 1963; Satinoff, 1978; Gordon, 1983; Briese, 1998). Importantly, for those that support the notion of a set-point, it is described to be an adjustable reference value and not a static set-point (Hammel *et al*., 1963; Hardy, 1965; Refinetti and Menaker, 1992). It also cannot be measured directly, but can be deduced according to the occurrence and direction of the thermoregulatory responses at the given time (Hammel *et al*., 1963).

Until relatively recently, the theory surrounding set-point was that there was a single thermostat or set-point (Hammel *et al*., 1963; Hardy *et al*., 1964; Cabanac and Massonnet, 1974). There are findings however, that do not agree with this idea. Numerous authors either do not agree with the concept of set-point theory or have suggested that the set-point model is one that complicates rather than simplifies the analysis of thermoregulation (Mitchell *et al*., 1970; Satinoff, 1978; Werner, 1980; Kobayashi, 1989; Romanovsky, 2004; Kobayashi *et al*., 2006; Romanovsky, 2007). These authors agree that there are inputs from the skin, brain and central body that are summed in a comparator, and that the output of the comparator is the error

signal, which initiates a response in the thermoeffector organs. Once a thermoeffector response is initiated, T_{core} is adjusted as is the error signal (Satinoff, 1978; Kobayashi, 1989; Romanovsky, 2004; Kobayashi *et al*., 2006; Kanosue *et al*., 2010).

Romanovsky (2004) challenged the theory of set-point, believing it all to be a bit vague. He suggested the substitution of balance point theory. He believed that the conclusion of a "change in set point" said nothing about the regulatory mechanism involved in the set-point response. The balance point theory suggests the balancing of active and passive processes of T_b control, rather than comparing T_b with a set point. Romanovsky's theory is supported by the work of Kobayashi and colleagues (2006, 1989), whose models suggest that thermoeffectors are wired to their own temperature sensitive sensory neurons, which have a neuronal firing threshold. Once that firing threshold is met, they generate a signal to effector cells. The sensory neurons can be peripheral or deep body thermal sensors. Kobayashi (1989) and Romanovsky (2007) have considered these interactions between thermal sensors and effector cells to be known as thermoeffector loops.

The theory on thermoeffector loops builds onto a model that was proposed by Satinoff (1978), based on the operation of multiple integrators arranged in a hierarchy. Satinoff (1978) suggested that the hypothalamus is not the solitary integrator of all the inputs of T_b, but rather the principal integrator that organizes other integrator functions at lower levels along the neural axis. An example of this is when hypothalamic lesions are present in rats, some autonomic and behavioural reflexes are completely abolished, whereas others remain unaffected, and Satinoff (1978) reported that animals without a hypothalamus are still able to generate thermal stress responses.

In the case of heat defence, skin vasodilation appears to occur first, and when this is not sufficient to reduce T_{core}, evaporative functions such as sweating and panting will be activated, as these mechanisms have higher thermoeffector thresholds (Kanosue *et al*., 2010). The hierarchical control of the system of Satinoff (1978) proposes that each of the thermoregulatory effector mechanisms (or thermoeffector loops as suggested by Kobayashi *et al*. (2006)) is not dependent on one control system, yet nor are they independent of one another. Satinoff (1978) suggests that the principal integrator, the hypothalamus, is at the top of the thermoregulatory hierarchy and that it facilitates and inhibits the structures at lower levels along the neural axis.

Satinoff tied the theory of set-point into the hierarchical control theory, by suggesting that the defence of the "neutral zone", termed for clarity as the "interthreshold zone" by Romanovsky (2004), is what defines the set-point. The interthreshold zone describes the range of T_{core} where metabolic and evaporative effector responses are minimal. This suggestion was supported by a study done by Keller (1963), who removed the entire hypothalamus in dogs, and found that the dogs still had the ability to thermoregulate. What had changed were the thresholds for the activation of thermoeffectors. The result was that although the dogs defended their T_{core} but at a wider interthreshold and Satinoff (1978) believed that that zone was indicative of set-point. The conclusion was that, when all integrative systems in parallel are fully functional in order of their hierarchical organization, the interthreshold zone will be as narrow as possible, giving the illusion of a single integrator for a single set-point.

1.1.4 Fever and hyperthermia

Fever is described in the Glossary (Thermal-Commission, 2001) as "*A state of elevated core temperature, which is often, but not necessarily, part of the defensive responses of organisms, to the invasion by live or inanimate matter recognized as pathogenic or alien by the host. The defended rise in core temperature is usually designated as due to a change in the thermocontroller characteristics resulting in an elevation of set-point body temperature.*" There are many studies that support this definition, with evidence that fever is established not by the overwhelming of thermoregulatory control mechanisms, but rather by an upward displacement of hypothalamic set-point (Hammel *et al*., 1963; Stitt, 1979; Gordon, 1983; Kozac, 1997; Roth *et al*., 2006; Fletcher *et al*., 2013). Fever is usually produced as a result of prostaglandin E_2 synthesis in the brain that is induced by exogenous pyrogens and pyrogenic cytokines (Fletcher *et al*., 2013).

According to the Glossary (Thermal-Commission, 2001) hyperthermia is "*The condition of a temperature regulator when T_{core} is above its range specified for the normal active state of a species*" *i.e*. T_{core} is above the normothermic temperature of a species. In different cases hyperthermia can be forced or unregulated. This forced hyperthermia can be caused by environmental factors such as exposure to or exercising in, very hot conditions where heat dissipation capacity is insufficient for thermal balance (Gordon, 1983; Thermal-Commission, 2001; Roth *et al.*, 2006). Forced hyperthermia can also be caused by either an increase in metabolic heat production, a response to a drug or anaesthetic, or an impairment of the thermoeffector mechanisms that are used to dissipate heat (Gordon, 1983; Thermal-Commission, 2001; Roth *et al*., 2006).

Hyperthermia can, however, also be regulated. Fever is an example of regulated hyperthermia and this is believed to occur by means of an upward displacement of set-point temperature (Stitt, 1979; Thermal-Commission, 2001).

1.1.5 Hypothermia, anapyrexia and torpor

Hypothermia as described by the Glossary (Thermal-Commission, 2001) is said to be "*The condition of a temperature regulator when core temperature is below its range specified for the normal active state of the species*" *i.e*. T_{core} is below the normothermic temperature of a species. Forced hypothermia can result by means of exposure to cold environmental temperatures, or be induced as for example during surgery due to the administration of anaesthetic. Hypothermia can result from an overwhelming or impairment of the thermoeffector mechanisms, without the presence of a pathological condition, where the organism is unable to maintain its T_{core} despite activation of heat-defence strategies (Kozac, 1997; Thermal-Commission, 2001). Hypothermia can also be a regulated response, as in the example of torpor or hibernation (Thermal-Commission, 2001).

Anapyrexia is a far less understood concept than fever. The term was coined by Cabanac and Brinnel (1987) and brought into the Glossary of Terms for Thermal Physiology by the IUPS Commission in the same year. In that edition of the glossary, the term anapyrexia was defined as "*A pathological condition in which there is an abnormal decrease in body temperature. Anapyrexia is distinct from hypothermia in that thermoregulatory responses indicate a defence of the antipyretic levels of core temperature."* However, the most recent edition of the glossary (Thermal-Commission,

2001) currently describes anapyrexia as "*A pathological condition in which there is a regulated decrease in core temperature. Anapyrexia is distinct from hypothermia in that thermoregulatory responses indicate a defence of the antipyretic levels of core temperature.*" Although there has evidently been some debate around whether the condition is regulated or not (abnormal), both definitions (Thermal-Commission, 1987; 2001) suggest that anapyrexia is pathological, the origin of which could be exogenous or endogenous. Adaptive cases of anapyrexia include hot flushes and alcohol intoxication (Cabanac, 2006; Thermal-Commission, 2001).

Anapyrexia has ultimately been defined around the same principles as fever but in reverse. Like fever, anapyrexia results from a change of set-point temperature, except that anapyrexia makes use of endogenous thermoregulatory control mechanisms that produce a downward displacement of hypothalamic set-point, thereby creating a defended decrease in T_{core} (Kozac, 1997; Branco *et al*., 2006). It is also described as pathological as in the case of fever. Anapyrexia has been put forward as the fifth major thermal state, the other four being fever, hyperthermia, hypothermia and normothermia (Kozac, 1997), but it has also been argued to be outdated, with one researcher debating whether the concept of anapyrexia exists at all (Romanovsky, 2004).

Kozac (1997) suggests that a common cause of anapyrexia via a Q_{10} effect to hypoxia. A reduction in T_b significantly reduces the body's need for oxygen (Branco *et al*., 2006). It is thought that this response to hypoxia is a regulated one and not due to insufficient oxygen supply for metabolism (Barros *et al*., 2001; Tattersall and Milsom, 2003), which supports the theory of Gordon (2001) that anapyrexia is a regulated response that results from the downward resetting of set-point.

The problem with the above is that although one could draw similarities between regulated hypothermia and anapyrexia, by definition anapyrexia is stated as a response under pathological condition whereas regulated hypothermia is not. As it stands, anapyrexia is technically defined as a different response from hypothermia (Thermal-Commission, 2001), unlike fever which has been accepted as a type of hyperthermia. Why this discrepancy of classification exists is unclear.

Torpor is an example of regulated hypothermia and is described as a temporary physiological state whereby the organism experiences a decrease in metabolic rate and a decrease in physical activity accompanied by a reduction in T_{core} (Hudson and Deavers, 1973; Hudson and Scott, 1979; Geiser and Ruf, 1995; Geiser, 2004). Torpor is similar to anapyrexia in that it is a controlled decrease of T_{core} due to the downward displacement of hypothalamic set-point (Wang and Wolowky, 1987; Barclay *et al*., 2001; Geiser, 2004) but torpor occurs more extensively. Core body temperature only needs to reach levels below the normothermic range to be classified as anapyrexia (Bicego *et al*., 2007). This makes the classification of anapyrexia dependent on the species-specific homeothermic range, an example of which is mice which have a range of 2-4^{o}C (Gordon, 2012a). T_{core} would only have to deviate outside of this range of the mouse to be classified as anapyrexic. Torpor is characteristically a much greater decrease in T_{core}, often great enough that minimum body temperature meets ambient temperatures (Hudson and Scott, 1979).

Torpor can be categorized into one of two classifications. One type of torpor is daily (heterothermic) torpor, bouts of which typically do not exceed 24 hours. During daily torpor, animals exhibit decreases in minimum T_{core} to between 10-30^{o}C (Geiser and Ruf, 1995). The other type of torpor is prolonged (hibernation) torpor, bouts of which

can extend from a number of days to a few weeks depending on the species. During prolonged torpor, T_{core} ranges between -3 to 16°C across 104 species being reported by Geiser and Ruf (1995). It is evident that there is a lot of interspecies variation which makes it difficult to determine where the state torpor begins and ends. There also appears to be some overlap between daily torpor and hibernation (Geiser and Ruf, 1995).

Confusion arises when researchers begin to interchangeably use the predefined terms, such as hypothermia and anapyrexia, or fever and hyperthermia. In some cases researchers have attempted to eliminate confusion by building onto these terms, as in "regulated hypothermia" and "regulated hyperthermia". By the definitions of other researchers, hypo- and hyperthermia are argued to be completely unregulated and uncontrolled responses (Stitt, 1979; Kozac, 1997; Fletcher *et al*., 2013).

The established "regulated" responses have thus been defined as anapyrexia and fever. The consequent problem with fever and anapyrexia is that they are defined as a regulated response, but they tend to lean towards a pathological origin and they don't allow for other conditions where the rise or fall in T_{core} is regulated but not pathological. Based on this reasoning, the definitions proposed by Gordon (1983; 2001) of regulated hypo- and hyperthermia seem appropriate. However, another problem then arises, in determining whether a condition is regulated or unregulated? At which point and under which circumstances can we classify a thermoregulatory state into one of these categories with confidence? These discrepancies seem to litter the thermoregulation literature.

1.2 The effect of ambient temperature on thermal balance

The ambient temperature at which an experimental test species is housed has a marked effect on both the physiology and behaviour of that species, influencing growth and development, metabolism, food and water intake, rest/activity state, cardiovascular function and immune function (Yamauchi *et al*., 1983; Gordon, 1993; Maloney *et al*., 2014).

As humans, it is characteristic of us to seek out temperatures that are most comfortable to us, most often within our thermoneutral zone (TNZ). The thermoneutral zone has been defined by the Glossary (Thermal-Commission, 2001) as "*The range of ambient temperatures at which physiological control of core body temperature is achieved by variations in sensible heat loss, that is without regulatory changes in metabolic heat production or evaporative heat loss*". The definitions of thermoneutral zone tend to vary, but in most cases they agree that it is the thermal zone in a homeothermic animal, which minimizes the metabolic energetic cost of maintenance of normal T_{core}. The lower critical temperature (LCT) and the upper critical temperature (UCT) delineate the lower and upper boundaries of the animal's TNZ, respectively. At temperatures lower than the LCT, heat production mechanisms are activated and at temperatures higher than the UCT, heat dissipating mechanisms are activated (Jessen, 2000).

For the most used strains of laboratory mice, the thermoneutral zone for single-housed mice is within the range of 27-31^{o}C (Herrington, 1940; Gordon, 1985; Gordon, 1993 Gordon, 2012), with most researchers settling at ~30^{o}C for the standard LCT. Mice will choose ambient temperatures that are concomitant with their metabolic TNZ (Gordon, 1983; Gordon, 1985; Gordon, 2012) and importantly, select temperatures

that are warmer than ambient temperatures typical of animal housing facilities (Gaskill *et al*., 2009; Gaskill *et al*., 2011). Even at the upper end of the animal housing temperature range (20-26^{o}C) set in the National Research Council Guideline (NRC, 2011), the mice are exposed to temperatures below their LCT, especially in the case of a single housed mouse.

Mice raised at typical animal housing temperatures (20-22^{o}C) have been found to be phenotypically different to those raised at their TNZ, with regards to their metabolism, thermal efficiency and have also displayed a decreased immune function (Yamauchi *et al*., 1983). A significantly increased metabolic rate (up to 50-60%) was observed in mice housed at standard housing temperatures when compared with their thermoneutrally housed counterparts (Gordon, 1985; Williams *et al*., 2002; Cannon and Nedergaard, 2011; Feldmann *et al*., 2009; Golozoubova *et al*., 2004) . In 1977 Besch and Woods defined the metabolic heat ratio (MHR) as the heat dissipation relative to basal metabolic rate (BMR) and measured this variable in rats held at room temperature (~24°C). Rats housed in typical animal facility conditions (~22-24°C) had a MHR that reflected a near doubling of the energy dissipated as heat when compared with those housed at thermoneutral temperatures.

Since differences in metabolic rate exist between mice housed in normal animal housing temperatures and mice housed at thermoneutrality, it is logical to infer that the thermal balance of the mice is different at these two conditions. Radiation, conduction and convection are dependent on the thermal gradient between a heat-emitting body and its ambient surroundings. If the ambient temperature is 22^{o}C, a mouse will easily lose heat to its environment. Whereas if a mouse is in its TNZ, the

gradient between the mouse's body and its environment becomes smaller and the drive for heat loss will therefore be reduced.

The TNZ and metabolic rate is also affected by the behavioural thermoregulation an animal initiates with the use of nesting material. Using nesting material an animal can control their microenvironment (Yang and Gordon, 1996). Gordon *et al*. (1998) found that the microenvironment of a cage had significant effects on heat loss. The effect of bedding, wood chips and a filter-top for the cage (provided more insulation than wire-top), markedly increased the operative T_a of the animal box from 19.2°C (bare acrylic cage) to 30.5°C, when housed at ~22°C. Nesting material has been found to affect food intake as well as body mass, with mice that had been provisioned with nesting material showing a lower food intake and a higher body mass (Dahlborn *et al*., 1996; Olsson and Dahlborn, 2002). Nesting material also yielded heavier litters and higher pup survival rates, and pushed forward the time of pup weaning (Olsson and Dahlborn, 2002), possibly due to its effect on the thermoregulatory ability of the offspring, which is limited up until weaning.

1.3 Circadian rhythm of body temperature and the Cosine waveform

There have been numerous methods proposed to assess variations in T_{core}, an animal's ability to thermoregulate and the combinations of heterothermic and homeothermic responses that an animal may utilise (Halberg *et al*., 1967; Nelson *et al*., 1979; McNab, 1983; Heldmaier and Ruf, 1992; Cooper and Geiser, 2008; Boyles *et al*., 2011). Body temperature and metabolic rate exhibit robust circadian rhythms. These rhythms are controlled by the suprachiasmatic nucleus (SCN) which is located in the anterior

hypothalamus (Borbély, 1982; Refinetti and Menaker, 1992). The SCN serves as the primary circadian pacemaker, receiving photic information from photosensitive retinal ganglion cells (Refinetti and Menaker, 1992; Meijer *et al*., 1998). This information is used to generate biological circadian rhythms that are characterized as oscillations that span a period of approximately 24 hours (Aschoff, 1979; Borbély, 1982; Refinetti and Menaker, 1992; Park and Tokura, 1998; Thermal-Commission, 2001; Refinetti, 2006).

The circadian rhythm of T_{core} is the variation of T_{core} across a 24-hour period and this variation is regulated (not forced) by means of physical activity and the sleep/wake cycle (Refinetti and Menaker, 1992). T_{core} increases during the waking hours with physical activity, and falls to its nadir during rest/sleep (Refinetti and Menaker, 1992; Leon *et al*., 2004). By measuring intraperitoneal temperature in rats, Briese (1998) found lower temperatures during resting periods (35.6-36^{o}C) and higher temperatures during active hours (37.8-38^{o}C), with the temperature exhibiting a circadian rhythm that approximated a cosine wave.

The amplitude of T_{core} is a reflection of the cyclic variation (peaks and troughs in the cosine waveform) in T_{core} around the mesor. In a homeothermic animal, this cyclic variation is said to be maintained within an arbitrarily defined species-specific limit, even in the instance of a much larger variation in T_a. Heterothermic responses describe any cyclic variability that may cause the amplitude of T_{core} to exceed this arbitrary range (Thermal-Commission, 2001; Refinetti, 2006). Refinetti (2006) highlights the difficulties of establishing an exact definition of the limits of homeothermy due to both inter-and intra-species variation. He does state however, that the acceptable range of variation must be included in the normal oscillation of the circadian rhythm of T_{core} for that species.

Under normal maintenance of body temperature in an endotherm, one can easily determine an average for T_{core} across a given period of time. However, T_{core} varies significantly across the 24 hour day, thus an average seems both unsuitable and inaccurate as it won't reflect this variation. During pregnancy, changes to T_{core} are an example of homeothermic thermoregulation and the circadian rhythm of T_{core} is cosinor-like in nature. As T_{core} is a biological time series and has circadian variation, a 'cosinor-rhythmometric' method is believed to be the most appropriate way to describe these data.

Cosinor-rhythmometry, as developed by Halberg *et al*. (1967) and reviewed by Nelson *et al*. (1979), defines the characteristics of a rhythm by fitting a mathematical formula to the data of a time series (in this case T_{core}) with a known period. The method uses a least-squares minimization approach to fit a cosine waveform to the data, showing the distribution of low and high temperatures (Halberg *et al*., 1967; Nelson *et al*., 1979; Refinetti and Menaker, 1992). The defining characteristics of this cosine wave are the mesor (mean), the amplitude (the height of the wave from the mesor), and the acrophase (the time of day at which the peak of the wave occurs) (Halberg *et al*., 1967; Nelson *et al*., 1979). By summarizing temperature data in this way, these characteristics can be used to compare the effects of an experimental treatment on the circadian pattern of T_{core}, or to describe the natural variation on T_{core}.

1.3.1 Circadian rhythm during pregnancy

During mammalian gestation, mothers experience substantial weight gain due to the growth of the foetal mass and the placenta(s). Aschoff (1982) showed that as the body mass of a non-pregnant organism increases, so its ability to store and dissipate heat

changes. One could infer that the same principles apply in the pregnant mammalian female, especially since the mother has to absorb the heat generated by the foetus in her uterus (Abrams *et al*., 1969; Schröder *et al*., 1988; Laburn *et al*., 1992; Schröder and Power, 1997; Faurie *et al*., 2001), essentially serving as a sink, into which the foetus dumps its metabolic heat. The majority of the mass gained through foetal growth occurs during the second half of pregnancy (Ibsen, 1928). Since the T_{core} of mice exhibits a circadian rhythm (Borbély, 1982; Refinetti and Menaker, 1992), it is logical to hypothesize that a change in body mass during gestation may change the mothers ability to store and dissipate heat, which in turn could affect the variation of circadian rhythm of T_{core}.

The daily oscillation of T_{core} decreases in amplitude with an increase in body mass in non-pregnant animals (Aschoff, 1979; Briese, 1998; Mortola and Lanthier, 2004). Since there is an increase in maternal body mass, this decrease in amplitude could also be the seen during pregnancy. It has been shown that rats (Kitrell and Satinoff, 1988) and hamsters (Scribner and Wynne-Edwards, 1994) exhibit a decrease in the amplitude of T_{core} towards the end of gestation. Body mass and thermal conductance have an inversely proportional allometric relationship, and as body mass increases, the thermal conductance of an animal decreases because of a reduced surface area-to-mass ratio (Hayssen and Lacy, 1985). A larger body is therefore able to regulate their T_{core} easier under normothermic ambient conditions, thus there is less fluctuation in T_{core} and this will be reflected in the variability of the cyclic variation, *i.e.* amplitude.

1.4 Thermoregulation and physiological adaptation during gestation

During pregnancy various aspects of the maternal physiology undergo a series of changes, particularly the cardiovascular and respiratory systems, as well as the female's thermoregulatory control (Fewell, 1995; Eliason and Fewell, 1997; Fewell and Tang, 1997), and a whole spectrum of changes to hormonal balance (Czaja and Butera, 1986).

Eliason and Fewell (1997) used a thermocline to investigate the preferred T_a range during pregnancy and lactation in rats. They showed that the preferred T_a (24-25°C), oxygen consumption and thermal conductance did not change across the gestation period and was not different from that of non-pregnant rats. However, during lactation, rats preferred lower ambient temperatures (~14°C) and their oxygen consumption and T_{core} increased.

1.4.1 Heat exchange between mother and foetus

During pregnancy there is an increase in the metabolic rate of the mother as energy demands increase. Heat is also generated *in utero* from metabolic processes of the developing foetus(es) (Gilbert *et al*., 1985; Schröder and Power, 1997; Edwards, 2006). In pregnant sheep, toward the end of gestation, as much as 15% of the total heat production originated from the foetus (Power *et al*., 1984). The oxygen consumption, and therefore heat production, of the foetus per kilogram is 1.5 times that of the mother (Asakura *et al*., 1990), and increases to almost twice the heat produced per kilogram near parturition (Bell *et al*., 1985).

The temperature gradient established between the body and its environment is a function of heat produced by a body per unit time and the conductance of the insulating shell of that body (Power *et al*., 1984). For a body to maintain a stable temperature, the amount of heat it generates must be equal to the amount of heat dissipated to the environment (Gilbert *et al*., 1985).

In the case of the gestating foetus, the foetus is the heat producing body and the mother is the environment. Before the foetus is born, it is not capable of regulating its own T_b (Asakura, 2004). This means the foetus is solely dependent on the mother for heat dissipation and temperature stability (Abrams *et al*., 1969; Schröder *et al*., 1988; Laburn *et al*., 1992, Faurie *et al*., 2001).

Heat moves from the foetus by two pathways; via convection through feto-maternal exchange in the placental circulation (approximately 84.5 ± 2.5%) or via conductance pathways through the foetal skin, across the amniotic and allantoic fluids and through the uterine wall (approximately 15.5 ± 2.5%) (Hart and Faber, 1965; Gilbert *et al*., 1985; Schröder *et al*., 1988; Edwards, 2006). Because the foetus dissipates the majority of its metabolic heat via the placental blood supply, any changes in the supply of blood will affect the rate at which the foetus dissipates heat (Schröder and Power, 1997; Laburn *et al*., 2002). The blood supply of both the mother and the foetus have such a close association that the foetus is thermally clamped to the mother, thus foetal temperatures should follow maternal temperatures very closely (Schröder *et al*., 1988). Foetal T_{core} of lambs followed in parallel with that of the mother, even with the peaks and troughs over the circadian cycle (Laburn *et al*., 1992; Laburn *et al*., 1994; Laburn *et al*., 2002).

For the two methods of heat transfer depicted above to occur, a feto-maternal temperature gradient must be established with the foetal temperature higher than that of the mother. In all species studied to date (including humans), during the late stages of pregnancy, foetal T_{core} was on average 0.5-0.6^{o}C higher than that of the mother (Laburn *et al.*, 1992; Laburn *et al*., 2002; Laburn *et al*., 2003; Faurie *et al*., 2004). Power *et al.* (1984) found a difference of 0.54^{o}C between the mother and foetus in sheep, whereas Hart and Faber (1965) and Abrams *et al.* (1969) found that foetal brain temperatures were 0.4-0.8^{o}C warmer than maternal aortic blood temperatures. Schröder and Power (1997) reported a range of 0.5-1^{o}C between the mother and the foetus after reviewing numerous studies.

Once this feto-maternal gradient is established, it remains fairly constant, with studies in lambs and goats indicating virtually no change in the last 5 weeks of pregnancy, regardless of the exponential increase in foetal mass (Laburn *et al*., 1992; Faurie *et al*., 2001). However, when pregnant ewes were exposed to mild (Laburn *et al*., 1992) and more severe (Laburn *et al*., 2002) heat (including exercise and environmental hyperthermia) or cold stress, the feto-maternal gradient changed in an adaptive manner. During heat stress, foetal T_{core} increases follow that of the mother but at a slower rate, leading to a decrease in the feto-maternal gradient. The same, but opposite effect was seen during cold exposure, where the feto-maternal gradient doubled (Laburn *et al*., 2002).

Any change in the uteroplacental blood supply will alter how much heat is lost or maintained in the foetal body. An increase in uterine blood flow when the mother is in hot climates will increase heat loss by the foetus, and a decrease in uterine blood flow when the mother is in cold climates will decrease heat loss by the foetus. Placental

blood flow increased by 50% in heat stressed rabbits (Lublin and Wolfenson, 1996) and it has been postulated that mechanisms exist that alter placental blood flow in response to thermal stress (Laburn *et al*., 1992; Laburn, 1996). Foetal protection by means of alteration in blood flow doesn't come without consequences, as an increase in blood flow to the uterus potentially diverts that blood from other important tissues in the mother, whereas a decrease in blood flow could jeopardise the supply of nutrients and oxygen to the foetus (Laburn *et al*., 2002).

1.4.2 Decrease in maternal Tcore during late gestation

There is mounting literature to show that maternal T_{core} decreases during pregnancy, reaching its lowest point just before parturition. This change in maternal T_{core} (designated ΔT for later purposes of this study) gradually develops across gestation and occurs in conjunction with the growth and development of the foetus. We know that the foetus is increasingly generating metabolic heat as it grows (Power *et al*., 1984; Bell *et al*., 1985; Gilbert *et al*., 1985; Schröder *et al*., 1988; Schröder and Power, 1997; Edwards, 2006), and it is possible that this adjustment to maternal T_{core} could be related to the process of foetal heat dissipation, that is, if the foetal temperature is the variable that is being regulated.

At the time of birth and within a matter of hours post-parturition, there is a rapid increase in maternal T_{core}. This pattern (a decrease across gestation and an increase post parturition) has been reported in rats (Kitrell and Satinoff, 1988; Fewell, 1995; Eliason and Fewell, 1997; Fewell and Tang, 1997; Cairns *et al*., 2004) ewes (Laburn *et al*., 1992; Laburn *et al*., 1994), goats (Faurie *et al*., 2001), dogs (Concannon and Hansel,

1977; Hoffmann *et al*., 1994) arctic ground squirrels (Williams *et al*., 2011), rabbits (Naccarato and Hunter, 1983), and humans (Lindqvist *et al*., 2003).

Fewell (1995) showed that the gestational decrease in T_{core} occurred during both the light and dark phases in Sprague-Dawley rats, suggesting that ΔT is incorporated into the natural circadian rhythm of T_{core}.

Probably the most pivotal piece of literature on this subject is that of Eliason and Fewell (1997) who found that the reversible decrease in T_{core} is a regulated response, which they determined by studying the preferred T_a of pregnant and non-pregnant rats in a thermocline. They found that, despite the decrease in maternal T_{core} across gestation, pregnant mice did not select different T_a to non-pregnant mice at any stage of pregnancy. This lack of use of thermotaxis against the change in T_{core}, indicates that the gestational decrease in T_{core} is a regulated response. Eliason and Fewell (1997) also found that the preferred T_a in lactating mice was much lower than in non-pregnant or pregnant mice, which indicates that the increase in T_{core} during lactation is an uncontrolled response, since the mice attempt to defend against the increase using thermotaxis.

Due to their findings, Eliason and Fewell (1997) decided to term the decrease in T_{core} during gestation a form of “regulated hypothermia”. Prior to these findings, the only examples of a regulated hypothermia were torpor and hibernation (Thermal-Commission, 2001), but if we consider the definition of hypothermia to be the scenario when T_{core} is decreased to a temperature that is below the normothermic, active-state range of a species, then the term proposed by Eliason and Fewell (1997) is quite well suited. This begs the question, is this decrease in T_{core} really below the normothermic

range of a pregnant female, or is the normothermic range temporarily redefined during gestation? Essentially, is maternal thermoregulation an example of normal homeothermy? Perhaps, when the mammalian female in question is pregnant, that decrease in T_{core} is included in a newly defined "gestational normothermic range", in which case, it wouldn't be an example of hypothermia at all. Something else to consider in this matter is that Eliason and Fewell (1997) found there was a downward shift and widening of the TNZ of rats near term of pregnancy, and that the TNZ of the lactating rat did not differ from that of a non-pregnant rat.

To bring together everything that is currently understood, we know that there exists both an internal thermal gradient between the mother and her foetus(es), and an external thermal gradient between the mother and her surrounding environment. These two gradients appear to exist independently of each other, but both need to be simultaneously maintained for the mother to regulate her own thermal balance. If foetal temperature is the regulated variable during gestation, hence it is the variable controlling the decrease in maternal T_{core} (by whichever hypothesized mechanism), then the process of heat dissipation from the mother to the environment should be independent of the heat being dissipated *in utero* by the foetus.

The T_a at which the mother is kept will influence her ability to dissipate heat to the environment. At lower ambient temperatures, the thermal gradient established between the heat producing mother and her environment is larger. A larger gradient allows the animal to offload heat to her surroundings more effectively. By altering thermal balance, potentially the extent to which ΔT occurs could be affected.

To summarise, there was a need to review the thermal physiology nomenclature because this study provoked debate around what to call the decrease in maternal T_{core} that occurs during late pregnancy. The available terminology options, as characterized by the decrease in maternal T_{core} were anapyrexia, torpor or hypothermia. It has already been defined as a form of anapyrexia by Kozak (1997).

By no stretch of the definition of torpor could the gestational decrease in T_{core} be defined as a state of torpor, since minimal gestational temperature does not reach even that of daily torpid temperatures (10-30^{o}C) (Hudson and Scott, 1979; Geiser and Ruf, 1995). Nor is there a substantial decrease in metabolic rate, as seen during torpor.

A third possibility comes from Eliason (1997) who has termed the gestational pattern in T_{core} a "regulated hypothermia". Since anapyrexia is not technically defined as a type of hypothermia (they are deemed separate concepts in the Glossary), anapyrexia and regulated hypothermia are then not the same thing and should not be used interchangeably. By its accepted definition, anapyrexia results from pathology. Regulated hypothermia therefore seems to be the best suited concept, as the gestational decrease in T_{core} does not appear to be pathological.

For the purpose of this study, the term "regulated hypothermia" will be particularised with reference to gestation, to call the response *gestational hypothermia*. Although hypothermia does, by definition, indicates that body temperature has decreased below the normothermic range of a species (Thermal-Commission, 2001), and it cannot be said with confidence what is defined as "normothermic" in a pregnant animal, out of the available terminology, "gestational hypothermia" still seems to be the most apposite term.

1.4.3 Theories on why gestational hypothermia occurs

i) Changes to ovarian hormones during pregnancy

Fewell (1995) suggested that the overall changes in T_{core} across gestation could be correlated with ovarian hormone adjustments. Ovarian hormones have been reported to affect T_{core} in several species including rats, mice, dogs, chimps, rabbits and humans (Grota and Eik-Nes, 1967). A circulating hormone of particular interest is progesterone, which has a known thermogenic effect as a result of increased thyroid activity in rats (Freeman *et al*., 1970). Increased plasma progesterone during and after the luteal phase, causes a rise in T_{core} (Ash and Heap, 1975), and if the animal successfully conceives, progesterone levels continue to increase steadily into the last trimester. This trend has been reported in ewes (Bassett *et al*., 1969), rats (Grota and Eik-Nes, 1967; Pepe and Rothchild, 1974), pigs (Ash and Heap, 1975) and dogs (Hoffmann *et al*., 1994), with all of these studies reporting an apparent decline in plasma progesterone in the final days leading up to parturition, spanning from two days in the rat to a week in the ewe.

The cessation of progesterone production allows the mother to give birth to the foetus, via the increase in other ovarian hormones, namely oxytocin and luteinizing hormone (Grota and Eik-Nes, 1967). Grota and Eik-Nes (1967) showed that by administering progesterone during this phase of plasma progesterone decline, the length of pregnancy was extended. This prepartum decrease in plasma progesterone levels has been linked to a decrease in T_{core} during gestation (Hoffmann *et al*., 1994).

ii) Teratogenic effects of hyperthermia

Gestational hypothermia has been suggested to serve a protective function with regards to the teratogenic effects of hyperthermia during pregnancy (Fewell, 1995). Regardless of how the hyperthermia comes about (whether by fever or environmental stress), an increase in T_{core} during gestation has been shown to have teratogenic effects on the foetus.

The duration, timing and extent of the hyperthermia dictates the severity and type of foetal complications (Edwards, 1969; Edwards, 1986; Shiota, 1988; Gericke *et al*., 1989; Dreiling and Carman, 1991; Edwards *et al*., 1995). Longer duration or higher temperatures result in embryonic death and abortions, whereas shorter durations and lower temperatures result in foetal resorptions and anomalies from hindered embryogenesis (Shiota, 1988; Schröder and Power, 1997; Graham *et al*., 1998, Asakura, 2004). Defects of the central nervous system (CNS) are the common result of gestational hyperthermia, often due to cell death or delay in the proliferation of somatic cells and neuroblasts (Edwards, 1969; Fisher and Smith, 1981; Edwards *et al*., 1995). If exposure occurs in any dose during the pre-implantation phase, embryonic death is likely, and if the exposure occurs during formation of the neural tube, neural tube defects are a consequence (Graham *et al*., 1998).

Despite the fact that the mother acts as a heat sink for the foetus during normal healthy pregnancy and that through this feto-maternal pathway the foetus in thermally protected, there are extreme circumstances where foetal temperature can be jeopardised. If the mother becomes heat stressed, either by environmental heat, heavy exercise or febrile infection, the foetus is put at risk (Edwards, 2006).

If maternal body temperature rises slowly, often the feto-maternal gradient can be maintained (Schröder and Power, 1994). However if the maternal body temperature rises rapidly above that of the foetus, the foetus would not be able to dissipate heat effectively to the mother and would experience a teratogenic rise in body temperature.

The teratogenic threshold range is reported to be ~ 1.5-2°C increase in maternal body temperature (Bell *et al*., 1985; Graham *et al*., 1998; Edwards, 2006). A feto-maternal thermal gradient of ~0.5-1°C is established during mid-late pregnancy and foetal temperatures have been reported to follow maternal temperatures closely (Power *et al*., 1984; Laburn *et al*., 1992; Laburn et al., 1994; Schröder and Power, 1997; Faurie *et al*., 2001). However, when under heat/cold stress, the foetal temperature will rise or fall, respectively, slower than that of the mother, altering the gradient to be smaller during heat stress and larger during cold stress (Laburn *et al*., 2002).

If the threshold for teratogenicity is a 1.5-2°C increase in maternal body temperature, the teratogenic threshold for the foetus will be dependent on a number of factors. These factors include the extent of the change in maternal temperature, the rate of change of maternal temperature, and the stage of pregnancy.

The stage of pregnancy at which hyperthermia occurs is important because there are stages at which the foetus is more susceptible, particularly during the first and third trimesters.

Interestingly, gestation has unique effects on the febrile response during the late pregnancy. Fever serves a purpose in survival from endotoxic insult by elevating body temperature in response in increase concentrations of prostaglandin E_2 (Aronoff and Neilson, 2001). It has been reported in sheep (Kasting *et al*., 1978), guinea pigs (Merker

et al., 1980) and rats (Martin *et al*., 1995), that in the few days leading up to parturition and the few hours postpartum the mother fails to develop a fever in response to a dose of endotoxin that would normally have a pyretic effect.

It could be possible that some form of trade off occurs in the late gestating animal exposed to endotoxic threat, in that the risk to the foetus caused by the rise in body temperature is not worth the protective result that fever provides in the first place.

Arginine vasopressin (AVP - also known as antidiuretic hormone) is an endogenous antipyretic hormone in the CNS and has been found to be elevated in the plasma of rats near the end of gestation and into lactation (Landgraf *et al*., 1991; Pittman *et al*., 1998). It is possible that AVP could explain the lack of pyretic response in late pregnant animals (Naylor *et al*., 1988; Pittman and Wilkinson, 1992; Pittman *et al*., 1998).

Fewell and Tang (1997) also reported that pregnant rats did not develop stress-induced hyperthermia in response to an open field experiment. They hypothesized that if the mothers had developed this induced hyperthermia, uterine and placental blood flow would be reduced by diverting blood flow to the thermoeffector organs in the mother, causing foetal hypoxia (Oakes *et al*., 1976). For the same reasons, this redirection of blood flow could play a role in why late parturient mothers do not develop fever when dosed with endotoxin.

iii) Hypoxia during parturition

Gestational hypothermia has been suggested to serve a protective function in potential foetal hypoxia (Wood, 1991; Fewell, 1995; Kozac, 1997). Hypoxia has an effect on the thermoregulation of an organism, resulting in a reduction of T_{core} (Barros *et al*., 2001; Tattersall and Milsom, 2003; Branco *et al*., 2006) by decreasing metabolic

heat production (reducing O_2 demands) and increasing sympathetic nervous system (SNS) activation of vasomotor action for heat loss mechanisms (Gordon, 1993).

Non-pregnant animals that are hypoxic select ambient temperatures in a thermocline that are lower than those selected under normoxic conditions, which is suggestive of a decrease in the thermoregulatory set-point (Gordon and Fogelson, 1991).

During gestation there is an increase in the energy demand of the feto-maternal unit, particularly towards the end of the pregnancy, when *in-utero* foetal growth is at its maximum and the rate of metabolic processes in the foetus is at its highest. This increased foetal energy demand requires high levels of O_2. Wood (1991), Fewell (1995) and Kozac (1997) all discuss the possibility that hypothermia during pregnancy could serve as a protective function against hypoxia by reducing foetal oxygen requirements. The Q_{10} effect describes a process whereby a decrease in body temperature of 1°C, reduces O_2 consumption by 11% (Wood, 1991; Schmidt-Nielsen, 1997). Fewell (1995) also suggested that hypothermia may play a role in counteracting the effects of parturition induced maternal hyperthermia which could potentially cause neuronal injury via asphyxia.

The question posed is this; does body temperature decrease in response to an increase in O_2 demand? Or is the decrease pre-emptive, serving as a protective measure against the development of hypoxia during parturition?

iv) Impairment of nonshivering thermogenesis in brown adipose tissue

Gestational hypothermia has been hypothesized to occur due to the deactivation of brown adipose tissue (BAT) during gestation (Villarroya *et al*., 1986; Trayhurn, 1989;

Speakman, 2008). Brown adipose tissue generates heat by increasing the metabolic rate after the ingestion of food, converting the chemical energy from food into heat (Foster and Frydman, 1979; Foster, 1986; Cannon and Nedergaard, 2004). BAT is a well vascularized tissue with high mitochondrial content. This autonomic thermoeffector organ is under control of the SNS, via noradrenaline interaction with β-adrenergic receptors and is essential for what is known as nonshivering thermogenesis (NST) (Foster and Frydman, 1979; Ricquier and Mory, 1984; Himms-Hagen, 1984; Cannon and Nedergaard, 2004). Activation of BAT occurs in cold exposed animals (cold-induced thermogenesis) and its heat production helps to maintain body temperature (Himms-Hagen, 1984). This tissue can also be activated during fever, arousal from hibernation, in neonates that are unable to adequately thermoregulate by shivering, and is particularly important in thermoregulation in small rodents.

Under normal activation of BAT in non-pregnant rats, extra energy obtained during periods of hyperphagia (overeating, dietary-induced thermogenesis) is dissipated as heat by increasing the metabolic rate (Rothwell and Stock, 1983). Hyperphagia would usually activate NST, but during gestation this is not the case. The opposite is seen to true, with hyperphagia peaking towards the end of gestation, occurring simultaneously with a decline in thermogenic activity of BAT (Rothwell and Stock, 1983; Trayhurn and Richard, 1985; Abalenda and Puerta, 1987).

The thermogenic activity of BAT is reduced during late gestation and lactation in rats, mice (Trayhurn, 1983; Trayhurn and Richard, 1985; Andrews *et al*., 1986; Speakman, 2008), squirrels (Nizielski *et al*., 1993), and hamsters (Wade *et al*., 1986; Trayhurn, 1989) even when fed *ad libitum*.

Major atrophy of BAT occurs during pregnancy, with interscapular BAT being substantially reduced (59% by day 15 of gestation Wade *et al.,* 1986), particularly towards the very late stages of gestation, just before parturition and into lactation (Andrews *et al*., 1986; Wade *et al*., 1986; Trayhurn, 1989). The protein content, tissue mass and mitochondrial mass were also reduced during late gestation, but especially during lactation (Wade *et al*., 1986; Villarroya *et al*., 1986).

Uncoupling-protein UCP1 (thermogenin) is of paramount importance in noradrenaline-induced thermogenesis (Cannon and Nedergaard, 2004). This protein is located in the inner mitochondrial membrane and uncouples the respiratory chain from ATP synthesis (oxidation from phosphorylation), yielding a low rate of ATP production, instead dissipating energy as heat (Nedergaard *et al*., 2001; Golozoubova *et al.*, 2004). Andrews *et al*. (1986), Villarroya *et al*. (1986) and Trayhurn (1989) all conducted studies that investigated mitochondrial GDP binding, which provides a good indication of proton conductance activity in the mitochondria and hence BAT activity. All three studies found GDP binding significantly decreased towards the end of gestation and into lactation. It is possible that NST in BAT is impaired during late pregnancy and lactation, is because although food intake is at highest, the energy obtained during these periods is required for the production of milk.

Trayhurn (1989) proposed that a reduction in BAT activity could be an energy saving mechanism to maximise milk production. Williams *et al.* (2011) suggested from their data in free-living arctic ground squirrels that the energy saving properties could be beneficial to animals in cold climates. Speakman (2008) argued this point, suggesting that it is not the energy saving property that increases milk production, but rather an increased ability of the mother to dissipated heat when BAT is deactivated. A reduced

heat burden on the mother would allow her to transfer her energy more efficiently, through her milk to her pups.

Sawa *et al.* (1991) suggested that the thermogenic activity of BAT is reduced towards the end of pregnancy due to the increased pre-birth circulatory levels of an inhibitor of nonshivering thermogenesis, the proposed inhibitor being adenosine. Adenosine has been found to be released by the placenta and inhibits brown fat lipolysis (Szillat and Bukowiecki, 1983; Iliou and Demarne, 1987; Ball *et al*., 1995), with foetal adenosine levels reaching four times higher than maternal adenosine levels in sheep (Sawa *et al*., 1991). Elevated concentrations of plasma adenosine have also been found in the maternal circulation, with pregnant women having 3.3-fold increase over that of non-pregnant women (Yoneyama *et al*., 2000). These findings suggest that adenosine could play a role in the inhibition of brown fat lipolysis that occurs both in the mother and the foetus, and could be linked to any gestational related hypothermia.

v) Angiotensin signalling pathway

It has been hypothesized that a specific thermoregulatory cooling pathway that utilizes ANGII as a signalling molecule could be the cause of gestational hypothermia (Cairns *et al*., 2004). Cairns *et al*. (2004) outlines the signalling pathway that supports that ΔT is a regulated mechanism and that the increase in T_{core} during lactation is forced. Rats given candesartan, an angiotensin type 1 receptor (AT_1) receptor antagonist, did not exhibit the expected ΔT during gestation, but seemingly still exhibited the same increase in T_{core} during lactation. Cairns *et al*. (2004) suggested that the reason the T_{core} of the candesartan-treated rats did not decrease during gestation, but still increased

during lactation could indicate that a specific intrinsic mechanism usually activated during gestation, may be impaired or inhibited. This impairment occurs when angiotensin action in the brain is blocked. Circulating angiotensin II (ANGII) levels increase during gestation (Sinnayah *et al*., 1999) and ANGII has been hypothesized to play a role in the homeostasis of body fluids and water drinking, acting as a signalling molecule in the brain during thermoregulation (McKinley *et al*., 2003).

The review of the literature it is evident that gestational hypothermia does occur in several mammalian species. Whether it occurs in mice is still unconfirmed. We believe that the input signal for gestational hypothermia is originating from the foetus, but there are various other mechanisms and signalling pathways that could potentially be activating or playing a role in gestational hypothermia.

CHAPTER 2 – INTRODUCTION

Ambient temperature affects the thermal balance of an animal by changing the rate of heat dissipation to the environment via convection, conduction, and radiation. This heat dissipation is dependent on the temperature gradient that exists between the animal and its surroundings. In hotter environments, the gradient is smaller, which reduces the animal's ability to dissipate heat, and when heat balance cannot be achieved, T_{core} will increase (Strunk, 1971; Pharo Gagge and Gonzalez, 2011). The opposite happens in colder environments, increasing the temperature gradient and the rate at which the animal dissipates heat.

When an animal is pregnant, the mother serves as the "environment" to the foetus, and these same rules of heat dissipation apply between mother and foetus.

As gestation progresses, heat is generated *in utero* from the metabolic processes of the developing foetus (Gilbert *et al*., 1985; Schröder and Power, 1997; Edwards, 2006). The foetus is solely dependent on the mother for the process of metabolic heat dissipation. Heat moves from the foetus to the mother by means of two pathways; 1) Via convection through feto maternal exchange in the placental circulation (~85%) or 2) via conductance pathways through the foetal skin, across the amniotic fluid and through the uterine wall (~15%) (Hart and Faber, 1965; Gilbert *et al*., 1985; Schröder and Power, 1997; Edwards, 2006).

There appears to be some benefit to keeping foetal temperature constant throughout gestation. If foetal temperature is constant then the rate at which the foetus dissipates metabolic heat should be proportional to the rate at which it is producing that heat.

The thermal gradient between the mother and her foetus should comply with this need, and as gestation progresses and foetal mass increases the mother's T_{core} should decrease accordingly to suit those thermal gradient requirements. This decrease in maternal T_{core} has been termed gestational hypothermia and has been reported in rats (Kitrell and Satinoff, 1988; Fewell, 1995; Eliason and Fewell, 1997; Fewell and Tang, 1997; Cairns *et al*., 2004), ewes (Laburn *et al*., 1992; Laburn *et al*., 1994), goats (Faurie *et al*., 2001), dogs (Concannon and Hansel, 1977; Hoffmann *et al*., 1994), arctic ground squirrels (Williams *et al*., 2011), rabbits (Naccarato and Hunter, 1983), and humans (Lindqvist *et al*., 2003). At the time of birth and within a matter of hours post-parturition, there is a rapid increase in maternal T_{core}. Eliason and Fewell (1997) studied the environmental temperature preference of pregnant and non-pregnant rats in a thermocline and concluded that gestational hypothermia is a regulated response. They found that there was no difference in the temperature preferences between pregnant and non-pregnant rats, indicating that ΔT was not as a result of the inability of the animal to regulate their T_{core}, but was rather a controlled response. During the hyperthermia associated with birth and lactation, rats chose considerably cooler ambient temperatures, indicating that the rapid rise in T_{core} after birth is forced and the animals were behaviourally thermoregulating to counteract it (Eliason and Fewell, 1997).

Why gestational hypothermia occurs remains elusive, but there are a few different hypotheses as to what causes the decrease in maternal T_{core}. Suggestions include the following theories; 1) gestational hypothermia occurs due to changes in ovarian hormones (Grota and Eik-Nes, 1967; Ash and Heap, 1975), 2) gestational hypothermia serves to protect the foetus from teratogenic effects of maternal hyperthermia (that

can occur either by an increase in T_a that overwhelms thermoeffectors, by heavy exercise or by maternal fever) or from foetal hyperthermia caused by compromising placental blood flow (Fewell, 1995), 3) gestational hypothermia occurs as a result of foetal hypoxia or in contrast as a protective function to reduce oxygen utilisation (by Q_{10} effect) and thereby prevent hypoxia (Wood, 1991; Fewell, 1995; Kozak, 1997), 4) gestational hypothermia occurs as a result of the inactivation and atrophy of brown adipose tissue (Villarroya *et al*., 1986; Trayhurn, 1989; Speakman, 2008), or 5) gestational hypothermia occurs as a result of an ANGII signalling pathway (Cairns *et al*., 2004).

CHAPTER 3 – AIMS AND HYPOTHESES

The aims of this experiment were:

1. To determine if gestational hypothermia occurs in mice
2. To determine if maternal mass, maternal food intake, foetal mass, and pup and litter growth rates differ between thermoneutral and sub thermoneutral temperatures (29°C and 22°C)
3. To determine whether the magnitude of gestational hypothermia (ΔT) differs between thermoneutral and sub thermoneutral temperatures (29°C and 22°C)
4. To determine if the foetal mass at the time of parturition (and by inference, the amount of heat produced by the foetal mass), is proportional to the magnitude of the gestational hypothermia that occurs during gestation (ΔT)

It was predicted that mice would exhibit gestational hypothermia.

It was also predicted that the mice kept at thermoneutrality (29°C) would have a different thermal balance to those kept below thermoneutrality (22°C), and hence have differing ability to dissipate heat. Presumably mothers would be able to dissipate less heat at their TNZ. This difference in heat dissipation influence maternal metabolic rate which might be reflected in maternal mass and food intake. In turn litter mass and pup growth rates after parturition could also differ between the two temperature groups for similar reasons.

Lastly it was predicted that the extent of the gestational hypothermia would be proportional to foetal mass, because in theory, litters with a larger foetal mass at the

end of gestation would generate more intra-uterine metabolic heat. That heat would need to be dissipated via the mother. Thus maternal T_{core} was expected to decrease to a greater extent in mothers with heavier litters.

CHAPTER 4 - MATERIALS AND METHODS

4.1 Animals

The experiments detailed below were approved by the Animal Ethics Committee of the University of Western Australia (RA/3/100/1218). Mice (ARC-Swiss) were obtained through the Animal Resources Centre (ARC, Canningvale, Western Australia, AU) and housed at the Pre-Clinical Facility (PCF) at the University of Western Australia. Thirty virgin females were used in two cohorts of 15 mice each. The mice initially weighed 28.3 ± 0.7 g (mean ± SEM). The mice were given an acclimation period of 7 days in the facility before the surgery and the experiments were initiated. The mice were housed individually, under a 12hr-light/12hr-dark cycle. Each mouse box had shaven aspen bedding, a toilet roll, a red Perspex house and shredded tissues. The mice were fed standard mouse chow (Specialty Feeds, Glen Forrest Stockfeeders, Glen Forrest, Western Australia, AU) and acidified water (between pH 2 and 3, to prevent bacterial infection) (Tanner and James, 1992) *ad libitum.*

4.2 Surgical procedures

The mice underwent a recovery surgical procedure for the implantation of temperature loggers. Each animal was anaesthetized via inhalation using Isoflurane (Attane, Provet WA, Bayer, Auckland, NZ) infused with oxygen. Induction was performed with the animal inside an airtight Perspex chamber filled with 4% isoflurane until the mouse lost its righting reflex. The mouse was then removed from the box and its head placed into a mask, through which inhalation of the Isoflurane continued for

the remainder of the surgery, at 1-2%, titrated to maintain a surgical level of anaesthesia as assessed by absence of withdrawal to toe pinch.

The animal's abdominal fur was clipped with an electric razor and scrub-sterilized twice to ensure aseptic conditions; first with povidine-iodine surgical scrub (Betadine®, Faulding Pharmaceuticals, Salisbury South, South Australia, AU) and then with a Chlorhexidene solution (VR ® Chlorhex C, Jurox Pty Ltd, Rutherford, NSW, AU). The animal was then covered with a fenestrated sterile drape. A midline incision, approximately 5-7 mm in length, was made and a precalibated, sterile, free-floating telemetry logger (see below) was implanted into the peritoneal cavity for the measurement of T_{core}.

Vicryl©, an absorbable, polyglactin suture of strand size 5-0 (Ethicon Endo Surgery, Inc. Ohio, USA) was used to close the incision. Two separate layers of sutures were used to close separately, the muscle layer and then the skin layer. Prior to the last stitch the isoflurane flow was stopped and the animal remained on 100% oxygen as it began to recover from the anaesthetic, which usually took 2-3 minutes. During this time, a single dose of analgesic, Buprenorphine, 0.01 mg/kg (Temgesic® Injection, Reckitt Benckiser, Slough, Berkshire) and a single dose of anti-inflammatory, Carprofen 5 mg/kg (Carpreive Injection, Norbrook Laboratories Pty Ltd, Australia) was administered by subcutaneous injection.

Initial recovery was monitored for 2-3 hours post-surgery, to assure that the sutures remained intact, and then every 3 hours for the remainder of that night. Thereafter the mice were observed and weighed twice daily for any indicators of pain (such as unkempt coat condition, hunched body posture, nose and eye condition, irritation of

the surgical site and lethargy) for one week. A second dose of Buprenorphine (0.01 mg/kg) was provided to animals exhibiting indication of pain during this recovery period. While they were recovering the mice were housed in individual animal boxes in a warm thermoneutral environment (~30°C) (Gordon, 1993). During recovery 4 mice were euthanased according to the approved ethics protocol as a result of post-operative complications.

4.3 Telemetry Thermologgers and Instrument Calibrations

Nano Loggers (Star Oddi, Iceland) were used to record T_{core} in each mouse. The loggers were approximately 5 mm in circumference and 15 mm in length, with a volume of 0.3 ml and mass of 1.3 g. The loggers were less than 5% of the average body weight of an adult, female mouse, which has been deemed safe for implantation in mice (Baumans *et al*., 2001; Leon *et al.*, 2004; Gaskill *et al*., 2013).

For the first cohort of mice (n=15) sampling intervals of the loggers were set at 15 minutes, which provided 54 days of logging before the memory capacity was exceeded. Insufficient time had been allowed for unsuccessful matings that required re-matings, and memory capacity was exceeded by the time of birth in all but four of the mice in this first cohort. Those four mice had been successfully fertilized at the first mating. For the second cohort of mice (n=15) a 60 minute sampling interval was used which provided us with 218 days of memory. From the original two cohorts of 15 animals each, memory capacity was reached before birth in eight mice, batteries failed in three of the loggers and post-operative complications resulted in the euthanasia of

four animals (two in each cohort), resulting in a final sample size for T_{core} data of 15 mice.

The loggers were calibrated in a water bath at a series of controlled temperatures (from 30°C to 42°C, 3°C intervals, for at least 6 readings at each temperature) against a mercury-in-glass thermometer that was certified by National Association of Testing Authorities, Australia (NATA), with an accuracy of 0.1 °C. The calibrations took place prior to surgical implantation and again after removal from the animals, for analysis of calibration drift and deviations of readings within and between loggers. The readings from the two calibrations were within of 0.1°C each other in all the loggers but one. In that logger the drift was 1.5°C and a correction was made to the data assuming a constant drift between the two calibrations.

4.4 Experimental Procedures

The mice were randomly assigned to two groups that were placed in different ambient temperature (T_a) controlled environments. One group was maintained at typical animal housing ambient temperature (n=8), where the average temperature was 21.7 ± 0.4°C (T_{22}) with an average relative humidity of 60% (10^{th} and 90^{th} percentiles were 50% and 70% respectively). The other group was maintained close to the lower critical temperature (LCT) of the thermoneutral zone (TNZ) (n=7) of mice, ~30°C (Gordon, 1993). The average temperature in this zone was 29.2 ± 1.1°C (T_{29}) with an average relative humidity of 40% (10^{th} and 90^{th} percentiles were 35% and 50% respectively). Room temperature and humidity levels were recorded by HOBO® Pro V2

Weatherproof Temperature & Humidity Data Logger (MicroDAQ.com, Ltd. Contoocook, New Hampshire, USA).

4.4.1 Daily Food intake

Food intake was measured in the second cohort of mice (n=13). From at least one week prior to gestation until the end of experiment, food intake was measured for each mouse by supplying the animal with a known amount of food and weighing the food remaining at midday the following day to within 0.01g (PGW2502e aeADAM®, Adam Equipment Co Ltd, Canningvale, Perth, WA).

4.4.2 Timed Mating and Gestational Timeline

Once the experimental groups were established, the mice were individually housed for the entire experiment except during mating. Prior to mating, reproductive status was monitored daily in each mouse via a validated vaginal smear cytology procedure (Caligioni, 2009). There are four stages, that span 4-5 days, in the oestrous cycle of a mouse; Pro-oestrous, Oestrous, Metestrous, and Diestrous. When oestrus was detected (Long and Evans, 1922), the female was placed into the cage of an adult breeder male, at the T_a specific for the female. When the female was removed from the male's box the following morning, she was checked for a copulatory plug.

The female's body mass was measured for the next three days to a resolution of 0.01g, using the same scales as used to measure food mass, and a rapid gain in body mass was taken to indicate a successful fertilization. If the female was not pregnant, vaginal smearing recommenced after approximately 3 days and the process was repeated.

Once pregnancy was confirmed, the day of detection of the copulatory plug was designated as pregnancy day 0 (P0). The mouse was then weighed every day until seven days post-partum. On the day of parturition (day 19 of pregnancy which was also day 0 of lactation [L0]), the pups were counted and weighed individually. The pups were then weighed again on day L2, day L4 and day L6. Any stillborn pups or acts of mismothering were noted.

Euthanasia was performed, according to the animal ethics criteria on day L6, by decapitation and via CO_2 asphyxiation for the pups and mothers, respectively. The loggers were retrieved and recalibrated and the data were downloaded for analysis using Mercury Application Software (Star-Oddi, Iceland).

4.5 Data Analysis

4.5.1 Cosinor Rhythmometric Analysis

A Cosinor Analysis was applied to the calibrated T_{core} data (n=15, n_{T22}=8, and n_{T29}=7). The T_{core} data are amenable to such analysis because the time series is an example of a biological time series that exhibits a robust circadian pattern (Refinetti and Menaker,1992; Refinetti, 2006). For each mouse a specific set of experimental time intervals was defined (see Table 4.1) during the gestation period, to which the cosinor analysis was applied, fitting a sinusoidal curve to each interval. The demarcated intervals were; Pre-Mating, Mating, Trimester 1, Trimester 2, Trimester 3, Pre-birth, Day of birth, and Lactation. At each time interval, the cosinor analysis provided us with the fitted wave's mesor, amplitude, and acrophase. The mesor is a measure of central tendency around an oscillating variable, and serves as the circadian-adjusted mean of

the cosine wave fitted to the raw temperature data (Refinetti, 2006). The amplitude is the difference between the peak/trough and the mesor of the cosine wave, and the acrophase is the time of day at which the peak of the cosine wave occurs (Refinetti, 2006).

Table 4.1 Description of gestational time intervals designated for the application of the cosinor analysis

Experimental Time Interval	**Specifications to interval**
1. Premating	Two day interval independent of the mating interval
2. Mating	Two day interval which includes the day/night of mating and the day the copulation plug was found, namely day P0 of gestation
3. Trimester 1	Two day interval in the middle of trimester 1, namely days P3 and P4 of gestation
4. Trimester 2	Two day interval in the middle of trimester 2, namely days P9 and P10 of gestation
5. Trimester 3	Two day interval in the middle of trimester 3, namely days P15 and P16 of gestation
6. Pre-birth	One day interval prior to parturition, typically day P18
7. Day of birth	One day interval over parturition, typically day P19 (Also day L0 of lactation)
8. Lactation	Two day interval during lactation, namely days L2 and L3

4.5.2 Repeated Measures Analysis of Variance

For the gestational time intervals described above, in addition to the mesor, amplitude, and acrophase of the core temperature rhythm, we also derived the daily maximum for T_{core}, and compared the daily maternal food intake, and the daily body mass of each mouse.

Statistical comparisons for both the cosine curve parameters and for the measured parameters of food intake and maternal mass were made by repeated measures analysis of variance (ANOVA).

Data were classified according to the two environmental temperature groups, namely normal animal housing ambient temperature (T_{22}) and thermoneutrality for mice (T_{29}), and the stages of gestation across time were treated as a repeated measure. The interaction between temperature treatment and time was also quantified. These repeated measures ANOVAs were performed in the Statistica 10 Software package (StatSoft, Tulsa, Oklahoma, USA). Significant main effects or interaction in the ANOVA were analysed further using the Tukey's HSD (honest significant difference) *post hoc* test for pairwise comparison of independent samples of unequal size.

4.5.3 Change in core body temperature and foetal mass parameters

From the measure of maternal mass and the individual pup mass on the day of parturition, a number of foetal mass parameters were determined. These parameters are summarised in Table 4.2. Each of the foetal mass parameters was compared

between the two housing temperature treatments by means of an unpaired, two-sample t-test.

The mesor values of T_{core} for first trimester and the day prior to birth were obtained for each animal from the cosinor analysis. Using these mesor values, the change in T_{core} (ΔT) between the first trimester and the day before parturition was quantified. Regressions were then performed to investigate the possible relationships between ΔT and i) the total foetal mass, ii) litter size and iii) the proportion of maternal mass that was accounted for by foetal mass. An arcsine transformation was applied to proportional data prior to analysis, to account for its non-normal distribution.

Table 4.2 Description of foetal parameters, derived from measurements of individual pup mass and maternal mass

Foetal Mass Parameter	**Specifications to Parameter**
1. Total litter mass	Total foetal mass or more specifically, the mass of the litter, weighed immediately post-parturition
2. Litter size	The number of pups that comprised each litter
3. Proportion of maternal mass	The proportion that the total foetal mass made up of the maternal mass just prior to birth

4.5.4 Maternal mass and growth rate analysis

Total litter mass was compared between the two housing temperature treatments, by means of an unpaired, two-sample t-test. Litter growth rate (g/day) and individual pup growth rates (g/day/pup) were determined, using the pup mass measurements that

were collected on lactation days L0, L2, L4 and L6. These two growth rate parameters were compared between the two temperature treatments ($n_{T22} = 8$, and $n_{T29} = 7$) using Welch's t-test (used for independent, unequal variance samples). To rule out the possibility of confounding effects of maternal mass on growth rate, maternal mass was also correlated with litter growth rates and the individual pup's growth rates, at each of the lactation intervals to investigate if there was any significant relationship between maternal mass and litter growth. A regression analysis was performed to determine if there was a relationship between the litter size and the mass of the individual pups for the overall sample.

All data and figures are presented as means ± standard error of the mean (SEM). Importantly, any differences between the means were considered significant at the 5% level, i.e. $P < 0.05$.

CHAPTER 5 – RESULTS

5.1 Cosinor analysis of body temperature

5.1.1 The mesor of body temperature

There was a significant effect of housing temperature on the daily mesor of T_{core} ($F_{1,13}$ = 8.7, P = 0.01). There was also a significant main effect of time ($F_{7,91}$ = 97.1, P<0.001) and an interaction between housing temperature and time ($F_{7,91}$ = 4.3, P<0.001). Despite the fact that on average, the mesor at T_{29}, was higher (at 37.3 ± 0.04°C) than the mesor at

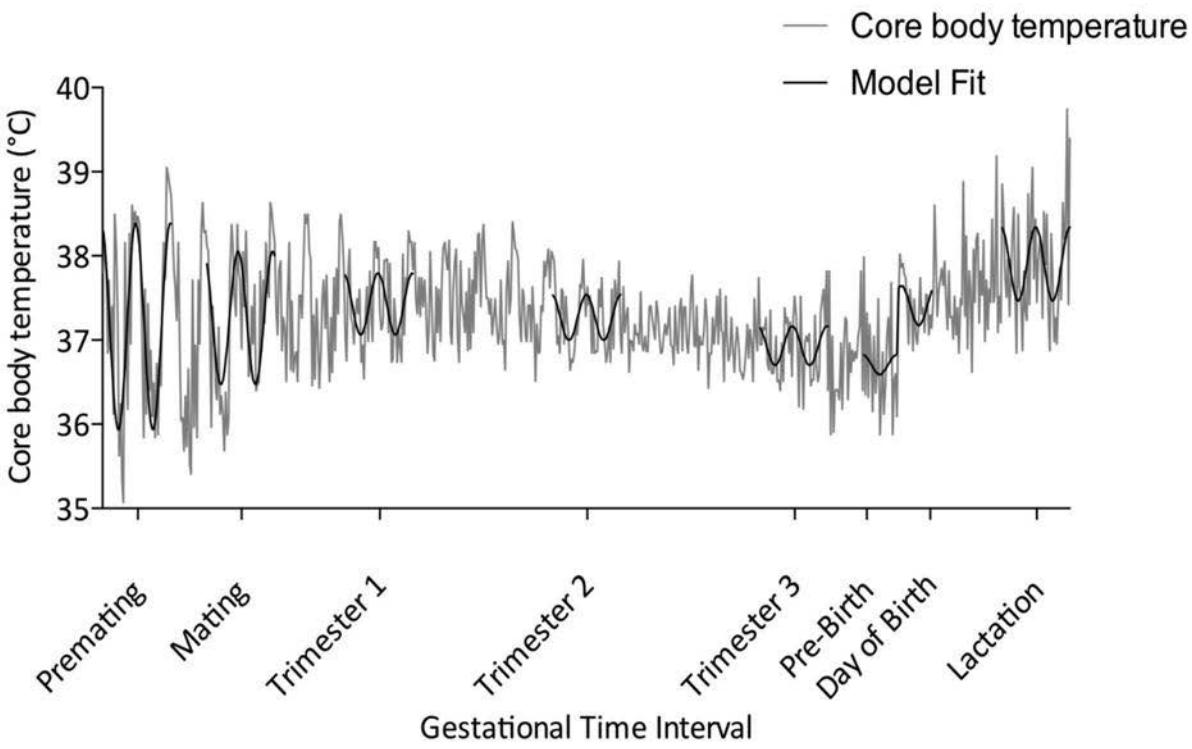

Figure 5 Example of core body temperature for a single representative mouse. Measurements were taken every 60 minutes over the course of the full gestation period, including lactation. This shows theoretical cosine wave that best fits the temperature data at each of the designated gestational time intervals (described in Table 4.1) as shown by the model fit, and provides the mesor, amplitude and acrophase for each period

T_{22} (37.1 ± 0.05°C)*, post hoc* analysis for the interaction (P = 0.01), revealed that the two groups were significantly different only during premating, when the mesor for T_{22} was lower than T_{29} by 0.4°C (P<0.001) and in trimester 1, when mesor for T_{22} was lower than T_{29} by 0.3°C (P = 0.03) (Figure 5.1A).

Post hoc analysis for the significant effect of time (Tukey's HSD test) revealed that overall, the mesor increased () between premating (37.1 ± 0.1°C) and trimester 1 (37.4 ± 0.1°C). The mesor for mating and trimester 1 was the same. There was then a significant decrease of 0.6°C (P=3.91) between trimester 1 and trimester 2, and a further decrease of 0.4°C (P<0.001) between trimester 2 and trimester 3. Thus overall there was a decrease of 1°C between trimester 1 and trimester 3, reaching a nadir of 36.7 ± 0.1°C on the day before birth. On the day of birth the mesor increased by 0.6°C (P = 0.001) and continued to increase into lactation by a further 0.4°C (P<0.001) (Figure 5.1A).

5.1.2 The acrophase of body temperature

There was no effect of either housing temperature ($F_{1,13}$ = 0.60, P = 0.45) or time ($F_{7,91}$ = 1.96, P = 0.07) on the time of acrophase. Despite the repeated measures ANOVA indicating a significant interaction between time and housing temperature ($F_{7,91}$ = 2.70, P = 0.013), the *post hoc* analysis revealed no significant differences between the T_{22} and T_{29} group at any of the gestational time intervals. The interaction was significant because on average the acrophase in the T_{22} group occurred 6 hours later during trimester 3, than it did during mating, trimester 1 and trimester 2. That single result is most likely due to one individual that showed a large change in

acrophase at trimester 3 (note the increase in SEM at trimester 3 of Figure 5.1B). On average, for the treatment groups combined, the acrophase occurred at 23h05 ± 03h30.

5.1.3 The amplitude of body temperature

There was a significant effect of time on the amplitude of T_{core} ($F_{7,91}$ = 19.4, P<0.001), with no effect of housing temperature ($F_{1,13}$ = 0.84, P = 0.37), or interaction ($F_{7,91}$ = 0.57, P = 0.78). *Post hoc* analysis revealed that the amplitude was largest during the premating interval, and decreased in the first trimester (P<0.001), from 0.7 ± 0.1°C during premating to 0.4 ± 0.1°C during trimester 1. The daily amplitude continued to decrease across gestation, with a decrease of 0.2°C between the first and third trimester (P = 0.004), at which point amplitude was the smallest observed at 0.2 ± 0.1°C. After trimester 3, the amplitude remained lower than it was prior to gestation, and the time intervals for the remainder of gestation and into lactation, did not differ from each other (Figure 5.1C).

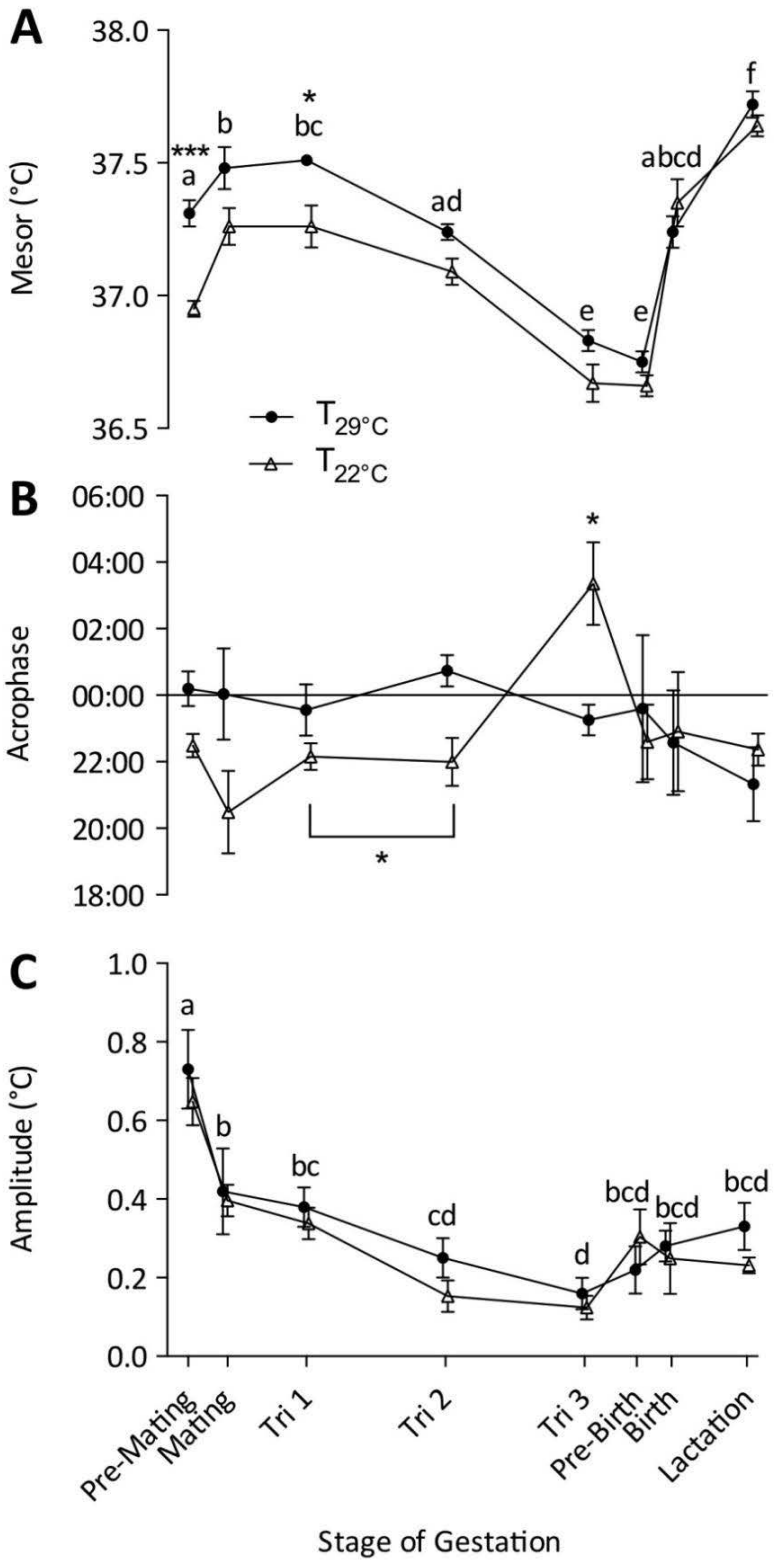

Figure 5.1 Cosinor analysis parameters of mesor (A), acrophase (B) and amplitude (C), across the designated gestational time intervals. *Data points represent the mean ± SEM of* the T_{22} group and the T_{29} group. Means with different superscripted letters differ significantly between time intervals (P<0.05). A significant difference between housing temperature is denoted by * and ***, indicating a p-value of P<0.05 and P<0.001, respectively. Significance was assessed by repeated measures ANOVA, followed by Tukey's HSD test for pairwise comparisons.

5.1.4 The maximum of body temperature

There was an effect of housing temperature on the daily maximum of T_{core} ($F_{1,13}$ = 10.4, P = 0.007), and a main effect of time ($F_{7,91}$ = 78.3, P<0.001) and an interaction between housing temperature and time ($F_{7,91}$ = 7.6, P = $3x10^{-7}$). The mean maximum temperature at T_{22} was lower (38.0 ± 0.1°C, P = 0.007) than at T_{29} (at 38.3 ± 0.1°C), p*ost hoc* analysis for the interaction revealed that the two groups were significantly different at only two of the gestational time intervals, namely during trimester 3, when the maximum for T_{22} was lower than T_{29} by 0.6°C (P<0.001) and during lactation, when the maximum for T_{22} was lower than T_{29} by 0.7°C (P<0.001) (Figure 5.2 A).

Post hoc analysis for the significant effect of time revealed that overall, from mating (38.5 ± 0.1°C) the maximum temperature decreased to reach the lowest maximum during trimester 3 (37.7 ± 0.09°C). The decrease in maximum temperature between each of the trimesters was significant, with a decrease of 0.3°C (P<0.001) between the trimester 1 and trimester 2 and a decrease of 0.3°C (P = 0.002) between trimester 2 and trimester 3. At birth the maximum temperatures increased, with an increase of 0.4°C between the day before birth and the day of birth (P<0.001) and a further increase of 0.8°C into lactation (P<0.001) (Figure 5.2 A).

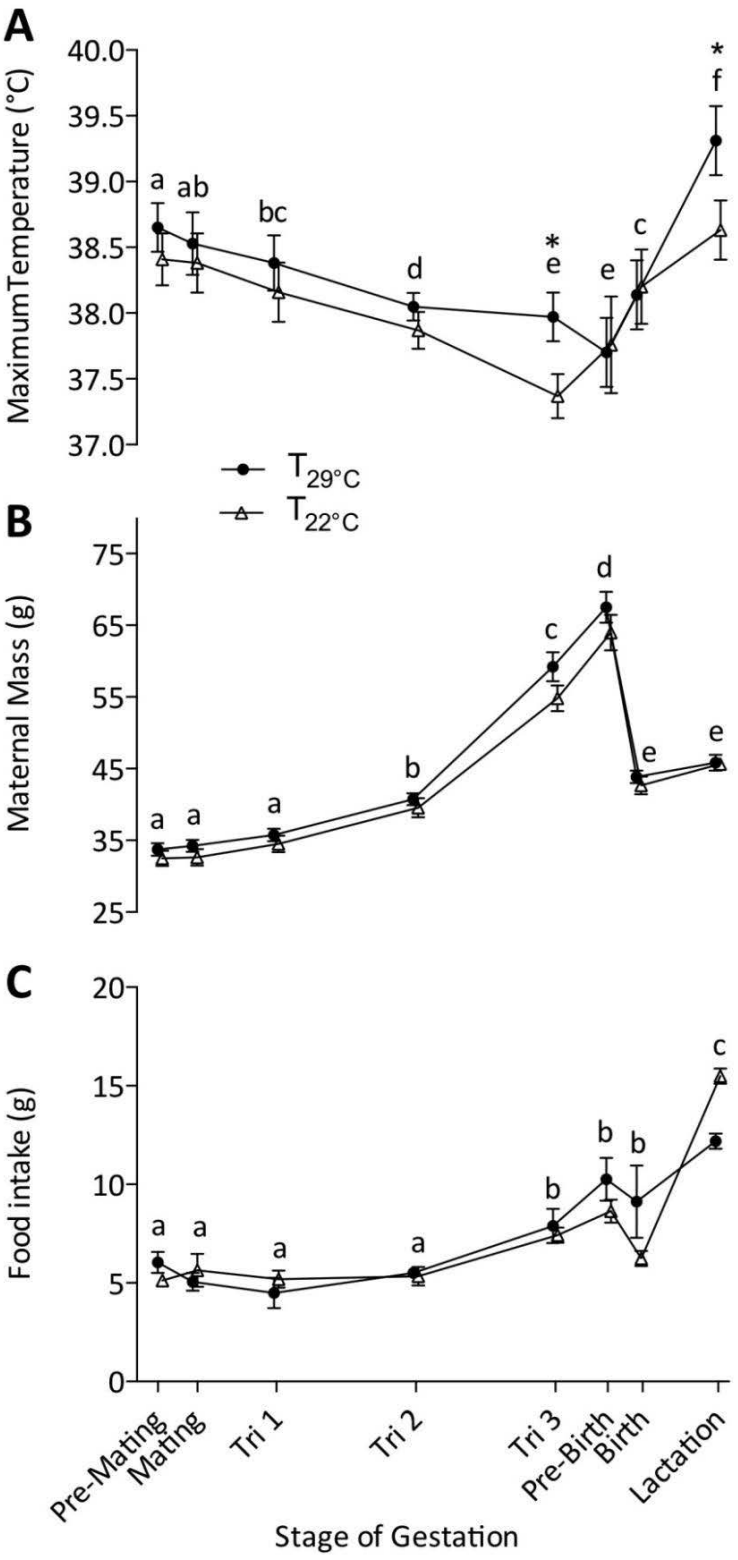

Figure 5.2 Maximum core temperature (A) (n_{T22} = 8, n_{T29} = 9), food intake (B) (n_{T22} = 5, n_{T29} = 6) and maternal body mass (C) (n_{T22} = 8, n_{T29} = 9), across the gestational time intervals. Data points represent means ± SEM. Means with different superscripted letters differ significantly between time intervals (P<0.05). A significant difference between the housing temperature is denoted by *, indicating a p-value of $1x10^{-4}$. Significance was assessed by repeated measures ANOVA, followed by Tukey's HSD test for pairwise comparisons.

5.2 Maternal body mass and food intake

5.2.1 Maternal body mass

Prior to mating, there was no difference in maternal body mass between the T_{22} mice (33.0 ± 1.1 g) and the T_{29} mice (34.8 ± 1.0 g) (P = 0.27). There was no effect of housing temperature on maternal body mass ($F_{1,15}$ = 1.3, P = 0.28), but there was an effect of time ($F_{7,105}$ = 396.9, P<0.001), and no interaction ($F_{7,105}$ = 1.4, P = 0.21).

Between trimester 1 and trimester 2, there was an increase in mass of 5.0 g (P<0.001) and between trimester 2 and trimester 3, there was an increase in mass of 7.0 g (P<0.001). The mice gained 8.7 g between trimester 3 and the day before birth (P<0.001). At birth there was a decrease in mass of 22.6 g (P<0.001) with the loss of the total foetal mass (Figure 5.2 C).

5.2.2 Daily Food intake

There was no effect of housing temperature on food intake ($F_{1,9}$ = 0.1, P = 0.78) but there was an effect of time ($F_{7,63}$ = 40.1, P<0.001), and an interaction ($F_{7,63}$ = 3.7, P<0.002). Despite the significance found for the interaction, *post hoc* analysis revealed no significant difference between the housing treatment groups at any of the time points. *Post hoc* analysis for the effect of time revealed that food intake stayed relatively constant from premating through to the second trimester, with no difference between the first 4 gestational time intervals. Daily food intake increased from 5.4 g/day to 7.7 g/day between trimester 2 and trimester 3 (P = 0.03), and increased again from 7.7 g/day to 9.5 g/day between trimester 3 and the day before birth (P = 0.02). On the day of birth, there was a decrease in daily food intake from 9.5 g/day to 7.8

g/day (P<0.001), followed by an increase into lactation from 7.82 g/day to 13.7 g/day (P<0.001) (Figure 5.2 B).

5.3 Changes in body temperature and foetal parameters

There was no effect of housing temperature on any of the foetal parameters, namely total litter mass (P = 0.47), litter size (P = 0.52) or proportion total of maternal mass that was foetal mass at term (P = 0.63). There was also no relationship between ΔT and any of the foetal parameters, namely total litter mass (R = 0.1, P = 0.70), litter size (R = 0.1, P = 0.71) or the proportion of total maternal mass that was foetal mass at term (R = 0.1, P = 0.74) (Figure 5.3).

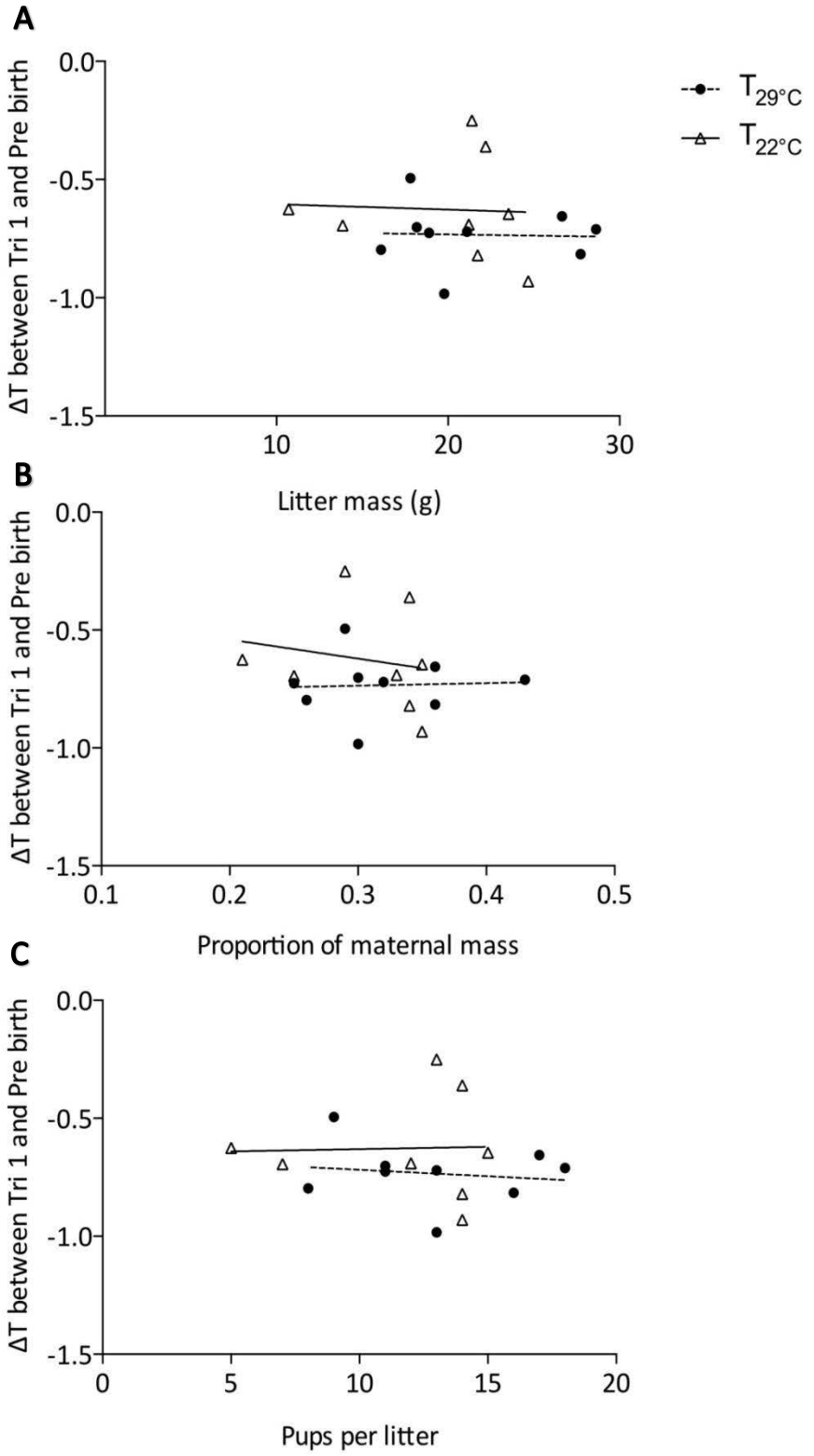

Figure 5.3 Linear regression analyses of the relationship between the change in T_{core} between trimester 1 and the day before birth (ΔT) as a function of (A) litter mass, (B) litter mass as a proportion of total maternal mass at birth and (C) number of pups per litter. Data points represent individual data of ΔT for each mouse with the corresponding parameter represented in each of the 3 panels, for the T_{22} group (n = 8) and the T_{29} group (n = 9).

5.4 Litter and individual pup growth rates

Immediately post-parturition the total litter mass was not different between the two housing temperature groups (P = 0.468).

Litter sizes between the two groups were not found to be different (P = 0.351) because there was no difference in the number of pups per litter, with the average litter size for T_{22} being 11.8 ± 0.8 pups/litter and for T_{29} being 12.9 ± 0.9 pups per litter. Of the 153 pups born in $T_{22,}$ one was found dead immediately after parturition. Of the 155 pups born in $T_{29,}$ five were found dead, one of which was partially cannibalized by the mother.

In the initial stages of lactation, on days L0-L2, the litters in the T_{22} group (5.2 ± 0.4 g/day) grew more than those in the T_{29} group (2.9 ± 0.4 g/day) (P = 0.002), with an overall difference in growth rate of 2.3 g/day (Figure 5.4). At the same lactation interval, individual pup growth rate was also faster (P = 0.003) in the T_{22} group (0.4 ± 0.1 g/day/pup) than in the T_{29} group (0.2 ± 0.1 g/day/pup), with an overall difference in growth rate of 0.2 g/day/pup. For lactation days L2 to L4 there was no difference between the temperature groups for either litter growth rate (P = 0.43) or individual pup growth rate (P = 0.31). Litter growth rate was again faster in the T_{22} group (6.0 ± 0.3 g/day) on lactation days L4-L6 (P = 0.01) than in the $T_{29°C}$ group (4.9 ± 0.2 g/day), with an overall difference of 1.1 g/day. Likewise, for the same lactation interval, individual pup growth rate was faster (P = 0.03) in the $T_{22°C}$ group (0.5 ± 0.1 g/day/pup) than in the $T_{29°C}$ group (0.4 ± 0.1 g/day/pup), with an overall difference of 0.1 g/day/pup (Figure 5.4).

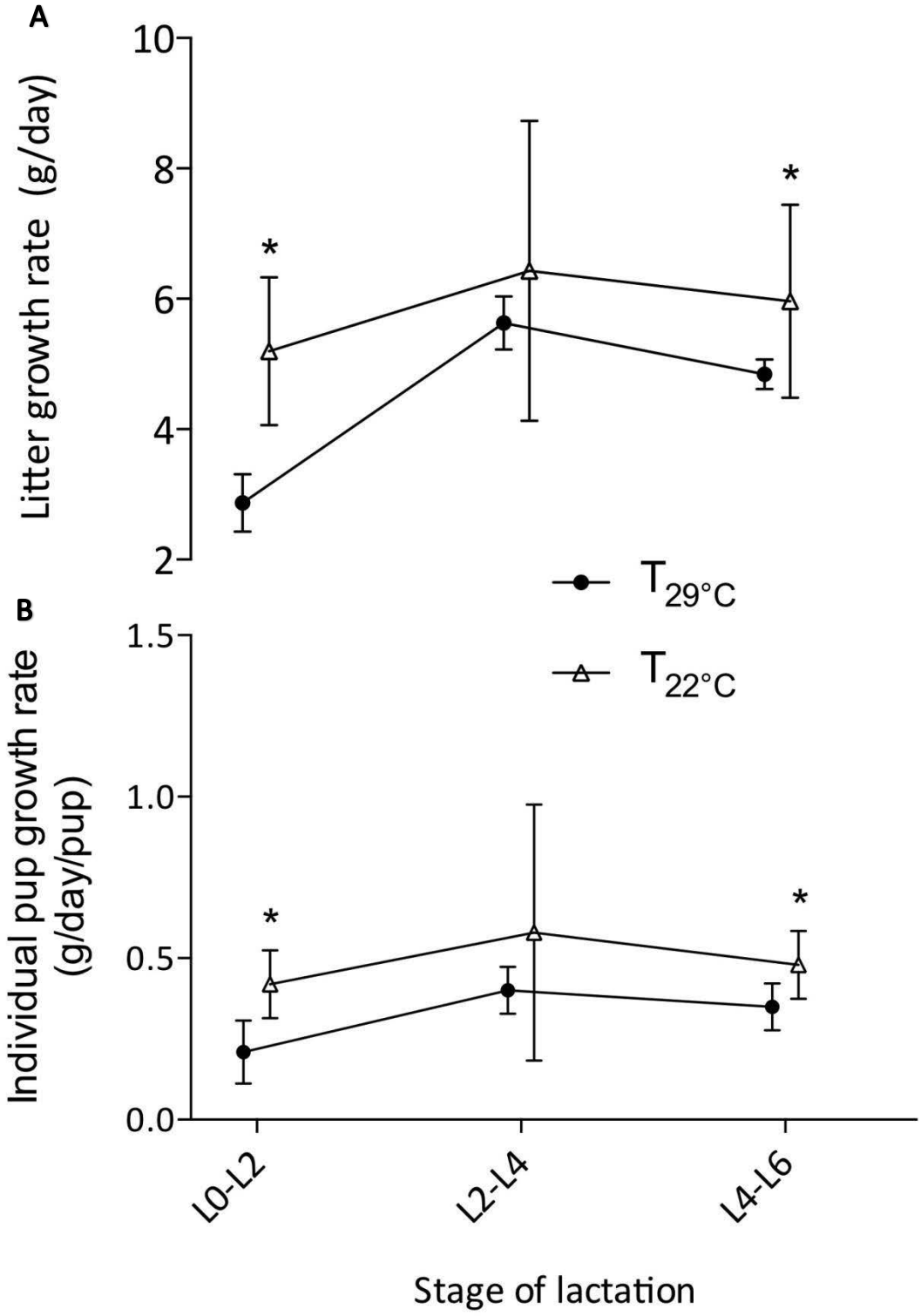

Figure 5.4 Growth rates for (A) litters and (B) individual pups, across the lactation period. Individual pup mass measurements were taken on days L0, L2, L4 and L6. Data points represent means ± SEM of the T_{22} group (n = 7) and the T_{29} group (n = 6). Significant difference between the two animal housing temperature groups at any particular time intervals are denoted by * indicating a p-value of <0.01.

There was no significant linear relationship between maternal mass and either the litter growth rate or the individual pup growth rate, at any of the lactation intervals.

Overall there was moderate inverse relationship between litter size and individual pup mass ($r = 0.85$, $P<0.001$) which indicated that as litter size increased, the average individual pup size decreased (Figure 5.4).

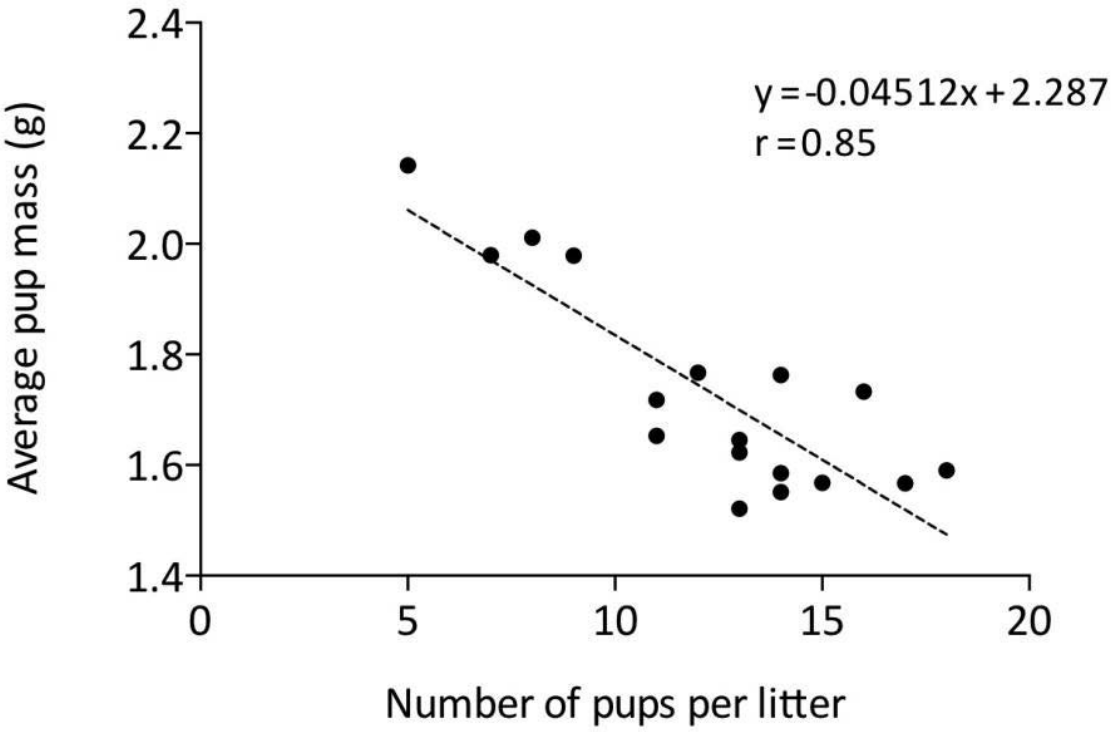

Figure 5.5 Linear regression of the relationship between the number of pups per litter and the average individual pup mass for that litter on the day of birth (n = 17).

CHAPTER 6 – DISCUSSION

6.1 The characteristics of core body temperature during gestation

The present study investigated T_{core} in mice during virgin mating, gestation and lactation. The first aim was to determine if gestational hypothermia, namely a decrease in maternal T_{core} (ΔT) during pregnancy occurred in mice, and it did, as demonstrated by the mesor data. Overall, maternal T_{core} exhibited a gradual decrease between trimester 1 and the day before birth, followed by a rapid increase after parturition. The same pattern has been seen in several other species, including rats (Kitrell and Satinoff, 1988; Fewell, 1995; Eliason and Fewell, 1997; Fewell and Tang, 1997; Cairns *et al.*, 2004), ewes (Laburn *et al*., 1992; Laburn *et al*., 1994), goats (Faurie *et al*., 2001), dogs (Concannon and Hansel, 1977; Hoffmann *et al*., 1994), arctic ground squirrels (Williams *et al*., 2011), rabbits (Naccarato and Hunter, 1983), humans (Lindqvist *et al*., 2003) and our data confirms unpublished findings in mice (Kozac, 1997).

The cosinor characteristics that describe the T_{core} data reveal an effect of housing temperature during premating and the first trimester of pregnancy, but the hypothermia in the third trimester and the hyperthermia during lactation did not differ between the housing temperatures. Why there was a lower mesor temperature in the T_{22} group during premating and trimester 1 and not during mating or for the remainder of gestation is unclear. After trimester 1 mesor temperatures converged to the same levels for the remainder of the pregnancy and lactation, indicating that both groups were able to thermoregulate their T_{core} to the same level, despite different ambient temperatures, perhaps eliciting behavioural thermoregulation to do so.

There was an increase in maternal T_{core} at the time of mating, similar to that reported by Gamo *et al.* (2013), who showed that in mice housed at 21°C, female T_{core} remained elevated for the entire 5 day period that they were housed with a male.

The only other difference between the treatment groups with regards to T_{core} was in their maximum temperatures. Interestingly, despite the fact that there was no difference in mesor temperature between T_{22} and T_{29} during the latter stages of pregnancy and lactation, the maximum temperatures were higher in the females housed at T_{29} than at T_{22} during trimester 3 and lactation. This could suggest one of two things. Firstly, it is possible that animals housed at T_{22} could be dissipating the heat of activity easier and thus their maximums do not reach as high temperatures. The other suggestion is that the maximum could be a representative of one reading, whereas mesor and amplitude are mean measures which tell us more about the overall rhythm of T_{core} and its variation. Thus a higher maximum could easily be indicative of transient increases in T_{core} being recorded at T_{29}. These maximum values could be as a result of unrelated spikes in T_{core}, and possibly an example of handling stress hyperthermia when researchers were measuring the body mass of the mice. If maximum body temperatures are reached during periods of activity (Refinetti and Menaker, 1992; Briese, 1998; Leon *et al*., 2004), and handling stress increases the activity of the animal, then it is possible that these maximums could be reflective of this handling period.

In the present study, the body mass of mice was measured daily around midday, meaning the animals were handled at least once per day. Even if we could determine that handling stress-related spikes in T_{core} were occurring, we do not have days on which the animals were not handled with which we could compare. Due to these

confounders, the maximum recorded temperatures cannot be definitively concluded to be reflective of daily maximum T_{core} of the animals under normal conditions.

There was no difference between the two housing temperatures for acrophase or amplitude. Acrophase is the timing of the peak of the T_{core} rhythm (Refinetti, 2006). The fact that there was no difference, tells us that the timing of the endogenous circadian rhythm is fairly rigid and that it is not affected by T_a or pregnancy. Therefore there is no observation of a phase delay or a phase advance of the circadian rhythm (Refinetti, 2006).

As amplitude is a measure of variance around the mesor temperature of the observed T_{core} rhythm, a decrease in amplitude shows that the variation in T_{core} progressively became smaller during pregnancy, to reach its lowest amplitude during trimester 3 in both temperature groups. As gestation progressed, the cosine rhythm became tighter, indicating that the thermoregulatory stability of the animals was improved, and T_{core} fluctuated less. It is possible that by reducing the fluctuations in maternal T_{core}, this may help to achieve this consistency in foetal T_{core}.

Amplitude also gives us an indication of the type of thermoregulation an animal may be utilising, *i.e.* homeothermy or heterothermy. Let us say that we consider a particular range of cyclic variation in a non-pregnant mouse to be normal homeothermy. We then observe in the pregnant mouse that the amplitude, and therefore cyclic variation, decreases as gestation progresses. Although cyclic variation has changed during pregnancy, it is still well within the range of normal homeothermy. Although homeothermy and heterothermy are technically defined as a dichotomy, based on these results it appears that the pregnant mice become more homeothermic.

Aschoff (1982) and Mortola and Lanthier (2004) found that in non-pregnant animals, an increase in body mass was correlated to a decrease in amplitude in T_{core}. Studies by Refinetti (1999) and Hetem *et al*. (2014) have refined the metrics of these comparative analyses and found that although amplitude varies within and between species, there is no relationship between amplitude and body mass. Because of this inconsistency, it is difficult to say whether changes in amplitude during pregnancy could be as a result of changes of body mass. In the case of the above studies, the scaling of amplitude to body mass works over several orders of magnitude, one where body mass is being compared between very large (*e.g.* elephant) and very small (*e.g.* mouse) homeotherms, and in the case of pregnancy, the changes in body mass are very small by comparison. In the present study, between trimester 1 and the day prior to birth there was a 50% increase in body mass from the pre-mating to day 18 of gestation.

Kitrell and Satinoff (1988) also reported that the amplitude of the T_{core} rhythm decreased during the final stages of pregnancy in rats and hamsters, and made brief references to changes in gestational hormone levels to explain these alterations to amplitude.

Interestingly amplitude did not change during birth or lactation in our study, instead it remained low. Kitrell and Satinoff (1988) found that amplitude increased during lactation. They did however, record values across the full four weeks of lactation, and do not state at which point they determined the amplitude or whether it was an average. The amplitude was analysed for only 6 days in the present study.

At this stage it is necessary to mention that the primary objectives and interests of this study surrounded the gestation period only, with particular mention to the final trimester and birth. The initial focus lay in the results of T_{core}, during the final stages of pregnancy and on the day of birth. As an extra measure, T_{core} was recorded into lactation for six days. Had measurements continued until the end of lactation, similar results to Kitrell and Satinoff (1988) may have been observed.

The higher rise in maternal T_{core} during lactation has been described as a form of forced hyperthermia, as shown by the work of Fewell (1995) and Eliason and Fewell (1997), where lactating rats preferred cooler ambient temperatures during lactation. One would expect that the amplitude of T_{core} may change during these forced hyperthermic periods of parturition and lactation. A forced hyperthermia results from the thermoeffector responses being overwhelmed, in this case by an increase in the amount of metabolic heat being produced by the mother and it would be reasonable to expect that this may cause larger fluctuations in $T_{core.}$ This would explain why Kitrell and Satinoff (1988) found an increased amplitude during lactation, however this was not the case with our findings. Changes in amplitude would also depend be dependent on when the measurements were taken during the 24 hour day. If the increased metabolism associated with lactation is sustained across the 24 hours day then daily amplitude may not be affected. It is unlikely that the suckling of pups which increases maternal metabolic rate is a continuous process, and so there would be expected decreases in the metabolism associated with bouts of time when the mother was away from her pups.

6.2 Is gestational hypothermia influenced by foetal heat production?

Once it was confirmed that mice did exhibit gestational hypothermia, further assessment was undertaken to determine whether foetal mass was proportional to the magnitude of ΔT (the decrease in maternal T_{core} from the 1st trimester until the day before birth).

There appears to be some benefit to keeping the T_{core} of the foetus constant, presumably at the same temperature of the mother at the start of gestation. But as the foetus grows it produces increasing amounts of metabolic heat. If the mother regulated her T_{core} as normal, then an increase in foetal metabolic heat production would cause the foetal T_{core} to rise. However, for the foetal T_{core} to remain at a constant temperature, the maternal T_{core} must decrease (ΔT) proportionally to maintain a large enough thermal gradient across which foetal heat can be dissipated.

In theory, a greater foetal mass should produce larger amounts of foetal metabolic heat, thereby creating a larger decrease in the maternal T_{core}. The current study found no relationship between ΔT and either total foetal mass, litter size, or the proportion of maternal mass that was made up by foetal mass at term. Therefore, it can only be implied that the magnitude of gestational hypothermia was not proportional to foetal metabolic heat production.

There was no effect of housing temperature on these relationships, thus maternal thermal balance had no effect on the extent of the ΔT. Since ΔT is independent of foetal mass, it seems unlikely that foetal temperature is the regulated variable. It appears the input signal for gestational hypothermia is not originating from the foetus

and must involve some other mechanism or signalling pathway. The next section will discuss what could be causing gestational hypothermia.

6.3 Possible causes of gestational hypothermia

6.3.1 Gestational changes to ovarian hormones

Gestational hypothermia has been linked to a prepartum decrease in plasma progesterone levels (Hoffmann *et al*., 1994; Fewell, 1995), as progesterone has known thermogenic properties (Freeman *et al*., 1970). Arguably, if progesterone has thermogenic effects and remains high until late pregnancy (Grota and Eik-Nes, 1967; Bassett *et al.*, 1969; Pepe and Rothchild, 1974; Ash and Heap, 1975; Hoffmann *et al*., 1994), then maternal T_{core} should in theory, remain high too.

In the aforementioned gestational studies, plasma progesterone levels are reported to decline only in the final days leading up to parturition. However, maternal T_{core} decreases gradually across the course of gestation (Eliason and Fewell, 1997). Thus it is unlikely that a "last minute" drop in the levels of plasma progesterone could be associated with this gradual decline in T_{core}.

Instead it seems more reasonable that the thermogenic effects of progesterone are being masked or nullified by the regulated gestational hypothermia occurring throughout gestation. When, as seen in the present study, the gestational hypothermia disappears leading into lactational hyperthermia, which has been suggested to be caused by an increase in corticosterone levels (Hoffmann *et al*., 1994; Fewell, 1995), the effects of progesterone may then be unmasked and thus contribute further to the hyperthermic state of the animal during lactation.

6.3.2 Foetal protection from teratogens

Initially it was believed that gestational hypothermia could play a role in protection of the foetus against teratogenicity in mice.

For the foetus to be at risk from teratogenicity, one of the following situations needs to occur; 1) There needs to be an increase in maternal temperature (to meet or exceed the teratogenic threshold of 1.5-2°C) (Bell *et al*., 1985; Graham *et al*., 1998; Edwards, 2006) that decreases the feto–maternal gradient enough for the foetus to be at risk (Laburn *et al*., 2002; Edwards, 2006), 2) this hyperthermia needs to occur at a stage of the pregnancy during which the foetus is susceptible to teratogenicity, namely the first (embryogenesis) or third trimester (organogenesis and growth) (Shiota, 1988; Dreiling and Carman, 1991; Edwards *et al*., 1995; Edwards, 2006) , or 3) placental blood flow must be compromised, impeding the primary pathway for heat dissipation by the foetus to the mother (Nelson and Grether, 1998; Graham *et al*., 2008).

In the present study, although an increase in maternal T_{core} at birth was observed, the maternal temperatures on the day of birth do not exceed those of premating levels, let alone meet the temperature extremes enough to deem it a hyperthermic event. It is unlikely that the increase in maternal T_{core} at birth would exceed the proposed maternal threshold for teratogenicity (1.5-2°C) (Bell *et al.*, 1985; Graham *et al*., 1998; Edwards, 2006), and our results show only a 0.6°C increase in maternal T_{core} at birth.

Importantly, even if this was considered to be a hyperthermic event, the most severe consequences of hyperthermia tend to occur in the first trimester during formation of the neural tube and embryogenesis (Shiota, 1988; Graham *et al*., 1998; Asakura, 2004) or in the middle of the third trimester during organogenesis and the final stages of growth (Dreiling and Carman, 1991). Core body temperature does continue to rise into

lactation, but by then the pups are no longer inside the uterus and are not susceptible to teratogenicity by maternal hyperthermia.

It has been shown that the febrile response in late pregnancy and early lactation is supressed in sheep (Kasting *et al.*, 1978), guinea pigs (Merker *et al.*, 1980) and rats (Martin *et al*., 1995). Maternal T_{core} reaches its lowest levels on the day prior to birth, the same time as when suppression of fever has been observed in the above mentioned studies. Thus even if gestational hypothermia was serving to protect the foetus from fever, fever at this stage of gestation is shown to be suppressed anyway.

The suppression of fever has been hypothesized to occur due to the antipyretic effect of the endogenous hormone arginine vasopressin (AVP). Arginine vasopressin is a neurotransmitter involved in central thermoregulatory pathways in the CNS and modulates febrile response by decreasing pyrogen-induced elevations in T_{core} (Kasting, 1989; Pittman and Wilkinson, 1992; Pittman *et al.*, 1998). When several sites of the brain were infused with AVP in non pregnant sheep (Kasting *et al.*, 1979) and rabbits (Naylor *et al*., 1985), the tissue site in which AVP that was found to be antipyretic was the ventral septal area (VSA) of the brain. Concentrations of AVP have been shown to be elevated in the VSA of the brain in late parturient females (Merker *et al*., 1980; Naylor *et al*., 1988; Pittman and Wilkinson, 1992; Landgraf *et al*., 1991).

When VSA AVP receptors are experimentally blocked, febrile response to prostaglandin E_1 occurs as normal. It appears that elevated levels of AVP in the VSA could be responsible for the suppression of febrile response in pregnant females; however the function of febrile suppression is still poorly understood. Fewell and Tang (1997) proposed that in the event of fever induced hyperthermia, foetal and uterine blood flow may be compromised to meet the needs of the maternal thermoregulatory

system. A pathological increase in maternal temperature would not only increase the use of oxygen by the mother, but would divert the available oxygen supply away from the foetus. Ultimately, it appears that a trade-off is occurring, where the risk to the foetus due to increased temperatures or oxygen supply, is given priority over the protective benefits the mother may receive from the development of fever in response to the endotoxin.

The only remaining teratogenic situation other than those posed by maternal hyperthermia and fever, is that of foetal hyperthermia caused by compromised placental blood flow. Acute occlusion of umbilical cord can reduce placental blood flow (Asakura, 2004) causing a fatal rise in foetal T_{core} in the following cases; when the umbilical cord is wrapped around the neck (tight nuchal cord), when the mother goes into shock, when the placenta separates from the lining of the uterus, when the umbilical cord becomes compressed during the birthing process, in the event of uterine rupture or during pre-eclampsia (Nelson and Grether, 1998; Graham *et al*., 2008). Most, if not all of the above situations occur during the birthing process. By the time parturition has commenced, maternal T_{core} will have begun to rise, as is indicated by our mesor data on the day of birth. It is therefore unlikely that gestational hypothermia would serve to protect the foetus in these cases.

The timing and situational context of all of the discussed teratogenic situations make it unlikely that the protection of the foetus from foetal and maternal hyperthermia is what is causing gestational hypothermia to occur.

6.3.3 Hypothermia in response to hypoxia

Bearing on the subject of O_2 supply brings us to our next hypothesis that gestational hypothermia occurs as a response to or in prevention of hypoxia.

In non-pregnant mammals, a reduction in T_{core} has been proposed to occur to conserve the available oxygen by reducing thermogenic metabolic processes (Barros *et al*., 2001; Tattersall and Milsom, 2003; Branco *et al*., 2006,). Thus far hypoxia has been reported to elicit a hypothermic state (Wood, 1991). The metabolic rate of a developing foetus is highest at the end of pregnancy; a stage where the largest amount of O_2 is required to fulfil foetal energetic demands. It could be the case that the gestational hypothermia occurs to reduce O_2 demands of the feto-maternal unit in order to prevent foetal hypoxia.

If, as suggested by Fewell (1995), gestational hypothermia is a controlled response, then it could not be the case that it is as a result of an insufficient oxygen supply, as this would indicate a forced response. For a Q_{10} value of 2.5, a decrease in T_{core} of 1^{o}C, from 37^{o}C to 36^{o}C results in an 8.7% reduction in metabolism and thus O_2 consumption (Dawson and Hulbert, 1970; Schmidt-Nielsen, 1997; Maloney *et al.*, 2011). It is therefore not unreasonable to infer that gestational hypothermia has the potential to work as a beneficial mechanism to reduce O_2 demands of the foetus and perhaps even of the mother as well.

Fewell (1995) suggested that the controlled hypothermia may counteract the effects of parturition induced hyperthermia which could potentially cause neuronal injury via asphyxia. Placental blood flow has been shown to be reduced in cases of umbilical cord occlusion, causing intrauterine hypoxia

There is no doubt from the existing literature that hypothermia reduces the oxygen demands, potentially serving as a protective mechanism against hypoxia (Wood, 1991; Barros *et al*., 2001; Tattersall and Milsom, 2003; Branco *et al*., 2006). However, it seems improbable that gestational hypothermia occurs as a response to an existing hypoxic state. Undoubtedly a decrease in maternal T_{core} would almost certainly facilitate the overall reduction in the amount of oxygen required for metabolism in the feto-maternal unit. However, whether this protective function serves as the driving force for gestational hypothermia is uncertain.

6.3.4 Deactivation of brown adipose tissue

A completely different hypothesis proposes that gestational hypothermia is not as a result of a controlled decrease in T_{core}, but rather due to impairment of thermoregulatory function (Naccarato and Hunter, 1983). The thermogenic activity of brown adipose tissue (BAT) declines significantly towards the end of gestation and during lactation in rats and mice (Trayhurn, 1983; Trayhurn and Richard, 1985; Andrews *et al*., 1986; Speakman, 2008) and in hamsters (Wade *et al*., 1986; Trayhurn, 1989).

Hyperphagia has been shown to increase the thermogenic activity of BAT (Rothwell and Stock, 1983; Trayhurn and Richard, 1985; Abalenda and Puerta, 1987), but this does not seem to be the case with gestational related hyperphagia. It is likely that gestational hyperphagia occurs in response to an increased energy demand during pregnancy. The energy obtained through an increased food intake could be being utilised to fulfil this demand, thus the mother has no need to activate BAT thermogenesis.

Gestational hypothermia has been suggested to be related to the inhibition of nonshivering thermogenesis via the deactivation of BAT, possibly to channel more energy from food to production of milk (Trayhurn, 1989). It has also been suggested that BAT thermogenic activity is inhibited to reduce the heat burden on the mother, making it easier for her to dissipate the metabolic heat being produced during lactation. By reducing the heat burden, she may better be able to transfer her available energy into milk, and hence into growth of her offspring. This concept was proposed by Król *et al.* (2007) and is further deliberated later in this discussion.

There is some evidence that circulating levels of plasma adenosine play a role in the inhibition of BAT thermogenesis during pregnancy and lactation (Sawa *et al*., 1991; Ball *et al.*, 1995). Adenosine has potent vasodilatory effects, metabolic suppressor properties, and modulates placental and uterine blood flow (Yoneyama *et al*., 2000). Adenosine is released by the placenta and inhibits foetal brown fat lipolysis (Szillat and Bukowiecki, 1983; Ball *et al.*, 1995). But plasma adenosine's antilipolytic effects are also seen in the mother, with a 3.3-fold higher level of adenosine in pregnant than non-pregnant women (Iliou and Demarne, 1987; Yoneyama *et al*., 2000), with pronounced levels being reported in the third trimester.

Although maternal adenosine levels are found to be four times less than that of the foetus, maternal plasma adenosine is still significantly elevated. If, as Szillat and Bukowiecki (1983) suggests is true and adenosine inhibits the thermogenic activity of brown fat, then it is possible that elevated plasma adenosine levels could explain inhibition of brown fat lipolysis in both the mother and her foetus. Interestingly, elevated concentrations of adenosine have also been found during hypoxia, possibly

because the vasodilatory and suppressive metabolic characteristics of adenosine may help to modulate the hypoxic condition (Winn *et al.,* 1981; Yoneyama *et al*., 2000).

Major atrophy of BAT occurs during late pregnancy and lactation, as evidence by observations of interscapular BAT mass being substantially reduced (Trayhurn and Richard, 1985; Andrews et al., 1986; Wade *et al*., 1986). Since this atrophy and reduced activity of BAT occurs in lactation as well as during late pregnancy, then if the atrophy is associated with gestational hypothermia, would it not be that the hypothermic state would persist into lactation? Instead we see a state of uncontrolled hyperthermia at the onset of parturition and lactation. Cannon and Nedergaard (2011) hypothesized that the brown fat atrophy is caused by foetal heat production during gestation, but by lactational heat production during lactation. Still this does not explain why at one stage the mother is hypothermic and in the other she is hyperthermic.

If BAT deactivation is what is causing gestational hypothermia, then we should see a more pronounced effect at T_{22} than at T_{29}. In non-pregnant animals, brown fat activation will occur at T_{22} where nonshivering thermogenesis is required (cold-induced thermogenesis), but will not occur at the TNZ because it is not needed (Foster and Frydman, 1979; Foster, 1986; Cannon and Nedergaard, 2004).

In theory if BAT activation only occurs at T_{22} (Foster and Frydman, 1979; Foster, 1986; Cannon and Nedergaard, 2004), then conversely, its deactivation during pregnancy should only make a difference to the thermoregulatory state of those housed at $T_{22.}$ Since the results of this study show the extent of the gestational hypothermia was not different at the two ambient temperatures, it is improbable that deactivation of BAT is the driver for gestational hypothermia.

6.3.5 Angiotensin signalling pathway

One of the more likely explanations for the control of gestational hypothermia originates from a study that investigates the gestational effects of candesartan, an angiotensin AT_1 receptor antagonist (Cairns *et al*., 2004).

Circulating levels of ANGII are shown to be elevated during pregnancy (Sinnayah *et al.,* 1999; Cairns *et al.*, 2004). Angiotensin has effects on thermoregulation, as well as fluid and electrolyte homeostasis by acting through AT_1 receptors in the brain (Mathai *et al.*, 2000; McKinley *et al*., 2003). These receptors, which influence vasomotor activity, are located in both afferent and efferent pathways of the peripheral autonomic nervous system (Allen *et al.,* 1998). When pregnant rats were given candesartan, gestational hypothermia did not occur. Candesartan crosses the blood-brain barrier and inhibits central angiotensin AT_1 receptors. It was therefore hypothesized that by means of AT_1 receptor blockade, a specific thermoregulatory cooling pathway that utilizes ANGII as a signalling molecule, was inhibited, and thus body cooling did not occur.

Interestingly, pregnant rats treated with candesartan still showed the marked increase in T_{core} during birth and lactation (Cairns *et al.*, 2004), which supported the notion that gestational hypothermia is a regulated mechanism (Eliason and Fewell, 1997) one hindered by candesartan. Lactational hyperthermia is “forced” and thus not affected by the candesartan or at least not controlled by the same signalling pathway as gestational hypothermia.

Undoubtedly, this is the most promising of the proposed hypotheses, however very little is understood about how candesartan inhibits gestational hypothermia and what signalling pathway/s could be involved. What we do know is that ANGII plays a key role in noradrenergic neuroeffector transmission and that it increases the rate of

noradrenaline (NA) synthesis and enhances NA release at presynaptic sites (Story and Ziogas, 1987; Allen *et al.*, 1998). It may therefore be that ANGII has its effects via NA.

It has been reported that central administration of NA, either by direct injection into the preoptic area of the hypothalamus (Cooper *et al*., 1976), or by intrathecal injection (Lopachin and Rudy, 1982) results in a dose dependent hypothermia. Cranston *et al.* (1972) also showed that when NA stores were depleted in the cat by inhibiting the re-uptake of NA by neurons, the result was a significant rise in T_{core}.

It is possible that when Cairns *et al*. (2004) blocked AT_1 receptors and inhibited the activity of ANGII, this down regulated the synthesis and release of NA; which would normally go on to cause the gestational hypothermia. The mechanisms may be completely unrelated, but interestingly, peripheral NA plays a role in cold-induced thermogenesis of BAT of non-pregnant animals by interacting with β-adrenergic receptors to activate brown fat lipolysis (Himms-Hagen, 1984). When non-pregnant rats were exposed to cold ambient temperatures, activation of BAT occurs, and an increase in NA release triggers heat production in BAT. Mory *et al.* (1988) showed that NA not only stimulates activity in BAT, but also causes an increase to the weight of BAT.

The presence of high ANGII, and hence NA levels, during pregnancy, but absence of BAT thermogenesis, suggests that some other mechanism involving sympathetic innervation of BAT must play a role. It is also possible that central and peripheral activity of NA will have markedly different effects. NA was administered centrally in the studies of Cooper *et al*. (1976) and Lopachin and Rudy (1982) where dose dependent hypothermia was observed. It may be that hypothermia develops as a

result of central activity of NA, whereas peripheral autonomic effects of NA, as in the case of BAT activity will have the opposite effect.

An important component of BAT that plays a role in adaptive adrenergic nonshivering thermogenesis is uncoupling protein 1 (UCP1) and the levels of UCP1 decrease during gestation (Nedergaard *et al*., 2001; Golozoubova *et al*., 2004). The concentration of UCP1 can be increased by NA administration (Mory *et al.*, 1988). Again, if NA levels are elevated in the pregnant animal, UCP1 concentration should be elevated and BAT activity should be activated, however this is not the case.

Another curious finding in the study of Cairns *et al*. (2004) was that despite the interruption of gestational hypothermia in the candesartan treated group, food intake, water intake, maternal body mass, litter mass and litter size were not different from the control group. If litter mass and litter size was not influenced by the absence of gestational hypothermia, this supports the lack of relationship found in the present study between gestational hypothermia and foetal/litter mass parameters. There was no report in the Cairns *et al*. (2004) study of any foetal deaths at birth or any foetal defects. If foetal parameters are unaffected by the absence of gestational hypothermia and there appears to be no risk to the foetuses of the candesartan treated animals, then why is gestational hypothermia happening at all? This finding may imply that gestational hypothermia does not occur to protect the foetus against teratogenicity.

If foetal temperature is the regulated variable, then the changes that are happening in the foetus should be proportional to the response that is causing the gestational hypothermia. If ANGII is what is causing the gestational hypothermia, then it is possible that whatever is signalling the increase in ANGII is originating from the foetus.

However, as we see that foetal parameters (foetal mass and litter size) are not affected by the absence of gestational hypothermia, it seems unlikely that the regulated variable is foetal temperature. This is supported by the findings of the present study, that the extent of gestational hypothermia is not correlated to the foetal mass.

Each of the proposed hypotheses that aim to explain the manifestation and function of gestational hypothermia appear to have deficiencies in their arguments. The mechanisms surrounding thermoregulatory control during pregnancy are yet to be fully understood. It could be that various connections exist between the discussed hypotheses, and that gestational hypothermia results by means of an intricate combination of all or some the proposed mechanisms.

6.4 The effect of maternal thermal balance on offspring growth rates

Although litter and pup mass were recorded as a side observation, interesting findings were noted from these measurements taken during the lactation period of the current study.

Lactation is a heat-producing process and is energetically more expensive than the gestation period, due to the additional factor of milk production and the conversion of daily food intake into milk that supplies the energy and substrates for growth of the offspring. In the present study daily food intake increased from the second trimester until the day before birth. On the day of birth, daily food intake decreased and then increased again into lactation to reach the highest recorded intake. Despite the fact that there was no difference in food intake between the two temperature groups during lactation, differences in pup growth rates were observed between the two

ambient temperatures. Litter mass was not found to be different at birth, but between days L0-L2 and days L4-L6, both the entire litters and the individual pups were grew at a faster rate at T_{22} than they did at T_{29}. Maternal mass was also uncorrelated with the growth rate of litters and individual pups, indicating that growth rate had nothing to do with the size of the mother.

It seems logical that since there is no correlation with maternal mass and there was no difference in food intake between T_{22} and T_{29}, that the difference in growth rate was as a result of T_a effects, on either the thermal balance of the mother and hence her capacity to produce milk, or on pups ability to convert milk into growth.

The results of our study show similarities with a cohort of studies that investigated the limiting factors on sustained energy intake during lactation in mice (Johnson *et al*., 2001a; Johnson *et al.,* 2001b, Johnson and Speakman, 2001c; Król and Speakman, 2003a; Król and Speakman, 2003b; Król *et al.*, 2007). In the early stages of their research, there were two hypotheses for the limiting factor on energy flow through the mother to the pups.

The first hypothesis was that the limitation caused by the size of the foetal body and the pressure it exerted on the digestive organs, which they termed a central limitation. The second hypothesis was that the limitation was the capacity of the mammary tissue to produce milk, which they termed a peripheral limitation. Initially, Johnson *et al.* (2001a) found that maternal energy intake in mice reached a peak, which they referred to as an asymptotic maximum of energy intake. They believed this classified a limit on maternal energy intake during lactation.

If the central limitation hypothesis was to be true, then under no circumstances would the mother be able to increase her food intake past the asymptotic value. If the peripheral limitation hypothesis was true, then food intake would be adjusted to the required milk production. Johnson *et al*. (2001a) manipulated litter sizes and found that mothers with larger litters did not increase their food intake past the proposed asymptotic maximum. But those with smaller litters reduced their food intake. Mothers with larger litters produced more milk than those mothers with smaller litters, but the milk had a lower energy content as the volume increased.

In agreement with the present study, Johnson *et al.* (2001a) found that larger litters produced smaller pups. The conclusion was that similar energy output by mothers result in fewer larger or many smaller pups, indicating that there must exist a limit on maternal investment. Other studies have shown that despite increased food intake of mothers with larger litters, pups were still smaller, indicating that individual pup energy allocation was not increased, and in most cases had actually decreased (Innes and Millar, 1981; Mattingly and McClure, 1982; Glazier, 1985).

Both of the proposed limitation hypotheses were supported by the findings of Johnson *et al*. (2001a), because mothers did not increase their food intake past the asymptote despite an increase in litter size (supporting the central hypothesis), but in smaller litters the mothers were able to adjust their food intake to suit milk energy output (supporting the peripheral hypothesis). Johnson *et al*. (2001b) then attempted to separate the two hypotheses by manipulating mice to be concurrently lactating and on their second pregnancy. During lactation of the first pregnancy and gestation of the second pregnancy, mice were fed a diet with low-energy content and consumed more

food, exceeding the asymptotic maximum, disproving the central limitation hypothesis. The gut was not the limiting factor by restrictions caused by the foetus(es).

For the peripheral hypothesis to be true, the maximal capacity of the mammary tissue to produce milk should not be altered by T_a. To test this theory Johnson and Speakman (2001c) housed lactating mice at 8°C and 21°C. At 8°C, mothers showed an increase in energy intake and an increase in the volume and energy content of milk, suggesting that the energy flow to the pups was increased when T_a was lower, partly showing that the mammary tissue was not a limit. But at the lower T_a there was a higher mortality in their pups. As a result there were smaller litter masses at the lower temperature. These results again disproved the central limitation hypothesis, because food intake increased, but also disproved the peripheral limitation hypothesis, because it showed that the mammary tissue had a finite capacity that limited energy flow. Increased milk energy could be as a result of increased energy demands of the offspring in the cold, but Johnson and Speakman (2001c) found upon weaning, that the pups at 8°C had a higher average body mass than at 21°C.

With both hypotheses found wanting, an alternative hypothesis was required and Król and Speakman (2003a) proposed the novel hypothesis that the capacity of an animal to dissipate the heat generated during lactation was the limit on sustained energy intake. They tested the theory by exposing lactating animals to thermoneutrality (30°C), which would reduce the thermal gradient available for the lactating mother to dissipate heat to its environment by limiting the heat flow across the thermal gradient. They recorded the same variables as in the Johnson and Speakman (2001c) study, and compared these results with the ones attained at 21°C and 8°C. The lactating mice kept at thermoneutrality had a lower asymptotic food intake than the females at both the

lower temperatures, had fewer and smaller pups and smaller litter masses than the mothers at 21°C, and had smaller pups than the mothers at 8°C. At 30°C, the milk produced had a lower energy content than at the two lower temperatures, both in terms of the volume of milk and the gross energy content (solids and fats) (Król and Speakman, 2003b). These results supported the heat dissipation limitation hypothesis, however they did not distinguish whether the limitation exists at the maternal level (intrinsically related to the heat dissipation capacity of the mother) or at the level of the offspring (extrinsically related to the heat dissipation capacity of the homeothermic pups).

Król *et al.* (2007) manipulated the maternal thermal balance by shaving the fur of lactating mice. Mammalian fur provides the animal with adequate species-appropriate insulation and reduces heat loss (Scholander *et al*., 1950; Hammel, 1955). Previous studies in hamsters that manipulated thermal insulation by means of shaving the fur, showed a marked increase in the total energy expenditure and a rise in food intake (Kauffman *et al*., 2003).

Król *et al*. (2007) housed two groups of mice at 21°C, and dorsally shaved one group to increase their capacity to lose heat to the environment by reducing the amount of thermal insulation provided by the fur. The shaved mice had a higher their food intake, exported more energy in the form of milk, and ultimately raised heavier litters and heavier pups than their unshaven counterparts. Maternal body mass did not differ between the shaved and unshaved mice and was not correlated with milk production. However, there was a correlation between milk energy output and the growth rate of both the pups and the litters.

So what does this mean for our study? The present study bares similarities to this cohort of studies. Having only recorded food intake and pup mass data to day L6 of lactation, accurate conclusions cannot be drawn on what may have occurred at peak lactation, but some of the more interesting congruencies are worth noting.

During the Król *et al*. (2007) study, there was an increase in the food intake of shaved mice compared to unshaved mice. The present study housed mice at thermoneutrality (T_{29}) and below thermoneutrality (T_{22}), which would have affected the thermal balance of the mice in a similar manner to shaving. However in the 6 days of lactation, there was no difference in food intake between the two ambient temperatures. Although no definitive conclusions could be made, perhaps if our mothers had been allowed to rear their young until weaning, a divergence in the food intake of the two groups may have been seen, possibly with an elevated food intake in the T_{22} mice.

Król *et al*. (2007) found that from day L12 onwards, litter masses were heavier in the shaved mice. Our study found an immediate difference in growth rate and litter mass between mice housed at T_{22} and T_{29}, with growth rates being faster and pups being heavier at the lower T_a. Why immediate effects on growth rate were observed in the present study, yet Król *et al.* (2007) only found differences from day L12 onwards is uncertain. The difference of the two housing temperatures could have had a more pronounced effect on thermal balance than having shaven and unshaven animals at the one temperature, yielding immediate effects on pup growth. Most important to our findings however, is that the litter growth rate was fastest at 21^{o}C in Król *et al.* (2007), which is what was found in the present study.

6.5 Limitations and caveats

As mentioned previously, a repeat of these experiments would carry the experiment to the end of lactation until offspring were due to be weaned. Although this study yielded some interesting data post-parturition that followed similar trends to other studies, the lactation data did not span the full length of lactation. Thus any observations that were made may not be an accurate representation of what data full lactation may have yielded in these mice.

Several studies to which reference has been made in this thesis have measured metabolic rate (by measuring oxygen consumption) and energy utilization (both input by food intake and output by milk energy content). Because lactation was not the focus of the present study these parameters were not measured. Although food intake and measures of pup growth rate were obtained, the metabolic rate, energy utilization and energy of milk output is important in the full understanding of the conversion of maternal daily energy intake into growth of her offspring.

In the present study there was no difference in food intake between T_{22} and T_{29}. Maternal mass was also not different at the two ambient temperatures. It is possible that this had something to do with the behavioural thermoregulation performed by mice with their nesting material.

As part of our ethics approval it was agreed that the mice would be housed with the facility-approved nesting material. Each mouse was provided with shaven aspen bedding, a toilet roll, a red Perspex house and shredded tissues. With this material the mice would easily have been able to build an effective nest, with which they could

behaviourally thermoregulate. Mice housed at lower ambient temperatures can behaviourally thermoregulate with the use of nesting material to effectively bring their ambient environment close to thermoneutrality (Gordon *et al*., 1998). In other studies, mice that were provisioned with nesting material at T_{22} did not have food intake higher than at TNZ (Dahlborn *et al.,* 1996; Olsson and Dahlborn, 2002), but we know that mice housed an normal animal housing ambient temperatures show a marked increase in their basal metabolic rate (Yamauchi *et al*., 1983; Gordon, 1985; Feldmann *et al*., 2009; Cannon and Nedergaard, 2011) and their food intake (Feldmann *et al.*, 2009; Golozoubova *et al*., 2004). The subsequent maternal and offspring parameters may have thus been different had there been no nesting material, namely we may have seen mothers of a larger mass at T_{22} due to an increase metabolic rate and food intake, and litter masses at the time of birth may also have been larger at the colder T_a.

It appears that nesting material has been a serious confounder to this study and although it may explain that no difference was observed in maternal food intake and body mass, it cannot explain why differences were still observed in the growth rate of the pups at the two ambient temperatures. The most logical explanation for these disparities is that during pregnancy the mice were making use of nesting material to adjust their microclimate because they were energetically challenged by the cold and still required the use of behavioural thermoregulation. However, during lactation when forced hyperthermia initiates and the maternal metabolic rate is highest, behavioural thermoregulation with nesting material may have been abandoned. If suddenly nesting material does not play a role in maintaining the microclimate of the mother, T_a would then affect maternal thermal balance.

Coupled with the heat load being generated by the pups, lactational hyperthermia has been found to influence the termination of nest bouts, with animals with higher T_{core} being found to decrease the amount of time spent in their nests with their pups (Scribner and Wynne-Edwards, 1993).

To accurately observe the effects of each T_a on the maternal thermal balance throughout gestation and lactation, one would need to completely remove the nesting material.

CHAPTER 7 – CONCLUSION

In summary, the data generated in the present study confirm that gestational hypothermia occurs in mice housed at both thermoneutral (T_{29}) and sub thermoneutral (T_{22}) ambient temperatures. Maternal T_{core} decreased to its nadir at the end of the final trimester of gestation, and then rapidly increased on the day of birth and into lactation.

The daily amplitude of the T_{core} rhythm decreased over the course of gestation, which implies that the mice ultimately become more homeothermic towards the end of gestation, indicating that their T_{core} is better regulated, with less overall fluctuations in T_{core} as gestation progresses. This supports the suggestion that gestational hypothermia is a regulated response.

Ambient temperature had no effect on the extent of the gestational hypothermia. Foetal mass and thus foetal heat production was not proportional to the magnitude of the gestational hypothermia. This tells us that foetal temperature is most likely not the regulated variable and that the input signal for gestational hypothermia is not originating from the foetus. It is therefore probable that gestational hypothermia is being caused by some other mechanism or signalling pathway, and the most promising of the ones discussed was that of the angiotensin signalling pathway.

Although litter size was the same at the two T_a at birth, pups grew faster at sub thermoneutral than at thermoneutral temperatures. It is possible that T_a affected the thermal balance of the mothers in such way that those housed at thermoneutral temperatures could not dissipate heat during lactation as easily as those at T_{22}. This

result suggests a thermodynamic limit on the ability to convert energy intake into pup growth during lactation.

Maternal mass and food intake exhibited the predictable trends associated with gestation, but were not different between the two ambient temperatures at any of the gestational stages.

Nesting material appears to have been a confounder in this study and likely influenced the thermal balance of the mice housed at T_{22}, especially during gestation. Had nesting material been removed, we might have seen effects of T_a on gestational hypothermia. We also expect that we would have seen a higher food intake and larger maternal body mass in the mice housed at T_{22}. We expect that had mice been housed at T_{22} been without nesting material from the beginning of the experiment, they may have started with an overall lower T_{core}. Thus the change in T_{core} due to gestational hypothermia may have been to the same extent in both T_a groups, but we would have seen an overall downward shift in the overall maternal temperatures at T_{22} compared to T_{29}.

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