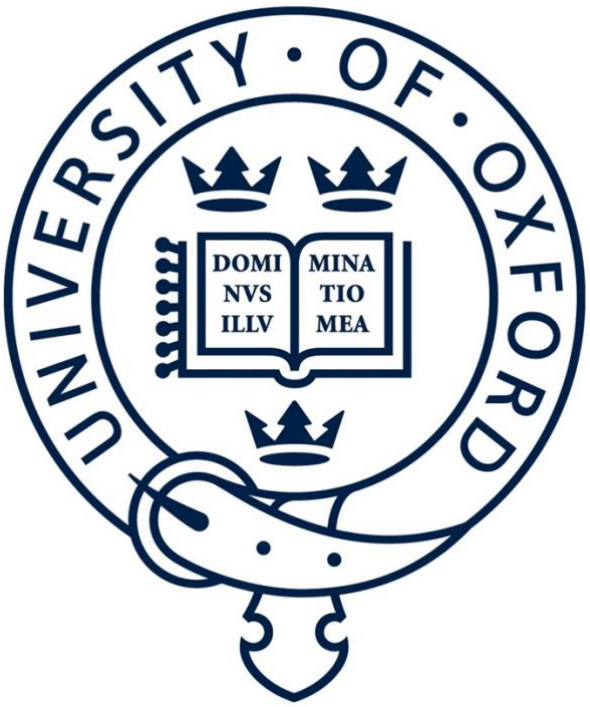
Fasting-induced torpor in mice: effects on sleep and behaviour



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Abstract

Torpor is a state of profoundly altered physiology, characterised by metabolic suppression and concurrent hypothermia. Relatively little is understood about torpor, and few researchers are aware that laboratory mice enter this state in response to limited food availability. The aim of this thesis was to further the understanding of torpor by investigating its interaction with sleep and the circadian system, and the effect of torpor on behaviour. Further, a secondary aim was to investigate the potential for torpor to be a cofounding factor in studies that use fasting techniques. First, this thesis characterised torpor induction and propensity in response to a common food restriction paradigm. Meal timing was found to significantly alter torpor characteristics, providing further evidence for the circadian control of torpor. As this food restriction protocol is commonly used within behavioural neuroscience, the effect of torpor induction on performance in behavioural tasks was investigated. This revealed that time of testing relative to torpor significantly impacted locomotor activity. Next, the relationship between sleep and torpor was investigated using chronic electroencephalography, and sleep deprivation and auditory stimulation techniques. Brain activity during torpor closely resembled sleep, although sleep-wake architecture was profoundly altered in the presence of torpor. An increase in sleep intensity following torpor was observed, suggesting that torpor may be a sleep depriving state. For the first time, auditory evoked responses were recorded in torpid mice, revealing a marked similarity with responses recorded during sleep, although response dynamics were found to differ between sleep and torpor. Finally, this thesis presents pilot data which suggests that water restriction does not result in torpor induction in mice, indicating that water restriction may be a suitable alternative to food restriction to avoid torpor. Overall, this thesis provides novel insights into the characteristics, neurophysiology, and regulation of fasting-induced torpor in mice. Moreover, the data presented here highlight the potential for torpor to be a potential confound in research using fasting techniques if not adequately controlled for.

Thesis word count: ~50,000

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Declaration and attributions

I declare that no part of this thesis has been, or is being, submitted for any qualification other than the degree of Doctor of Philosophy at the University of Oxford.

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Glossary and abbreviations

Circadian rhythm: a biological rhythm, approximately 24 hours in length (from Latin, about a day)

Ectotherm: any animal whose regulation of body temperature depends on external sources

EEG: electroencephalography

EMG: electromyography

Endotherm: an animal that is capable of internally generating heat

Fasting-induced torpor: the induction of torpor in response to limited food availability

FFT: Fast Fourier Transform

Heterotherm: the ability of an animal to alter its body temperature set point

Homeotherm: maintenance of body temperature at a constant level

NORT: novel object recognition task

NREM sleep: non-rapid eye movement sleep

OFT: open field task

Photoperiod: the period of light over a 24 hour day

Photoperiod induced torpor: induction of torpor in response to a shortened photoperiod

Preceding sleep-wake history: vigilance states/behaviours occurred prior to the time of interest

REM sleep: rapid eye movement sleep

RER: respiratory exchange ratio

SCN: suprachiasmatic nucleus/nuclei – the master circadian clock

Sleep homeostasis: regulation of sleep need

Slow oscillation: bi-stable slow (<1 Hz) oscillatory alternation in membrane potential

Slow wave: regular waveform occurring at the SWA frequency range

Spectral analysis: how much specific frequencies contribute to a signal

Spectral power density: the measure of signal's power content versus frequency

SWA: slow wave activity (0.5-4 Hz)

SWE: slow wave energy; the cumulative SWA as a percentage of the maximum SWA

Synaptic plasticity: change in the strength of synapses

The 3RS: the principles of replacement, reduction, and refinement of the use of animals in research

Torpor: a sustained and controlled suppression of metabolic rate, accompanied by hypothermia

Two-process model: propensity to sleep is determined by the interaction between both a circadian (process C) and a homeostatic component (process S)

Vigilance state: states can be classified as wake, NREM sleep, or REM sleep

VO_2: volume of oxygen consumption

Zeitgeber: cues from the environment (giver of time)

Chapter 1: Introduction

1.1 An introduction to torpor

The term ‘endothermy’ or ‘endothermic’, is used to refer to an animal’s ability to internally generate body heat via metabolic reactions, as opposed to ectotherms that rely of external heat sources from the environment. Endothermic homeotherms maintain a constant core body temperature around a homeostatic set point, typically between 35-37ºC, allowing for optimal function of physiological processes to be maintained. Maintaining a constant core body temperature is metabolically demanding and requires sufficient energy levels to be maintained, either through food consumption or via the breakdown of energy stores. This can be especially challenging for smaller species which have a high surface area to volume ratio, and therefore lose a larger degree of body heat to the environment (Geiser, 2004). Despite these challenges, maintaining a high core body temperature has enabled endothermic homeotherms to exploit a wide variety of ecological niches that ectothermic species cannot.

A subset of endotherms across multiple taxa, termed heterothermic endotherms, have developed the ability to enter torpor, during which thermoregulatory and metabolic set points are significantly lowered (Geiser, 2004; Melvin & Andrews, 2009). These periods of metabolic suppression allow for energy requirements to be reduced by up to 90% in some species (Heldmaier et al., 1999), therefore providing a significant evolutionary advantage for surviving periods of harsh environmental conditions. Indeed, a study that investigated extinction rates across different lineages suggested that the ability to enter torpor was able to predict the likelihood of a species becoming extinct, with fewer extinctions occurring in lineages where torpor is utilised (Geiser & Turbill, 2009; Ruf & Geiser, 2015).

There are multiple types of torpor that exist across endothermic heterothermic species; the ones of most relevance to this thesis include hibernation, photoperiod-induced daily torpor, and fasting-induced daily torpor. Although torpor itself refers to an active suppression of basal metabolic rate, accompanied by a concurrent drop in body temperature (Figure 1A), these types of torpor have important differences in their expression, regulation, and underlying physiology.

Hibernation, sometimes referred to as multi-day torpor, is characterised by torpor bouts being sustained over multiple consecutive days lasting from weeks to months in response to seasonal changes (Ruf & Geiser, 2015). The long torpor bouts may also be interspersed with brief interbout arousals during which metabolic rate and body temperature return to pre-hibernation levels (Daan et al., 1991). The purpose of these interbout arousals is unknown but they have been suggested to be important for maintaining optimal immune function and prevention of infections (Bouma et al., 2010), and for sleep (Daan et al., 1991) . Indeed, brain lesions that prevented the occurrence of interbout arousals was found to be fatal (Satinoff, 1967). Due to the length of torpor bouts during hibernation, metabolic rate and body temperature reach much lower levels when compared to shorter forms of torpor. For example, core body temperature has been reported to drop as low as -2.9^{o}C in hibernating arctic ground squirrels (*Spermophilus parryii*) (Barnes, 1989).

In contrast to hibernation, other species enter much shorter bouts of torpor. This form of torpor is termed daily torpor, due to bouts typically lasting less than 24 hours (Ruf & Geiser, 2015). Bouts of daily torpor follow a predictable cyclical pattern, occurring at approximately the same time each day. It should, therefore, be unsurprising that torpor has been shown to be closely linked to the circadian system (Heller & Ruby, 2004) and appears to be entrainable to both light and food timing cues (Paul et al., 2004; van der Vinne et al., 2018). Daily torpor can be

further categorised into photoperiod-induced torpor and fasting-induced torpor. Photoperiod-induced torpor describes the occurrence torpor in response to a short photoperiod such, as an 8:16 light-dark cycle. A short photoperiod is associated with winter conditions and is therefore considered to be a response to anticipated shortage of food, thus requiring the employment of energy saving mechanisms. It is thought that the advantage of this form of daily torpor compared to hibernation is that it maximises available opportunities to forage for food.

Fasting-induced daily torpor occurs in response to an actual shortage of food that results in the animal being in an energy deficit. In order to reduce the energetic needs of the animal, torpor is induced until energy balance is restored (Heldmaier et al., 2004); critically, fasting-induced torpor is not seasonal and occur in response to any food shortage. It has been well established that the house mouse (*Mus musculus*), including laboratory mice, enter short, and relatively shallow, bouts of torpor in response to fasting (Hudson & Scott, 1979). Despite mice being one of the most popular model species within biomedical research, with the UK Home Office reporting that ~70% of returned procedures in 2021 were conducted in mice (Annual Statistics of Scientific Procedures on Living Animals Great Britain 2021), the implications of torpor induction in response to fasting have not been well characterised. This is of note as fasting and food restriction are common techniques used within behavioural neuroscience, metabolic studies, and drug studies, where the ability of mice to enter torpor may not be common knowledge. Consequently, the extent to which inadvertent torpor induction in mice is impacting on the data generated during these studies is mostly undetermined. As such, torpor in laboratory mice will be the focus of this thesis; however, comparisons to torpor in other species will be included where relevant, or where data in mice are limited.

1.1.1 Torpor in the laboratory mouse

Mice, including laboratory mice, are a species that enter daily torpor in response to an actual food shortage (Jensen et al., 2013). Torpor in mice is driven by significant metabolic suppression, with reports of basal metabolic rate being reduced by up to 70% compared to baseline (Brown & Staples, 2010), which is accompanied by hypothermia that can reach close to ambient temperature, with a nadir of 20^{o}C have been previously reported (Hudson & Scott, 1979). The drop in core body temperature is thought to occur due to a suppression of thermogenesis and an alteration to the thermostatic set point of the animal (Sunagawa & Takahashi, 2016). The hypothermia observed during torpor bouts differs mechanistically from other forms of hypothermia and the two states show different dynamics (Figure 1). For example, torpor is controlled, and the onset is significantly quicker than for hypothermia (Geiser et al., 2014). Moreover, torpid animals rewarm using non-shivering thermogenesis via increased brown adipose tissue activity (Nowack, Giroud, et al., 2017), whilst hypothermia requires passive rewarming from the environment (Geiser et al., 2014).

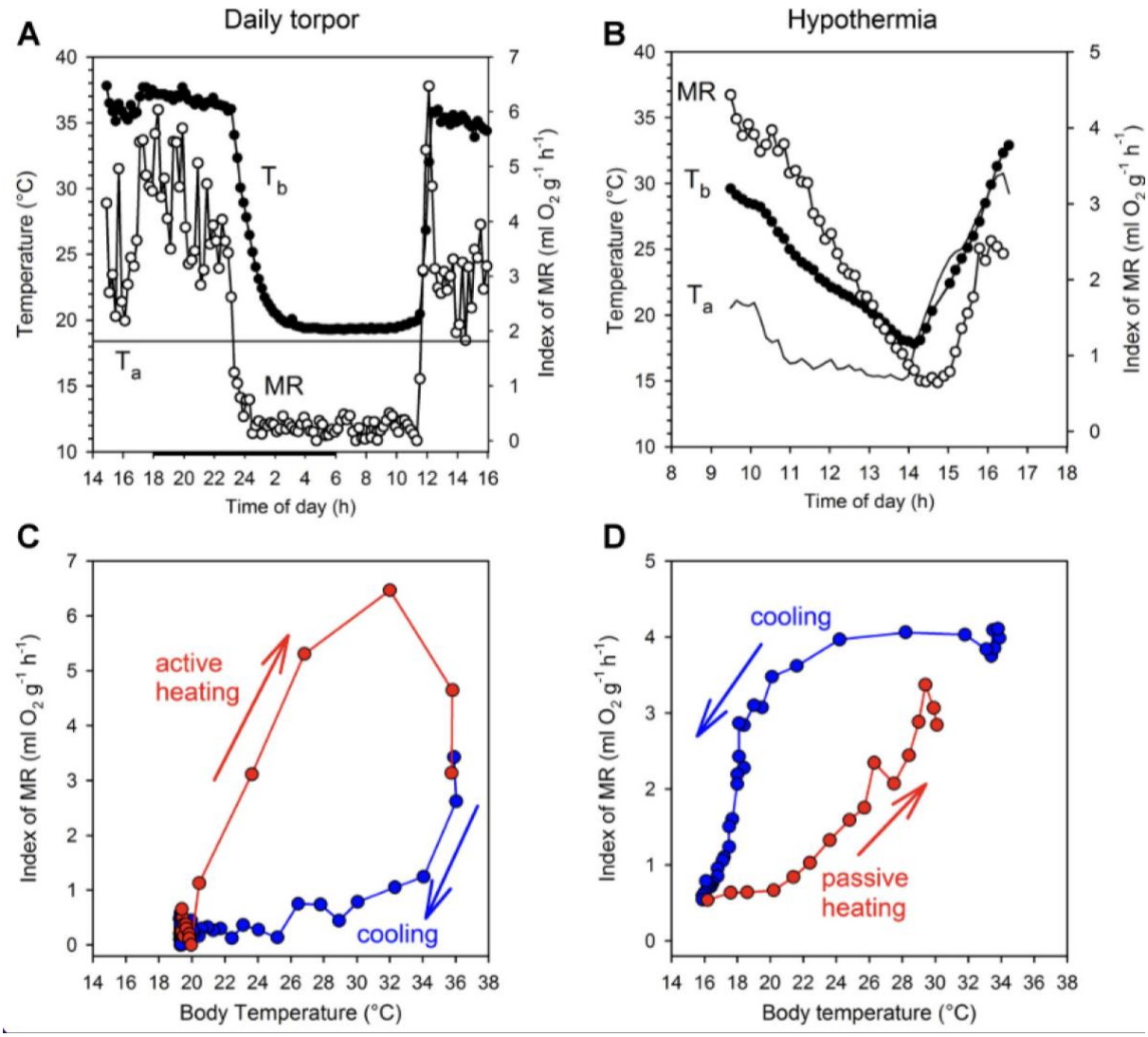

Figure 1: Comparison of metabolic rate (MR) and body temperature dynamics (Tb) between daily torpor in a heterothermic species of dunnart (A,C) compared to hypothermia in a strictly homeothermic species of rat (B,C). Figure from Geiser et al 2014.

Due to the small size of mice, a relatively large proportion of their internally generated body heat is lost to the environment with an estimated 30% of a mouse's energy budget spent on thermogenesis at ambient temperatures of 22ºC (Abreu-Vieira et al., 2015). Moreover, laboratory mice are typically housed well below their thermoneutral zone of ~30ºC (Lodhi & Semenkovich, 2009), which therefore increases the degree of passive heat loss and the metabolic demand required to maintain body temperature near the homeostatic set point. As such, laboratory mice are particularly sensitive to food shortages with complete removal of food reportedly resulting in torpor within as little as 5-6 hours (Hudson & Scott, 1979; Jensen

et al., 2013). Further reducing ambient temperature to 15^{o}C in combination with fasting has been reported to induce torpor in mice within 2 hours of cold exposure (Hitrec et al., 2019; Oelkrug et al., 2011). Other methods of increasing energetic demands will also result in torpor induction, for example, a work-for-food paradigm in which mice were required to run on a wheel to obtain food resulted in torpor when the number of wheel revolutions required to earn food was increased (Schubert et al., 2010).

The physiology of a torpid mouse is profoundly altered at the molecular, cellular, and systemic level, compared to a euthermic mouse (Melvin & Andrews, 2009). It is unclear which of these physiological changes are responsible for triggering torpor, and which occur as a result of torpor, or if there is a bidirectional relationship. For example, levels of circulating metabolic hormones such as leptin, ghrelin, neuropeptide-Y, and thyroid hormones, are significantly altered during torpor. However, these hormones can also modulate torpor propensity and depth, and may also play a role in controlling entry into torpor. Initiation into, and emergence from, torpor requires modulation of sympathetic and parasympathetic outputs, with sympathetic activation of white fat (Braulke & Heldmaier, 2010; Swoap et al., 2006; Swoap & Weinshenker, 2008). Conversely, administration of leptin prevents fasting induced torpor (Gavrilova et al., 1999), whilst leptin-deficient Ob/Ob mice have been reported to spontaneously enter torpor during ad libitum feeding (Webb et al., 1982). Conversely, ghrelin, the so called ‘hunger hormone’, deepens torpor bouts (Gluck et al., 2006). Moreover, a study investigating glucose availability reported that circulating glucose levels were low prior to entry into torpor, and then rose during arousal from torpor (Lo Martire et al., 2018). These findings suggest that nutritional status plays an important role in the induction and modulation of torpor bouts in response to fasting. Further, these findings highlight the complex and interrelated nature of physiological processes during torpor that will be occurring during

studies that use fasting in mice. It is unclear whether this profoundly altered state of physiology is being accounted for when investigating metabolic processes, or the pharmacokinetic/pharmacodynamic properties of novel pharmaceuticals.

Due to the characteristic metabolic suppression that occurs during torpor, a notable depression of the cardiovascular and respiratory systems also occurs. Heart rate drops from ~600 bpm during euthermia to ~150 bpm during torpor (Swoap & Gutilla, 2009), a much greater reduction than what can be achieved with pharmacologically induced hypothermia (Vicent et al., 2017), indicating that active suppression of heart rate is occurring during torpor rather than being a by-product of metabolic depression (Vicent et al., 2017). Similarly, the respiratory system has been shown to be supressed during torpor in pocket mice (Withers, 1977) and dormice (Elvert & Heldmaier, 2005), with as little as 30-40 breaths per minute occurring during torpor in the dormice, compared to 300-350 breaths per minute during euthermia.

During torpor, there are significant changes to the expression of certain genes (Gautier et al., 2018) and proteins (Hindle et al., 2014), allowing cellular and physiological processes do adapt to the significant metabolic suppression and hypothermia during torpor, without causing ill effect such as hypoxia or reperfusion injury. For example, cold-inducible RNA-bindings proteins are upregulated during torpor, which are thought to facilitate protein synthesis under conditions that would usually be too cold for optimal translation, and to have neuroprotective properties (Andrews, 2019). Further, there is also a shift in metabolic gene expression resulting in a switch from carbohydrate metabolism to in favour of fatty acid metabolism during torpor, likely to enable the utilisation of stored energy in the absence of food (Carey et al., 2003).

This change in fuel utilisation during torpor can be measured via a respiratory quotient (RQ) determined using indirect calorimetry. The RQ is a ratio between carbon dioxide production and oxygen consumption, which drops from 1 at euthermia to ~0.7 during torpor (Diedrich et al., 2015; Heldmaier et al., 1999; Nestler, 1990; Satoh et al., 2006). An RQ close to 1 indicates that carbohydrates are being used as the main energy source whereas an RQ of 0.7 indicates that energy is being generated from lipolysis instead (Even et al., 2012; Speakman, 2013).

There is also evidence that torpor has significant central effects, in addition to the peripheral effects previously described. Studies investigating synaptic and dendritic morphology surrounding torpor bouts indicate a significant change in plasticity in both hibernators, and hamsters entering photoperiod-induced daily torpor. These studies report a transient reduction in the number of dendritic spines and connections between synapses in the hippocampus (Popov et al., 1992), hippocampal mossy fibres (Popov & Bocharova, 1992), and in higher regions of the brain, including the cortex (Von Der Ohe et al., 2006).

The advent of new technologies has enabled the investigation of underlying neural circuits that may be involved with initiating and modulating torpor. For example, recent studies have used chemogenetic techniques to identify neurones in the preoptic area (POA) of the hypothalamus that are active during torpor and when stimulated, induce a hypothermic state similar to torpor (Hrvatin et al., 2020; Zhang et al., 2020). The POA has previously been associated with thermoregulation (Zhao et al., 2017) and contains leptin receptors so is sensitive to changes in nutritional status (Park & Ahima, 2015), making the POA an ideal candidate for central torpor regulation. Moreover, chemogenetic activation of neurons in the dorsal medial hypothalamus, which receives projections from the POA, resulted in modulation of torpor depth, length, and propensity (Ambler et al., 2022). Finally, a study utilising DREADDS (designer receptors

exclusively activated by designer drugs) to target activation of hypothalamic pyroglutamylated RFamide peptide (Qrfp) expressing neurons in the medial POA and the anteroventral periventricular nucleus, resulted in a torpor-like state in mice, characterised by prolonged hypothermia and cardiorespiratory suppression (Takahashi et al., 2020).

It is unclear how these central effects translate to behavioural changes during and surrounding torpor bouts. Moreover, although the structural changes in synaptic and dendritic morphology observed during torpor are rapidly reversed within two hours following arousal from torpor (Popov & Bocharova, 1992; Von Der Ohe et al., 2006), it is unknown whether there are any long term impacts on behaviour. Published work investigating the effect of torpor on behaviour is mixed depending on the species and paradigm used, with very few studies focusing on fasting-induced torpor in mice. One study that did use fasted mice investigated spatial working memory using a Morris water maze, wherein mice were trained to find a submerged platform, during the test trial, the platform was removed, and swimming patterns were analysed. The mice used were fasted at a cool ambient temperature (19^{o}C) to induce a single bout of torpor and were found to perform as well as non-fasted controls that had not entered torpor. Conversely, mice that were fasted at warm temperatures, and non-fasted mice housed at cool temperatures, both of which did not enter torpor, performed worse in comparison to mice that had entered torpor, therefore suggesting that torpor protects against memory disruption (Nowakowski et al., 2009). However, a study in Djungarian hamsters found that recognition memory was impaired during a novel object recognition task when hamsters were tested shortly following torpor (Palchykova, Crestaet al., 2006) . A more recent study conducted in hibernating golden mantled squirrels found that torpor did not significantly influence spatial memory when animals were tested in a Barnes maze following hibernation that they had previously been trained in before entering hibernation. However, this study reported a high

degree or variation in performance which may have contributed to the inconclusive outcome (Hensleigh et al., 2022). In contrast, a long bout of hibernation over the winter in a similar species, the European ground squirrel, was found to significantly impair performance in a previously learned spatial memory maze task in which the squirrels had to find a food reward via the shortest path with no errors in <1 minute. A control group of squirrels that had not entered hibernation required significantly fewer trials to relearn the task in comparison, suggesting that hibernation negatively affected memory retention (Millesi et al., 2001).

Taken together, these studies demonstrate how profoundly altered torpid physiology is compared to non-torpid physiology. As such, physiological measurements taken from a torpid and a non-torpid mouse will not be comparable, however, the lack of awareness that mice are able to enter torpor may very well mean that such comparisons are often being made inadvertently.

1.1.2 Measuring and defining torpor

Despite torpor representing a significantly altered state of physiology, a robust set of criteria for defining torpor have not been agreed upon within the field (see Barclay et al., 2001 for a more in depth overview). Attempts at defining torpor often apply a binary outlook onto an animal as being in torpor or not, whereas in reality torpor exists as a continuum. For example, Huang et al. reported that fasted mice show shallow dips in their peripheral body temperature which increase in length and depth as fasting progresses (Huang et al., 2021). Consequently, a binary definition disregards torpor as a variable state with intermediate values, and so transition states are ignored.

One of the most popular methods for defining torpor is by measuring body temperature, due to the predictable bout of hypothermia that occurs. Methods of obtaining body temperature measurements vary from more invasive, such as surgically implanted telemeters (Bechtold et al., 2012; Sunagawa & Takahashi, 2016) or rectal probes (Geiser & Masters, 1994), to less invasive thermal imaging either as snapshots (Hitrec et al., 2019) or as continuous measurements (Huang et al., 2021; van der Vinne et al., 2020). Each method has its own set of pros and cons, as reviewed in detail by Meyer and colleagues (Meyer et al., 2017). However, for definitions of torpor, continuous non-invasive methods are preferred in order to capture the progressive entry into and exit from torpor without disturbing the animal.

A number of torpor studies use surgically implanted temperature-sensitive telemeters to allow for continuous recordings of core body temperature (Tb). Many of the common definitions of torpor have been developed using this method with some examples including, a Tb of <34^{o}C when preceded by 15 minutes of cooling (Iliff & Swoap, 2012), a Tb of <32^{o}C (Diedrich et al., 2015), a Tb of <31^{o}C (Kato et al., 2018a), a Tb of <31^{o}C that is sustained for at least 30 minutes (Brown & Staples, 2010) and a Tb of <26^{o}C for at least 6 hours (de Veij Mestdagh et al., 2021). Despite these studies using core body temperature to define torpor via a similar method, these definitions are very different from one another, therefore highlighting the lack of consensus that exists in the literature.

Temperature telemeters may also be limited by the requirement for surgical implantation by highly trained researchers, and the need for sufficient time for the animals to recover from surgery, therefore limiting throughput. Moreover, invasive surgical procedures negatively impact the welfare of the animal, requires researchers to be highly trained to perform surgical

procedures, and may require animals to be singly housed following surgery to ensure healing, depending on the institution (Meyer et al., 2017).

More recently, methods of recording peripheral body temperature (Tskin) to detect torpor are starting to become more prominent in the literature due to their non-invasive nature, therefore improving welfare and removing the need for surgery. However, thermal imaging often also necessitates single housing and is less well established for torpor detection which has resulted in a lack of consensus for defining torpor using this method. Recent examples used visual assessment of infrared thermal images to determine whether mice were in torpor (Hitrec et al., 2019). However, this method does not use a clear criteria for defining torpor and is up to the discretion of the observer, therefore limiting replication and reproducibility. Other users of thermal imaging have attempted to establish a threshold for torpor, similar to definitions using core body temperature measurements. For example, Huang et al defined torpor as when peripheral body temperature had decreased by >3 standard deviations below the median euthermic Tskin for >1 hour (Huang et al., 2021), whereas Ambler et al used a threshold of a reduction in peripheral body temperature by >4 standard deviations below the mean euthermic temperature for >1 hour (Ambler et al., 2022). An additional benefit of these definitions is that it allows for individual thresholds to be established for each mouse, enabling inter-individual variation in torpor characteristics, due to differences in size or body composition, to be accounted for.

Another widely used method for torpor detection uses the measurement of metabolic parameters, such as energy expenditure or oxygen consumption. As torpor is thought to be driven by the active suppression of metabolic rate and precedes hypothermia, some view metabolic recordings as the gold standard for measuring torpor. However, measuring metabolic

rate often requires specialist equipment with limited throughput, therefore reducing its utility. Similar to definitions using body temperature measurements, a large degree of variation exists when using metabolic rate. One definition states torpor to be when metabolic rate is 25% lower than expected for normothermic inactive animals measured during the daytime at the same ambient temperature (Hudson & Scott, 1979), which requires initial metabolic phenotyping to establish what the expected metabolic rate is. Another study noted that metabolic rate increases to a peak just prior to torpor entry when metabolic rate starts to decrease and used this peak to mark the beginning of torpor in hamsters (Heldmaier et al., 1999). Others combine metabolic and temperature recordings, such as a study by Oelkrug et al who determined mice to be in torpor when oxygen consumption had decreased to <25 ml per hour for more than two hours, in addition to when Tb <30ºC (Oelkrug et al., 2011).

Overall, there is little consensus for defining torpor and definitions can vary greatly depending on the species, the method being used to measure torpor and induce torpor, and the study design and experimental question. Despite this, commonalities include a significant drop in body temperature or metabolic rate for an extended period of time in order to prevent daily fluctuations in these parameters being defined as torpor. As such, conceptualisation of a working definition of torpor should attempt to capture these components.

1.2 Torpor, sleep, and the circadian system

1.2.1 The relationship between sleep and torpor

Torpor is often described as an evolutionary extension of sleep due to both states being adaptive, reversible states, in which a stereotypical posture is assumed, and sensory acuity is reduced (Heller & Ruby, 2004; Schmidt, 2014; Silvani et al., 2018). Moreover, sleep represents

a modest energy saving strategy owing to a small reduction in body temperature and metabolic rate, whilst torpor provides a more specialised method for energy conservation (Lesku & Schmidt, 2022; Schmidt, 2014). Further, electrophysiological recordings of brain activity using electroencephalography (EEG) have revealed that torpor is entered via sleep, specifically non-rapid eye movement (NREM) sleep (Vyazovskiy et al., 2017). In addition, brain activity during torpor closely resembles NREM sleep which is predominantly comprised of slow oscillations between 0.25-4 Hz (Deboer & Tobler, 1995; Huang et al., 2021). The frequencies present in the EEG during torpor are slightly slower than during euthermic sleep. Despite this, it is often not clear where sleep ends and torpor begins when using EEG recordings alone, and so body temperature or metabolic recordings must be used also (Huang et al., 2021).

Despite the similarities between sleep and torpor, it has been suggested that torpor is not conducive to enabling the restorative processes that are thought to typically occur during sleep. For example, Silvani and colleagues argue that the frequency shift in brain oscillations during torpor results is a non-physiological state that is distinct from NREM sleep, despite being visually similar (Silvani et al., 2018). This is supported by experimental evidence in hamsters which showed that, following torpor, there is a period of high intensity NREM sleep which dissipates over time (Vyazovskiy et al., 2017). Such sleep dynamics are usually associated with prolonged wakefulness and an accumulation of sleep pressure, following which a 'sleep rebound' is observed (Deboer & Tobler, 1994). Moreover, multi-day torpor during hibernation is interspersed with brief interbout arousal periods, which are typically spent in deep sleep (Daan et al., 1991; Trachsel et al., 1991). Further, rapid-eye movement (REM) sleep is virtually absent during deep torpor which is thought to be due to REM sleep being a temperature dependent process (Deboer, 2002; Deboer & Tobler, 1995); therefore, the hypothermia that accompanies torpor is not conducive for the expression of REM sleep. REM sleep is a normal

part of a typical sleep cycle and, although we are not sure of the exact purpose of REM sleep, it is highly conserved between species, indicating its importance. Selective deprivation of REM sleep is associated with deficits in cognitive processes such as learning and memory (Aleisa et al., 2011; Wiesner et al., 2015). Altogether, the sleep rebound following torpor and the absence of REM sleep has led to the hypothesis that sleep pressure accumulates during torpor which must then be cleared during deep sleep upon arousal, which would also explain the deep sleep observed during interbout arousals in animals undergoing long periods of hibernation.

Sleep deprivation is associated with a variety of poor health outcomes including reduced motor performance (Varga et al., 2014), poor cognitive performance in learning and memory tasks (Palchykova et al., 2006), and physiological disruption in both animal models and humans. Moreover, chronic sleep disruption is a risk factor for metabolic disease, including diabetes (Knutson et al., 2007), dysregulation of the immune system (Besedovsky et al., 2012), cardiovascular disease (Nagai et al., 2010), and neurodegenerative and neuropsychiatric disease (Fifel & Videnovic, 2021). Data from post-torpid hamsters indicates that torpor impairs memory to a degree that is comparable with memory deficits following sleep deprivation (Palchykova et al., 2006), therefore supporting the hypothesis that torpor in not equivalent to sleep. However, the effect of torpor on memory remains unclear due to mixed findings as previously discussed. It is unclear from the literature whether fasting-induced torpor is consistent with the sleep depriving effects of hibernation and daily torpor, and how this translates to the behavioural phenotype. If fasting-induced torpor is sleep depriving, then this would suggest that laboratory mice on food restriction or fasting paradigms may be in a state of chronic sleep deprivation, which in itself would significantly impair optimal cognitive and physiological function, therefore confounding any outcomes measuring related parameters.

1.2.2 The circadian system and its interaction with sleep and torpor

Like sleep, torpor is thought to interact with the circadian system. Circadian, deriving from the Latin ‘about a day’, refers to the internally generated rhythmic oscillations in physiology and behaviour that occur across the 24 hour day. Such rhythms in behaviour and physiology enable animals to anticipate predictable changes in the environment, which is thought to provide a selective advantage (Mistlberger, 2005). These circadian rhythms are controlled by a centrally located ‘master clock’, termed the suprachiasmatic nucleus (SCN), which receives environmental inputs to entrain the ‘clock’ by regulation of a transcription-translation feedback loop (Takahashi, 2017). The environmental inputs that influence circadian timing are known as zeitgebers (from German meaning ‘time giver’), the strongest of which is light. However, a number of other factors such as food timing are also able to influence certain rhythms, with food availability being one of the strongest zeitgebers after light (Mendoza, 2007; Stephan, 2002).

Sleep and wake are temporally regulated to predictable parts of the 24-hour daily cycle. It is hypothesised the sleep-wake architecture, including their timing, duration, and intensity are regulated by the dynamic interaction of two processes. The first, Process S, represents a sleep-dependent homeostatic process which accumulates with prolonged wakefulness and dissipates during subsequent sleep, as indicated by the intensity of slow wave activity (frequencies <4 Hz) (Borbély, 2022; Borbély, 1982). This is why an increase in the intensity of sleep is observed following sleep deprivation due to the additional accumulation of sleep debt. The second, Process C, represents a sleep-independent circadian process that controls the timing of physiology and behaviours to ensure that an organism’s behaviours and physiology occur at a time point that is the most ecologically advantageous. Physiological examples of the circadian system include body temperature and melatonin rhythms, and arousal (Borbély et al., 2016).

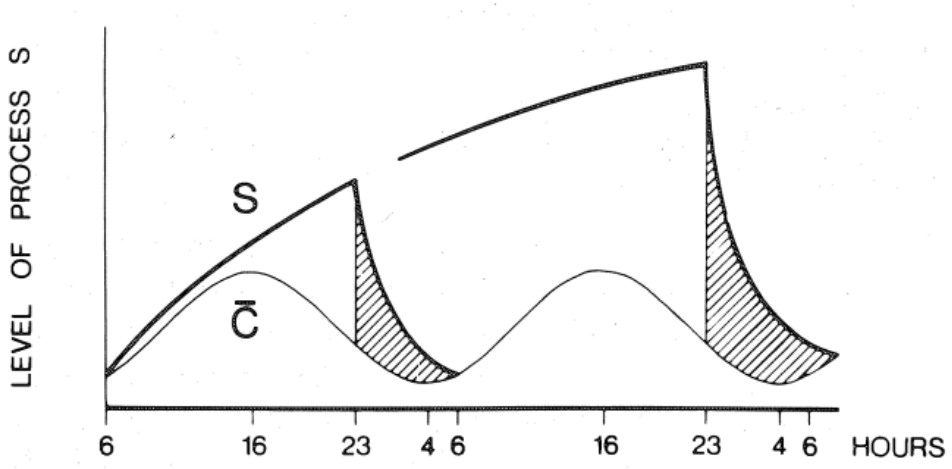

Figure 2: Schematic of the two process model of sleep regulation following regular and extended waking periods. The homeostatic process of sleep (Process S) shows the exponential increase accumulating sleep debt, and thus increased sleep propensity, during wakefulness. Extended periods of waking results in increased sleep debt. Process S rapidly decreases upon entry into sleep, with the sleep intensity being proportional to time spent awake. The circadian process, shown here as the negative function (C), reflects the time course of wake propensity throughout the day. Figure from (Borbély, 1982).

Torpor also appears to be under circadian control and will typically occur at the same time each day, with mice typically entering torpor during the latter half of the dark phase which corresponds to their active phase (van der Vinne et al., 2018, 2019). Moreover, work in genetically modified mouse lines in which the genes associated with circadian timing are mutated demonstrated that circadian timing was the major determining factor of when torpor occurred in mice (van der Vinne et al., 2018). Notably, this work also reported that torpor bouts were transiently inhibited by food availability, indicating that feeding time is also able to influence torpor timing. Further, hamsters with SCN lesions demonstrated lost rhythmicity in their torpor timing. However, torpor bout timing was able to be re-entrained using a predictable feeding schedule (Paul et al., 2004; Ruby et al., 1989). These studies suggest that torpor timing is strongly influenced by both light and food cues.

However, to allow for torpor to be expressed, reorganisation of the sleep-wake cycle must occur to facilitate torpor induction whilst also ensuring that behaviours necessary for survival such as feeding and euthermic sleep can be accommodated (Northeast et al., 2020). Fasted mice, for example, show increased consolidation of wake and sleep bouts when torpor is reliably being induced, whereas non-fasted mice will typically 'nap' throughout the light and dark phases (Huang et al., 2021). Moreover, fasted mice show increased activity levels during the light phase, which is usually their rest phase, when food is provided during this period therefore counteracting the typical circadian organisation of wake and sleep (Gabloffsky et al., 2022; Northeast et al., 2019).

1.3 Torpor and the 3Rs

As discussed above, torpor is associated with profound changes in physiology and at the molecular, cellular, and systemic levels, resulting in significant alternations to metabolism, body temperature, activity patterns, and hormone levels. Further, torpor has been shown to impact the morphology of the central nervous system, disrupt sleep, and influence behaviour. Despite the widescale effects of torpor, fasting-induced torpor in mice has received surprisingly little attention in neuroscience literature, and is largely unknown outside the torpor field.

Mice are one of the most widely used model species in preclinical research, with food restriction and deprivation being common techniques across several fields of study (Barkus et al., 2022). This is exemplified by a PubMed search using the terms "fasting" OR "food restriction" AND "mice" OR "mouse" which returned 207,555 results published in the last 5 years alone (returned 16th June 2023). Consequently, it is likely that many experiments using a form of food restriction may be inadvertently inducing torpor. Moreover, the circadian timing of torpor means that bouts typically occur during the night, so would be easily missed by

researchers if parameters such as body temperature or metabolic rate were not being continuously monitored. As such, it is unlikely that the impact of torpor on physiology and behaviour is being appropriately recognised and controlled for during food restriction studies. As the variables of interest in many food restriction studies are physiological samples or behavioural parameters, it is likely that these measures will be confounded by the presence of torpor and may vary significantly within and between individuals, and between studies, depending on the torpor history of each mouse.

Animal research in general has been under scrutiny over the last few years due to growing concerns that much of the published literature is not reproducible, and therefore may not be truly representative. For example, some studies have suggested that many published works are unreliable due to a mixture of bias, poor experimental design, and over reliance on novel positive findings (Ioannidis, 2018; Wacholder et al., 2004). One survey estimated that 70% of researchers have been unable to reproduce the results of another scientist's study (Baker, 2016). Estimates based on empirical data suggest that irreproducibility may be even higher at between 75-90% (Begley & Ioannidis, 2015)

The consequences of false positives and false negatives are far reaching and impact research at every level from basic to clinical. For example, unreliable data contributes to the high failure rate when transitioning from animal to human models during drug development (Seyhan, 2019), resulting in a translational bottleneck in which few novel compounds are found to be sufficiently efficacious during human studies. Moreover, animal studies often suffer from high levels of variability which limits the ability to reach meaningful conclusions and further develop the research question ("Troublesome Variability in Mouse Studies," 2009). In this context, it is possible that the induction of torpor in fasted mice may be contributing to inter-

and intra-individual variability, in addition to confounding experimental outcomes, and contributing to poor reproducibility.

Another important consideration when conducting research in animals is the impact of procedures on welfare from an ethical perspective of minimising animal suffering and minimising the number of animals suffering. High levels of variability require larger numbers of animals to be used to have a sufficient effect size, resulting in unnecessary animal studies being conducted in efforts to reproduce poor quality data.

To address these ethical considerations, animal research worldwide is guided by the principles of Replacement, Refinement, and Reduction, termed 'the 3Rs', which were first introduced in 'The Principles of Humane Experimental Technique' by Russell and Burch in 1959 (Russell & Burch, 1959). These principles are now embedded into national legislation in the UK with the 1986 Animals (Scientific Procedures) Act using the 3Rs as a foundation to ensure higher welfare standards in UK laboratories. Better husbandry and more robust experimental design have also been shown to produce more reliable science. Notably, a study by Hurst and West found that improved training in husbandry and handling techniques reduced stress in experimental rodents and improved experimental outcomes (Hurst & West, 2010); this study was later corroborated by other groups (Henderson et al., 2020; Singhal et al., 2014). These findings are not surprising as stress is known to significantly alter physiological variables and behaviour (Gaskill & Garner, 2017).

Overall, fasting-induced torpor represents a potential confounding factor in research involving fasted mice due to the well-established alterations to physiology that occur at every level. Moreover, the potential sleep-depriving effects of torpor suggest that torpor may be resulting

in long term alterations to physiology and behaviour which will not be as readily reversable or as transient as hypothermia and hypometabolism. Consequently, data generated from fasted mice many not always be robust or reliable enough for answering the experimental questions being investigated.

1.4 Hypothesis and project aims

The main hypothesis for this thesis is that torpor is readily induced in food restricted mice and acting as a confound due to the effects of torpor on physiology, sleep and behaviour. As such, torpor and its impacts on other physiological processes will be investigated from both a scientific and 3Rs perspective, to further our understanding of torpor and related states, whilst exploring how this may be impacting the mouse models used within research. It is hoped that this work will help to identify strategies for generating more robust and reliable data. To this end, this thesis will attempt to provide insight into sleep and torpor processes by further investigating the relationship between sleep and torpor in addition to their regulation. Doing so will help to further our understanding of sleep and torpor as neurophysiological states, in addition to determining whether fasting-induced torpor is a sleep depriving state and how this may be impacting experimental data. Moreover, this thesis will investigate the effects of torpor on behaviour and cognitive processes to determine whether torpor is confounding behavioural data.

The individual aims of this thesis are as follows:

1. Determine whether a commonly used food restriction paradigm is sufficient to induce torpor in mice, and the impact of feeding time on torpor characteristics.
2. Investigate whether torpor is affecting behavioural task outcomes by altering cognitive processes, altering activity, or contributing to variability.

3. Investigate the relationship between sleep and torpor, including the interaction and regulation of these states.
4. Determine whether water restriction associated with a drop on body weight is sufficient to induce torpor in mice whilst making comparisons to fasting-induced torpor induced by food restriction.

Chapter 2: General Methods

2.1 Experimental animals

Adult male C57BL/6J mice (University of Oxford Biomedical Services or Charles River Laboratories, UK) were used throughout this thesis (see individual methods sections for details regarding number and ages of the mice used). A single sex (male) was used throughout this thesis in order for the results to be more applicable to the wider research landscape, as males are still used in the majority of mouse-based research (Beery & Zucker, 2011). Female mice more readily enter torpor compared to male mice (Kato et al., 2018; Sunagawa & Takahashi, 2016; Swoap & Gutilla, 2009) therefore, the data presented in this thesis are likely to be representative of females; however, future work should aim to replicate the results in female mice, also.

During Chapters 3, 4, and 8, mice were individually housed in open top cages to allow for individual thermal imaging and food restriction to be performed. Cages were housed in custom-made light-tight chambers to minimise disturbances.

For electrophysiological recordings (Chapters 5-7), mice were individually housed in custom-made clear plexiglass cages (20.3 x 32 x 35 cm). Cages were housed in ventilated, light, and sound attenuated Faraday chambers (Campden Instruments, Loughborough, UK, two cages per chamber).

Mice were housed under entrained light conditions, to enable investigation of torpor timing relative to Zeitgeber time (Heller & Ruby, 2004; van der Vinne et al., 2018). All animals received 12:12 light-dark conditions (light level, 120-180 lux) although the exact timing of

lights on/off differed between experiments. If the light-dark cycle was shifted for experimental purposes, mice were given at least 1 day for every 1 hour shifted to allow for complete entrainment to the new cycle.

Room temperature and relative humidity were maintained at 22±1°C and 50±20%, respectively, in compliance with standard laboratory conditions. Water was provided ad libitum throughout, except in Chapter 8 during which a subset of mice underwent water regulation. Mice were given at least 3 days to habituate to their new environment before commencing experiments. All procedures were performed under a UK Home Office Project License and conformed to the Animals (Scientific Procedures) Act 1986 in accordance with institutional guidelines.

2.2 Food restriction

To induce torpor in mice, a chronic food restriction protocol was performed as previously described (van der Vinne et al., 2018). Briefly, all food was removed ~2 hours before feeding time. Mice were then provided with a ration of food once daily throughout the food restriction period (Teklad global 16% protein diet, Envigo, Blackthorne, UK); the food restriction period never exceeded 4 weeks. The amount of food provided was initially calculated as 70% of the average daily food intake during ad libitum conditions for each individual mouse, which was determined by weighing all food in the cage daily for ~3 days; this amounted to 2.0-2.5 g of food. Time of feeding is detailed in individual chapters.

Mice were weighed ~1.5 hours following feeding to allow time for mice to eat the provided food. Bodyweight was maintained at ~85% of the individual mouse's free feeding bodyweight which was taken the day before the start of food restriction. If an individual's weight dropped below 85%, additional food was provided at weighing time and the subsequent daily food ration

was adjusted. Food restriction was terminated if bodyweight dropped below 80% of free feeding bodyweight.

2.3 Thermal imaging

Body temperature is commonly used as a method for detection of torpor bouts, due to the hypothermia that accompanies the period of metabolic suppression (Swoap et al., 2006). Despite metabolic suppression being the driver of torpor induction, body temperature can be more easily measured with less specialised equipment and with higher throughput.

Here, non-invasive thermal imaging cameras (Optris Xi 80 with wide angle 80° x 80° lens, Optris GmbH, Berlin, Germany) were used to continuously record peripheral body temperature (Tskin). Cameras were installed ~20 cm above the animal's cages and were programmed to measure two cages, each containing one mouse, simultaneously. Tskin was determined by recording the hottest pixel every 1 s using the manufacturer's software (Optris PIX Connect, Optris GmbH); the ambient temperature was also determined by measuring the coldest pixel every 1 s.

The thermal imaging readout was prone to artefacts and noise due to the nature of peripheral recordings. For example, movement, piloerection, and moving out of the camera view contributed to this noise. To minimise noise, temperature measurements >35ºC and <18ºC were considered to be artefacts and were removed from subsequent analysis. In addition, temperature data were smoothed over 1-minute intervals, and a moving 20-minute average was calculated (Figure 3). To prevent mice from going out of view, a Perspex block was used to stop mice from hiding beneath the cage's food hopper, in addition to bedding material being limited to ~10 g per mouse.

The thermal cameras used in this thesis was optimised and validated by Vincent van der Vinne as part of a previous study (van der Vinne et al., 2020). This work found that thermal imaging cameras provide an underestimation of core body temperature, as expected, and were highly individual to each mouse, thus Tskin measurements cannot be used as a direct proxy for core temperature. Despite this, the thermal cameras were found to accurately measure relative changes in body temperature and were able to reliably detect periods of euthermia and hypothermia. As such, it was determined that the benefits of using a non-invasive system that allowed for continuous undisturbed recordings outweighed the limitations.

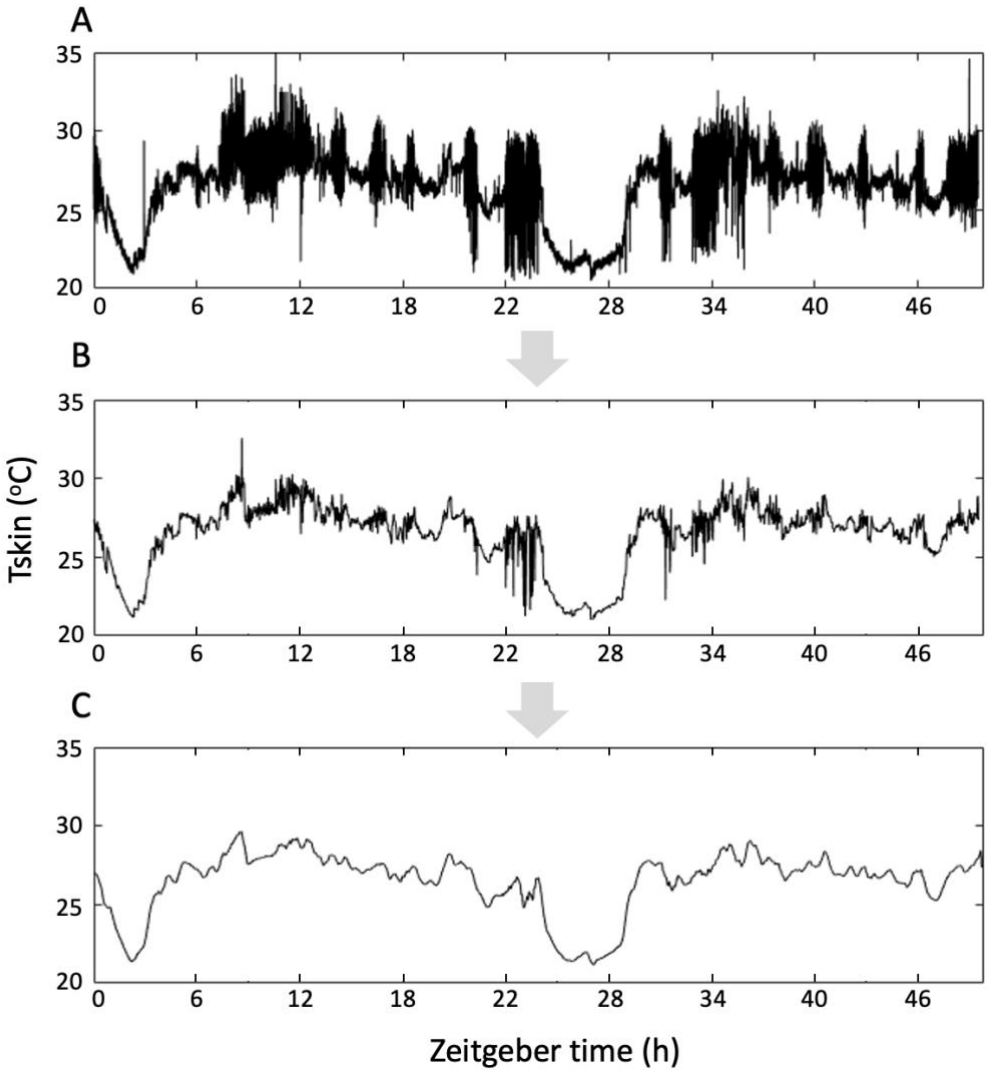

Figure 3: Smoothing of thermal imaging data. (A) Shows the raw thermal imaging data over 48 hours from a representative mouse entering torpor. (B) Thermal imaging trace following smoothing over 1-minute intervals to remove noise. (C) Additional smoothing using a 20-minute moving average to further remove noise and artefacts.

2.4 Torpor detection

Previous work on torpor has predominantly used metabolic or core body temperature recordings to develop criteria for detecting torpor bouts. Consequently, no criteria for torpor detection using peripheral body temperature recordings have been established. It has been shown that thermal imaging data cannot be used as a proxy to extrapolate core body temperature (van der Vinne et al., 2020), thus a new working definition for torpor was required for this thesis.

Previously, thermal imaging has been used to detect torpor by visually inspecting snapshots of thermal images (Hitrec et al., 2019). However, this method does not provide the temporal resolution required to characterise bouts for the purpose of this thesis, nor does it provide an objective method for torpor detection that can be easily replicated. Other studies have used a temperature threshold, similar to how core body temperature recordings are used to detect torpor. For example, a study in Djungarian hamsters (*Phodopus sungorus*) defined torpor as when peripheral body temperature had dropped below 25^{o}C (Ruf et al., 1991). However, this was not optimal for the data presented in this thesis as it was determined that using a temperature threshold alone would not be reliable due to inter-animal variability and the presence of circadian fluctuations in body temperature.

Instead, the criteria outlined in Huang et al 2021 was deemed to be the most applicable due to the commonality in methods, in such that peripheral Tskin measurements recorded via continuous thermal imaging was used to detect torpor (Huang et al., 2021). This study defined torpor as when Tskin dropped by more than two standard deviations below median ad libitum Tskin, for more than 1 hour. Here, this threshold was adapted to a decrease of >3 standard

deviation below the median euthermic Tskin, recorded on the last day of ad libitum feeding, for each individual mouse. This drop in Tskin was required to be sustained for >1 hour to be considered torpor. Entry into, and exit from, torpor, was determined as when Tskin crossed the 3 standard deviation threshold. Visual inspection of the Tskin traces with the threshold applied was performed to ensure that the threshold was appropriate. Three standard deviations, rather than two used in Huang et al, was used due to to high levels of noise observed in the thermal data. This definition has several advantages as it accounts for inter-animal variation as the temperature threshold is individualised for each mouse. Further, the drop in Tskin is sufficiently large so that mild hypothermia or circadian fluctuation is not included. The time threshold of >1 hour further prevents minor transient temperature fluctuations being included.

2.5 Implantation of EEG electrodes

Mice undergoing electrophysiological recordings (Chapters 5-7) were implanted with custom-made cranial head stages for chronic EEG/EMG recordings under aseptic conditions as described previously (Krone et al., 2021; McKillop et al., 2021; Milinski et al., 2021). Head stages were composed of stainless-steel screws (shaft diameter 0.86 mm, InterFocus Ltd, Cambridge, UK), stainless-steel wires, eight-pin and 90° connectors (Pinnacle Technology Inc, Kansas, USA, model 8415-SM). Connections were soldered and sealed with dental acrylic cement. Following conductivity testing, head stages were sterilised for aseptic surgery using 70% ethanol.

Isoflurane at 3-5% at an oxygen flow rate of 2-3 L/min was used to induce anaesthesia until righting reflex was lost. Mice were then transferred to a heat pad and a nose mask administering 2-3% isoflurane. The head and neck areas were shaved with clippers and the exposed area was

cleaned with ethanol and providone iodine washes, before administering analgesia sub-cutaneously (Metacam® (meloxicam, 5 mg/kg, Boehringer Ingelheim Ltd., Bracknell, UK) and Vetergesic® (buprenorphine, 0.1 mg/kg, Sogeval UK Ltd., York, UK).

Mice were then fixed in a stereotactic frame using ear bars (David Kopf Instruments, California, USA; Model 900 Small Animal Stereotaxic Instrument), placed on a heat pad and a rectal probe inserted to ensure core body temperature was maintained at ~36ºC during surgery. Isoflurane was adjusted to 1-2% at an oxygen flow rate of 1-1.5 L/min for maintenance; isoflurane concentration was titrated throughout the surgery so that a stable breathing rate of 50-90 breaths per minute was maintained. Artificial tears (Viscotears, Alcon Laboratories Ltd, UK) were applied regularly to prevent the eyes from drying out.

After ensuring the absence of the pedal reflex, an incision was made down the midline of the head running from the eyes to just above the neck muscle. Skin was retracted using bulldog clips to expose the skull. Connective tissue was removed using a cotton swab and cleaned using ethanol and saline. Phosphoric acid etching gel (37%, Henry Schein Inc, USA) was applied to the skull surface, rinsed with saline, and scratched gently with a scalpel blade to ensure all tissue was removed so that the head stage would firmly attach to the bone.

Following identification of Bregma, EEG electrode coordinates were marked using a sterilised surgery-safe pen. Three EEG electrodes were implanted above the following regions as follows (coordinates are relative to Bregma): (i) frontal EEG (motor area, anteroposterior (AP) 2 mm, mediolateral (ML) 2 mm); (ii) occipital EEG (visual area, V1, AP -3.5–4 mm, ML 2.5 mm); and (iii) cerebellar reference EEG (2mm behind Lambda, ML ±0.0 mm). A fourth free-standing

screw was implanted contralaterally to the occipital EEG (AP -3.5 to 4.0 mm; ML -2.5 mm), to act as stabilising anchor for the head stage.

A surgical drill with a 0.6 mm diameter carbon steel burr (InterFocus Ltd, Linton, UK) was used to create holes in the skull in the designated sites. Screw electrodes were epidurally implanted using plyers and a small screwdriver, so that that the electrodes were just touching the surface of the dura without damaging the brain. All EEG screws were fixed with dental cement SuperBond® (Prestige Dental Products Ltd, Bradford, UK). Finally, two single stranded wires were implanted bilaterally into the nuchal muscle for electromyography (EMG) recordings. All screws and cables were secured using a final application of dental cement, and the skin of the neck sutured where necessary.

The electrode configuration used was chosen as these recording sites are routinely used for standard chronic recordings of electrical brain activity (Huber et al., 2000; McKillop et al., 2021). Moreover, these regions are particularly useful for sleep recordings as slow wave frequencies begin at the front of the brain, thus predominating the frontal derivation, whilst theta activity dominates in the occipital region. Slow and theta frequencies are the main frequency bands used for characterisation of vigilance states, therefore recording from these regions facilitates scoring of state transitions.

Sterile saline was administered subcutaneously (5 ml/kg per hour of surgery) at the end of surgery to compensate for fluid loss. Mice were transferred to a heated chamber (30^{o}C initially, 25^{o}C after regaining righting reflex) and monitored closely following termination of anaesthesia. Mice where then individually housed in clean individually ventilated cages for a recovery period of 7-10 days. In the first 3 days of recovery, all mice were given oral

Metacam® suspended in a strawberry jelly to provide post-operative analgesia; i.p injections of Metacam® and Vetergesic® were administered as required. Mice were monitored daily and scored for altered natural and provoked behaviour, appearance, respiratory rate, and signs of grimace (Langford et al., 2010).

2.6 Electrophysiological recordings

2.6.1 Data acquisition

Electrophysiological signals were acquired using multichannel neurophysiology recording system (Tucker-Davis Technologies Inc., Alachua, FL, USA), and the electrophysiological recording software Synapse (Tucker-Davis Technologies Inc., Alachua, FL, USA). Data was stored on a local computer at a sampling rate of 305 Hz and filtered between 0.1-100 Hz. EEG signals were obtained from the frontal and occipital derivations and referenced against the cerebellum EEG. EMG was obtained as a differential between the two EMG leads.

2.6.2 Signal processing and scoring of vigilance states

EEG and EMG signals were resampled offline at a resampling rate of 256 Hz using custom written MATLAB scripts (The MathWorks Inc, Natick, Massachusetts, USA), before being transformed into European Data Format (EDF) using the software, Neurotraces, as previously described (McKillop et al., 2021).

Data were then visualised offline using SleepSign for Animals (version 3.3.6.1602, SleepSign Kissei Comtec Co., Ltd., Nagano, Japan). Vigilance states were manually scored by visual inspection of the frontal, occipital, and EMG signals, partitioned into 4s epochs. Waking was defined as low voltage, high frequency EEG with high EMG activity. NREM sleep was defined

as high amplitude, low frequency, with the occurrence of slow waves (1-4 Hz), and low EMG activity. REM sleep was defined as low voltage and high frequency EEG, but with low level or absent EMG. Epochs with recording artefacts were scored as the respective vigilance state but were not included in subsequent analysis. Brief awakenings of ≤16s were scored as movement artefacts and were not included in further analysis. Torpor is not defined using electrophysiological criteria and thus could not be scored using EEG signals. Brain activity during torpor bouts, as determined by body temperature measurements, resembles low amplitude NREM sleep and so was scored as such. SleepSign software was used to calculate power spectra of each of the scored epochs, using a Fast Fourier Transform (FFT) routine (Hanning window) with a 0.25 Hz resolution and exported in the frequency range between 0-30 Hz for spectral analysis.

2.7 Statistical procedures

Data were processed using MATLAB (version R2022a; The MathWorks Inc, Natick, MA, USA) and analysed using Prism (GraphPad, version 9). All data were tested for normality using a Shapiro-Wilk test prior to statistical analysis. Reported averages are mean ± SEM; non-parametric data are reported as median values. Details of the statistical tests and the numbers of animals used in each of the analyses are stated in the methods section for each chapter.

Chapter 3: Induction of torpor in laboratory mice and the effect of feeding time on torpor characteristics

3.1 Introduction

As introduced in Chapter 1, an energy deficit due to limited food availability will trigger a hypometabolic and hypothermic state of torpor to conserve energy in laboratory mice (Ambler et al., 2021). Several paradigms have previously been reported to induce torpor in mice, including acute fasting (Kato et al., 2018), fasting combined with low ambient temperature (Oelkrug et al., 2011), increased energetic demand through foraging (Schubert et al., 2010), and food restriction over several days (van der Vinne et al., 2018). Many of these paradigms are used routinely in behavioural, metabolic, circadian, and pharmacological studies, amongst others. However, the potential for torpor induction as an experimental confound is rarely considered, despite increased attention and awareness of the lack of reproducibility in animal research (Baker & Penny, 2016).

One of the most prevalent techniques used when conducting behavioural tasks is chronic food restriction. This technique involves providing animals with a reduced ration of food once per day to induce weight loss of 10-15%, which is maintained throughout the study period. It is thought that this degree of food restriction is optimal for increasing engagement as the animals can earn a food reward during the task (Makowiecki et al., 2012). Animals can be trained to complete the task without the use of food restriction to receive a palatable food reward (Carroll et al., 1989), however, this is less common as it may not always be successful and can prolong the training period (Lattal & Williams, 1997).

Behavioural data are subject to high levels of inter- and intra-individual variation therefore requiring large sample sizes. Moreover, reproducibility within and between studies is often limited making it challenging to draw conclusions of translate findings to a clinical or other research settings (Begley & Ioannidis, 2015; Mandillo et al., 2008). This chapter investigated whether a common behavioural food restriction paradigm is sufficient to induce torpor in mice, and how regularly torpor occurs. The induction of torpor in this setting may suggest that torpor is a source of variation and a confound within behavioural data.

There is little standardisation in the food restriction protocols used for behavioural studies, and the majority do not report when food was provided, or whether food timing was consistent between animals and between days. It appears that feeding schedule is overlooked as a variable to be controlled but it may be necessary for reproducing results. Food is a powerful zeitgeber for circadian rhythms and can influence activity patterns, and hormone rhythms (e.g., metabolic hormones (leptin, ghrelin, insulin), glucocorticoids) (Mendoza, 2007; Northeast et al., 2020). Moreover, torpor has been suggested to be under circadian control, with torpor typically occurring in the latter half of the active, dark phase, with internally generated circadian rhythms being the primary driver (Hitrec et al., 2019; van der Vinne et al., 2018). Feeding time and food intake can influence torpor timing in circadian mutant mice (van der Vinne et al., 2018), and food timing was found to be the primary driver of torpor bouts in SCN-ablated hamsters (Paul et al., 2004), further indicating that feeding schedule may be a source of variation due to its influence on torpor.

Taken together, these previous findings led to the hypothesis that torpor is likely being induced in response to this common food restriction paradigm. In addition, it was hypothesised that the lack of standardisation of feeding schedules during behavioural experiments may be resulting

in variation in the circadian timing of torpor between studies, and potentially within and between individuals in the same experiment. Furthermore, determining how feeding schedule affects torpor propensity and characteristics may help to refine food restriction paradigms. For example, food could be provided at a time that would cause torpor bouts to be shifted away from the time at which testing typically occurs, therefore providing a window of opportunity.

3.1.1 Experimental aims

To address these hypotheses, the main aims of this chapter were:

i) To determine whether food restriction to ~85% of free feeding bodyweight will be sufficient to induce torpor in mice

ii) To investigate the effect of feeding time on torpor characteristics

3.2 Methods

3.2.1 Animals and recording conditions

Adult, male C57BL/6J mice were used in this study (University of Oxford Biomedical Services; n=8; aged 11 weeks). Mice were individually housed in conventional wire-top cages (M3 cage, 48x15x13 cm) on a 12:12 h light-dark (12:12 LD) cycle for the duration of the experiment. Cages were housed in custom made light-tight chambers (LTCs; Figure 4B), with four cages per chamber (Fisher et al., 2012; Tam et al., 2021). LTCs were illuminated by warm white LED strips (200 lux at the cage floor) during the light phase of the 12:12 LD cycle. Ambient room temperature and relative humidity were maintained at 21 ± 1°C and 60 ± 1%, respectively. Water was provided ad libitum throughout.

Peripheral skin temperature (Tskin) was non-invasively recorded using continuous thermal imaging cameras, mounted ~20 cm above the cage base (Optris Xi 80 compact spot finder thermal imaging camera with 80° wide angle lens, Optris GmbH, Berlin, Germany; one camera per two animals). Custom Perspex blocks (23 × 12 × 3.2cm, Aquarius Plastics, Surrey UK) were used to block access to the area beneath the food hopper, to minimise the chance of mice being out of view of the thermal camera. Enrichment items, such as tunnels, were removed to prevent obstruction of view of the cameras, in line with previous studies (van der Vinne et al., 2020). Bedding material was reduced for this reason also, but a minimum of 10 g was provided to ensure mice could sufficiently thermoregulate (Gaskill et al., 2011, 2012, 2013).

Tskin was determined by recording the hottest pixel every 1 s and processed as previously described in Chapter 2. In brief, a high-pass filter was applied at 18^{o}C and low-pass filter at 35^{o}C to remove artefacts that occurred due to movement and changes in animal posture. The filtered data were then binned into 1-minute intervals, and a 20-minute moving average applied to smooth the data and further remove noise. The processed thermal imaging data was used to calculate mean, minimum, and maximum Tskin values for each 24-hour recording, across all days of food restriction.

Tskin measurements were used to detect torpor and determine torpor characteristics, as described in Chapter 2. Briefly, a custom algorithm was developed, based on the criteria described by Huang et al., 2021, to define torpor as when Tskin dropped by >3 standard deviations below the median euthermic Tskin for >1h for each animal. The start and end of torpor were determined as when Tskin crossed this threshold.

3.2.2 Experimental protocol

Mice were assigned to one of two experimental groups to assess the effect of feeding time on torpor characteristics: the morning fed group and the night fed group (n=4 for each group). Power calculations were not used to determine sample sizes for this study, as the effect size was expected to be large due to a torpor bout typically involving a considerable (>5^{o}C) drop in body temperature that is easily detected (Huang et al., 2021; van der Vinne et al., 2020). In addition, there are few existing studies of a similar nature which limits the ability to accurately perform power calculations.

Mice were singly housed and moved to the recording chambers where they were allowed to habituate to their new environment for 2 weeks before 1 week of baseline measurements were recorded, after which food restriction was initiated by removing all food. A single ration of food (2.0-2.5 g) was provided at the same time each day; the initial amount was determined by calculating 70% of individual average ad libitum daily intake, and then titrated based on bodyweight (van der Vinne et al., 2018). Mice were maintained at ~85% of their free feeding bodyweight, which was determined by taking a daily bodyweight measurement ~2 hours after food was provided. Additional food was provided following weighing if bodyweight had dropped below 85%, and the following day's food ration increased. Conversely, the following day's food ration was reduced if bodyweight was >85%.

During food restriction, the morning group received food one hour after lights on, at zeitgeber time 1 (ZT 1). The night group received food one hour after lights off, at ZT 13, which was performed under dim red light due to feeding occurring after lights off. The night group were kept on a reverse light-dark cycle (lights on at 7 pm), allowing both groups to be fed in parallel to minimise disturbances (Figure 4A). Mice were given one day per every 1 hour shifted to

habituate to the new light-dark schedule (~12 days). It had initially been planned to reverse the feeding treatments, to allow for a within-subjects design; however, food restriction had a profound effect on bodyweight which was found to be closely related to torpor induction. As such, it was determined that the animal's physiology had been altered to such a degree that the starting point after the switch would not be comparable to the start of food restriction.

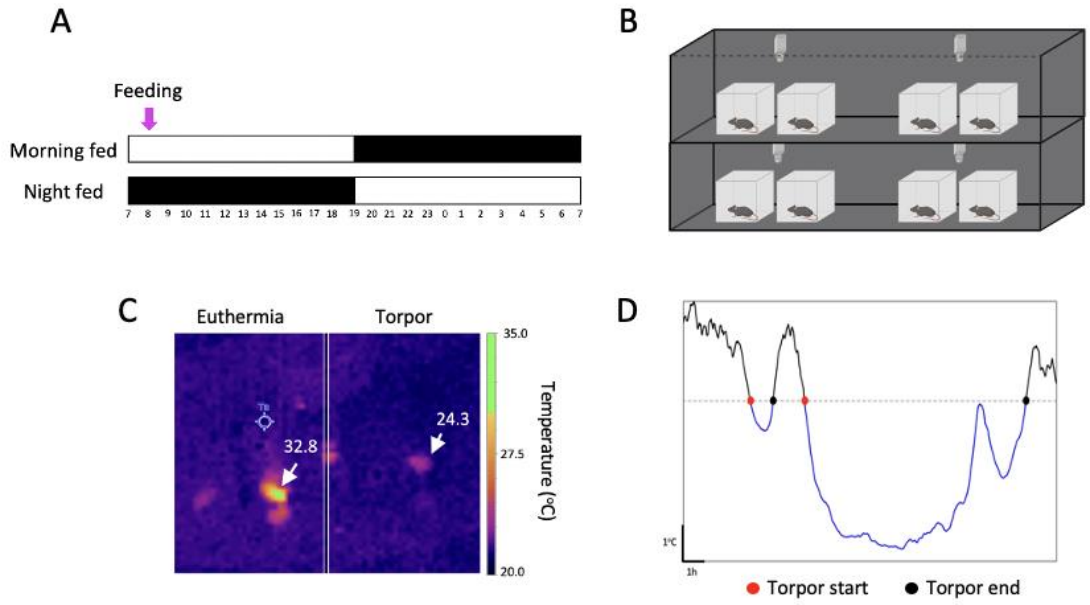

Figure 4: Experimental design and set up. (A). Feeding schedule during food restriction for both groups. The morning fed group (n=4) received food at one hour after lights on (zeitgeber time (ZT) 1, 8AM). The night fed group (n=4) were kept on a reverse light-dark cycle so that food could be provided in tandem (i.e., at 8AM) but was one hour after lights off for these animals (ZT 13). (B). Set up during recording. Mice were individually housed, and cages kept in one of two custom made light-tight chambers (LTCs), with 4 mice per chamber. Thermal imaging cameras were installed ~20 cm above the cage floor to allow for continuous thermal measurements to be recorded. One camera was used to record thermal measurements from two cages simultaneously. (C). Example thermal images recorded from two mice, one at euthermic body temperature (left), and one during torpor (right). (D). Overview of torpor detection using peripheral temperature (Tskin) measurements. The median Tskin during ad libitum feeding for each mouse was used to calculate a threshold for torpor of >3 standard deviations below the median euthermic Tskin. When Tskin dropped below this threshold for >1 hour, mice were considered to be in torpor. When Tskin rose above the threshold then torpor was considered to have ended.

3.2.3 Statistical analysis

Data were processed using MATLAB and analysed using Prism (GraphPad, version 9). Data are presented as mean values ± SEM unless otherwise stated. Data were tested for normality using a Shapiro-Wilk test prior to analysis. Analysis of parameters over time were conducted using two-way ANOVAs with repeated measures, with a Geisser-Greenhouse correction. Due to variables of interest changing dynamically over time, between group comparisons were conducted using data from the final 6 days of food restriction as this was the point at which the parameters stabilised. These comparisons were performed using an unpaired t-test, or a nonparametric Mann-Whitney U test as appropriate. Simple linear regression analysis was performed to determine the relationship between variables of interest. Within group comparisons of Tskin were performed using paired t-tests, whilst between group comparisons were performed using unpaired t-tests.

3.3 Results

Bodyweight did not differ significantly between the two groups before the start of food restriction (Figure 5B; Morning: 24.9 ± 1.51 g; Night: 27.6 ± 0.246 g; $p=0.129$, $t(6)=1.76$; unpaired t-test). Unsurprisingly, average bodyweight significantly dropped over days of food restriction with a main effect of day of food restriction ($F_{(3.73,22.4)}=112.2$ $p<0.0001$; two-way ANOVA with repeated measures) and group being found ($F_{(1,6)}=9.55$, $p=0.0214$; two-way ANOVA with repeated measures), but no significant interaction between time*group found ($F_{(17,102)}=1.41$, $p=0.1456$; two-way ANOVA with repeated measures) (Figure 5A).

Due to the dynamic effect of food restriction on bodyweight, between group comparisons of bodyweight, expressed as a percentage of baseline, were performed on the final 6 days of food

restriction once bodyweight and stablished and mice were no longer undergoing a period of weight loss. This revealed that morning-fed mice weighed slightly, but significantly, more than night-fed mice, despite the aim of all mice being maintained at ~85-90% of free feeding bodyweight (Figure 5C; Morning: 86.8 ± 0.729%; Night: 84.1 ± 0.281%; p=0.0147, t(6)=3.39; unpaired t-test). However, this may be explained by bodyweight measurements being taken relative to feeding time, resulting in very different circadian times for weighing between groups, and bodyweight is known to fluctuate over the 24-hour day (Refinetti, 2020).

Next, the change in body temperature following initiation of food restriction was compared between the two groups. It has been shown that absolute Tskin recordings can vary greatly between individuals (van der Vinne et al., 2020); therefore, to enable more direct comparisons between groups, the change in Tskin relative to a baseline day was calculated. The day prior to the start of food restriction was chosen as the baseline day, due to this day being the closest to food restriction and being after the habituation period. Body temperature was found to decrease significantly over days of food restriction (Figure 5D; $F_{(3.04,18.3)}$=32.3, p<0.0001; Two-way ANOVA with repeated measures), with a maximal decrease in mean Tskin of over -4^{o}C from baseline being recorded. No difference in change in mean Tskin was found between groups (Figure 5E; Morning: -2.58 ± 0.280 ^{o}C, 95% CI [-3.47 -1.68]; Night: -2.80 ± 0.277 ^{o}C, 95% CI [-3.68 -1.92]; p=0.586, t(6)=0.575; unpaired t-test). There was also no difference in the change in minimum Tskin between groups (Figure 5F; p=0.4284, t(6)=0.8493; unpaired t-test).

Previously, a relationship between body temperature and bodyweight in heterothermic species that enter torpor under fasted conditions has been reported (Kato et al., 2018; Solymár et al., 2015). As such, the relationship between bodyweight and Tskin was investigated and compared between groups. To this end, the average change in mean Tskin for every 1% change in

bodyweight (i.e., 85-85.9% etc.) was calculated, followed by simple linear regression analysis for the morning and night group. A significant positive relationship was found between bodyweight and Tskin for both groups (Figure 5G; Morning: r^2=0.5922, p<0.0001; Night: r^2=0.6225, p<0.0001), indicating that Tskin decreased in line with bodyweight for both groups.

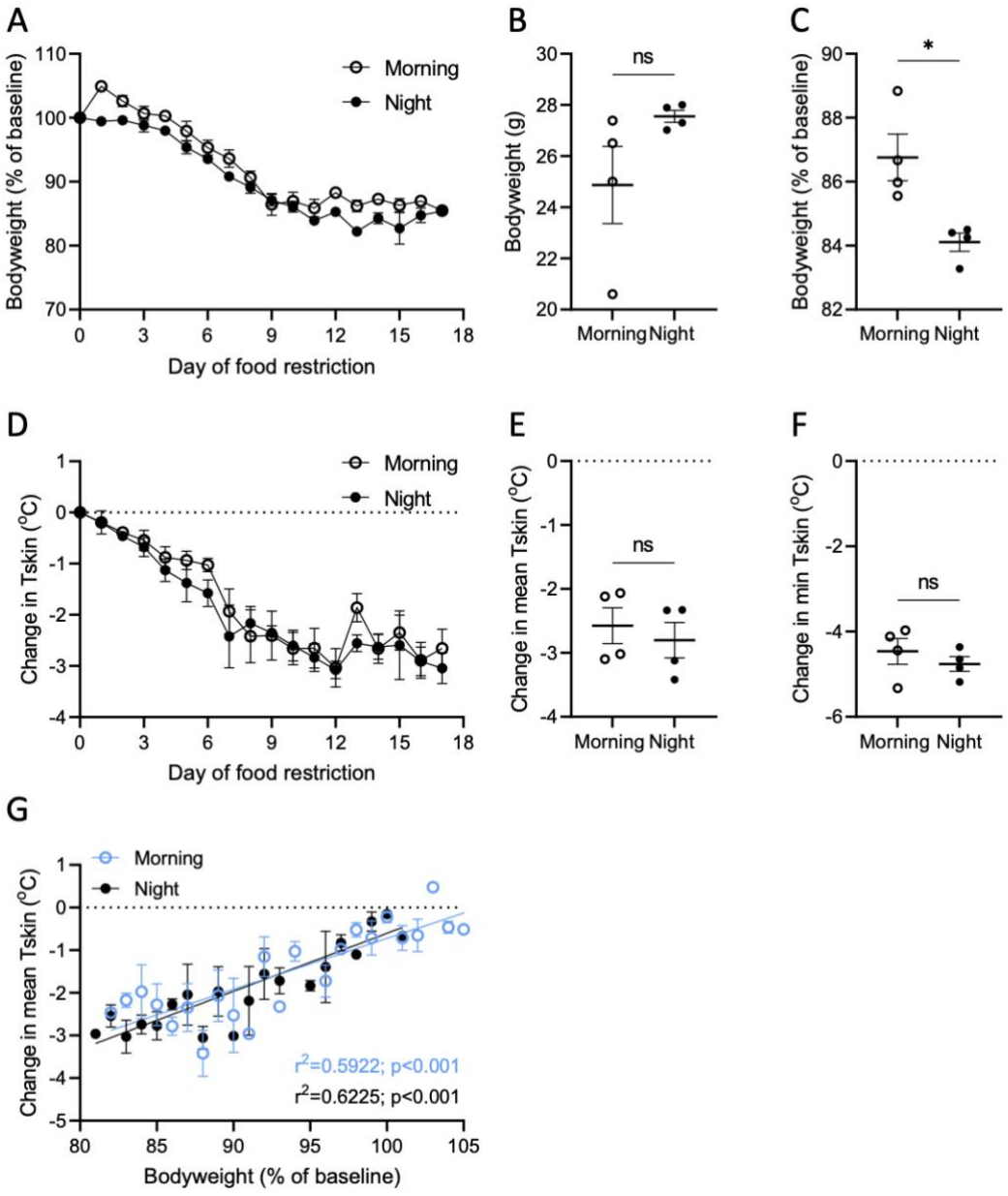

Figure 5: Effect of food restriction on bodyweight and body temperature during two feeding schedules. (A) Bodyweight decreased during food restriction at a similar rate for mice fed in the morning (n=4), and mice fed at night (n=4). Weight stabilised at ~85-90% of free feeding bodyweight at day 8 of food restriction. (B) Absolute bodyweight did not significantly differ between groups before food restriction commenced. (C) Bodyweight, as a percentage of baseline, significantly differed between groups during the last 6 days of food restriction, despite the aim of being maintained at 85-90%. (D) Change in daily mean Tskin, relative to baseline euthermic Tskin, over days of food restriction. (E) Comparison of the change in mean Tskin and (F) minimum Tskin between groups on the last 6 days of food restriction. No significant difference was found between groups was found for mean Tskin and min Tskin. (G) A significant positive relationship was found between bodyweight and change in Tskin for both the Morning group and the Night group (Morning: r^2=0.5922, p<0.0001; Night: r^2=0.6225, p<0.0001; simple linear regression). N=4 for each group. Data are represented as a mean value ± SEM. ns: non-significant (p>0.05), *p<0.05.

The decrease in body temperature as a function of bodyweight can be explained by the induction of torpor. Plotting the distribution of Tskin measurements recorded over 24 hours before and during food restriction shows a shift from a unimodal distribution, which would typically be observed in endothermic species maintaining core body temperature around a set point, to a bimodal distribution which is indicative of heterothermy (Figure 6B). Moreover, visualisation of raw temperature traces revealed large, sustained drops in body temperature in mice in both groups, which were reliably induced from ~day 8 of food restriction (Figure 6A).

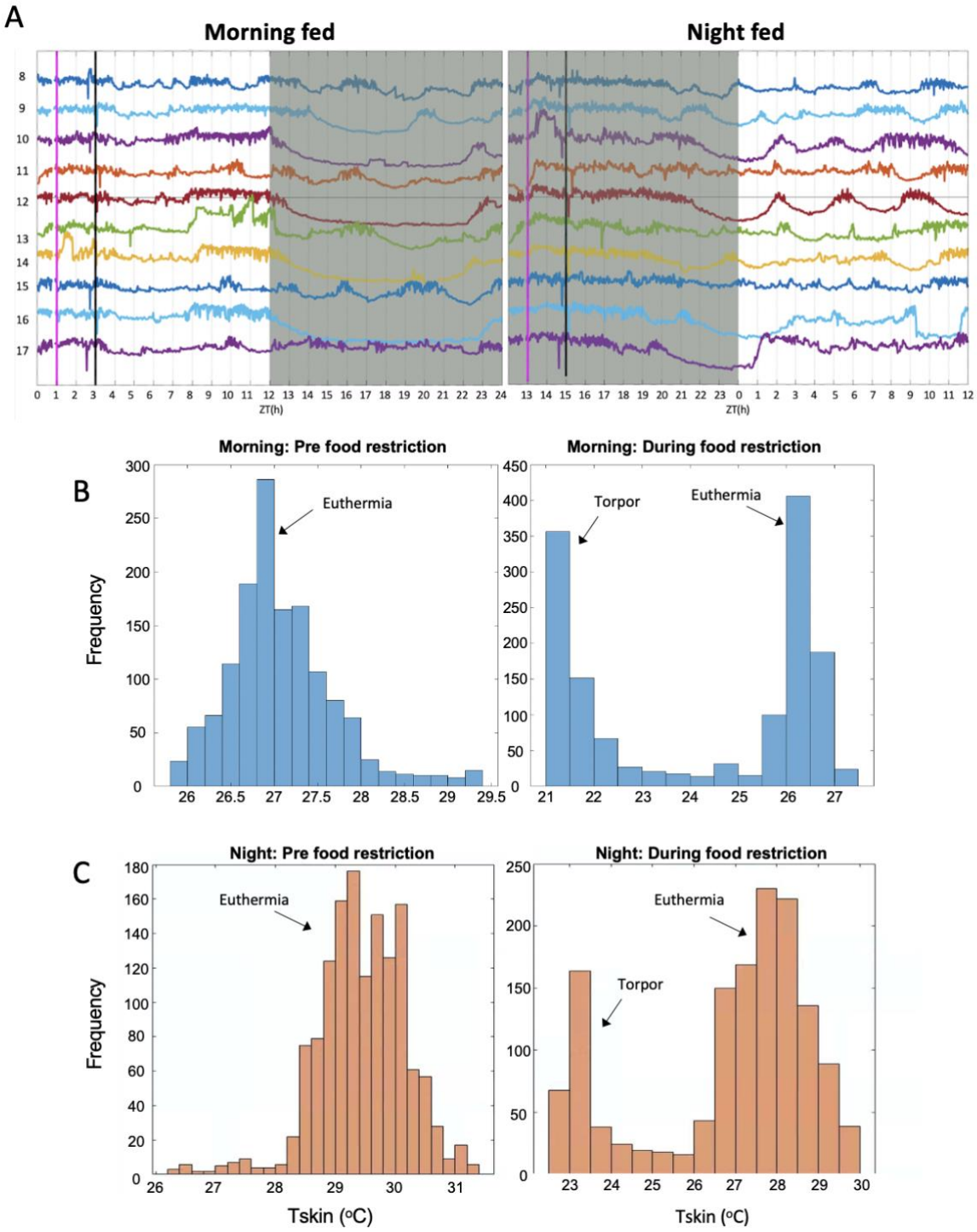

Figure 6: (A) Raw temperature traces over consecutive days of food restriction from a representative morning fed mouse (left) and a representative night fed mouse (right). Feeding time is represented by the pink line, and the black line shows the time at which bodyweight measurements were taken. (B) Distribution of Tskin measurements over 24 hours on an ad libitum fed day (left), versus a food restricted day (right) from a representative morning fed mouse, and a representative night fed mouse (C). Note the two peaks observed during food restriction representing euthermia and hypothermia.

Further analysis showed that the number of torpor bouts being induced each day increased over days of food restriction, with all mice entering torpor by day 8 of food restriction, coinciding with the large drop in body temperature observed in Figure 5A (Figure 7A; main effect of day of food restriction: $F_{(3.14,18.8)}$=10.1, p=0.0003; main effect of group: $F_{(1,6)}$=7.78, p=0.0316; interaction between day of FR*group: $F_{(17,102)}$=1.26, p=0.234; 2-way ANOVA with repeated measures). Between group comparisons of the average number of torpor bouts per day, across the final 6 days of food restriction once bodyweight had stabilised, found that night fed mice entered significantly more torpor bouts compared to morning fed mice (Figure 7B; Morning: 2.75 ± 0.222 bouts; Night: 4.70 ± 0.526 bouts; p=0.0142, t(6)=3.416, unpaired t-test).

The mean duration of torpor bouts increased over days of food restriction for both groups, although no interaction between day of FR*Group was observed (Figure 7C; main effect of day of food restriction: $F_{(3.06,18.4)}$=7.87, p=0.0013; main effect of group: $F_{(1,6)}$=5.10, p=0.0647; interaction between day of FR*group: $F_{(17,102)}$=0.0978, p=0.489; 2-way ANOVA with repeated measures). Comparisons of longest torpor bout and mean torpor once bodyweight had stabilised also found no significant difference between groups (Figure 7D, E; longest torpor bout: morning 437.0 ± 79.3 mins, night 361.8 ± 47.6 mins, p=0.4473, t(6)=0.813; mean torpor bout: morning 4.37 ± 0.739 h, night 2.59 ± 0.1359, p=0.0558, t(6)=2.366; unpaired t-tests). In line with these data, the percentage of time spent in torpor over each 24-hour day was found to significantly increase over days of food restriction (Figure 7F; main effect of day of food restriction: $F_{(17,51)}$=15.6, p<0.0001; main effect of group: $F_{(1,3)}$=4.15, p=0.1343; interaction between day of FR*group: $F_{(17,51)}$=0.6792, p=0.8089; 2-way ANOVA with repeated measures). These findings represent the increasing metabolic challenge as food restriction progresses that eventually results in the induction of torpor to conserve energy.

Next, the depth of torpor bouts over days of food restriction was calculated by determining the area beneath the threshold used to detect torpor for each individual mouse, in line with methods presented previously (Huang et al., 2021). In agreement with the previous findings discussed above, the depth of torpor increased over days of food restriction, likely due to the increasing number and length of torpor bouts (Figure 7H; main effect of day of food restriction: $F_{(3.56,21.4)}$=12.5, p<0.0001; main effect of group: $F_{(1,6)}$=1.31, p=0.2960; interaction between day of FR*group: $F_{(17,102)}$=0.5358, p=0.9286; two-way ANOVA with repeated measures). There was no difference in torpor depth between groups across the final 6 days of food restriction once bodyweight has stabilised, despite the night group entering significantly more torpor bouts per day (Figure 7I; p=0.4390, t(6)=0.8287; unpaired t-test). The lack of difference in torpor duration may explain why depth is similar, and further supports that the increased number of bouts observed during night feeding help to mitigate the bouts being shorter on average, therefore optimising energy savings.

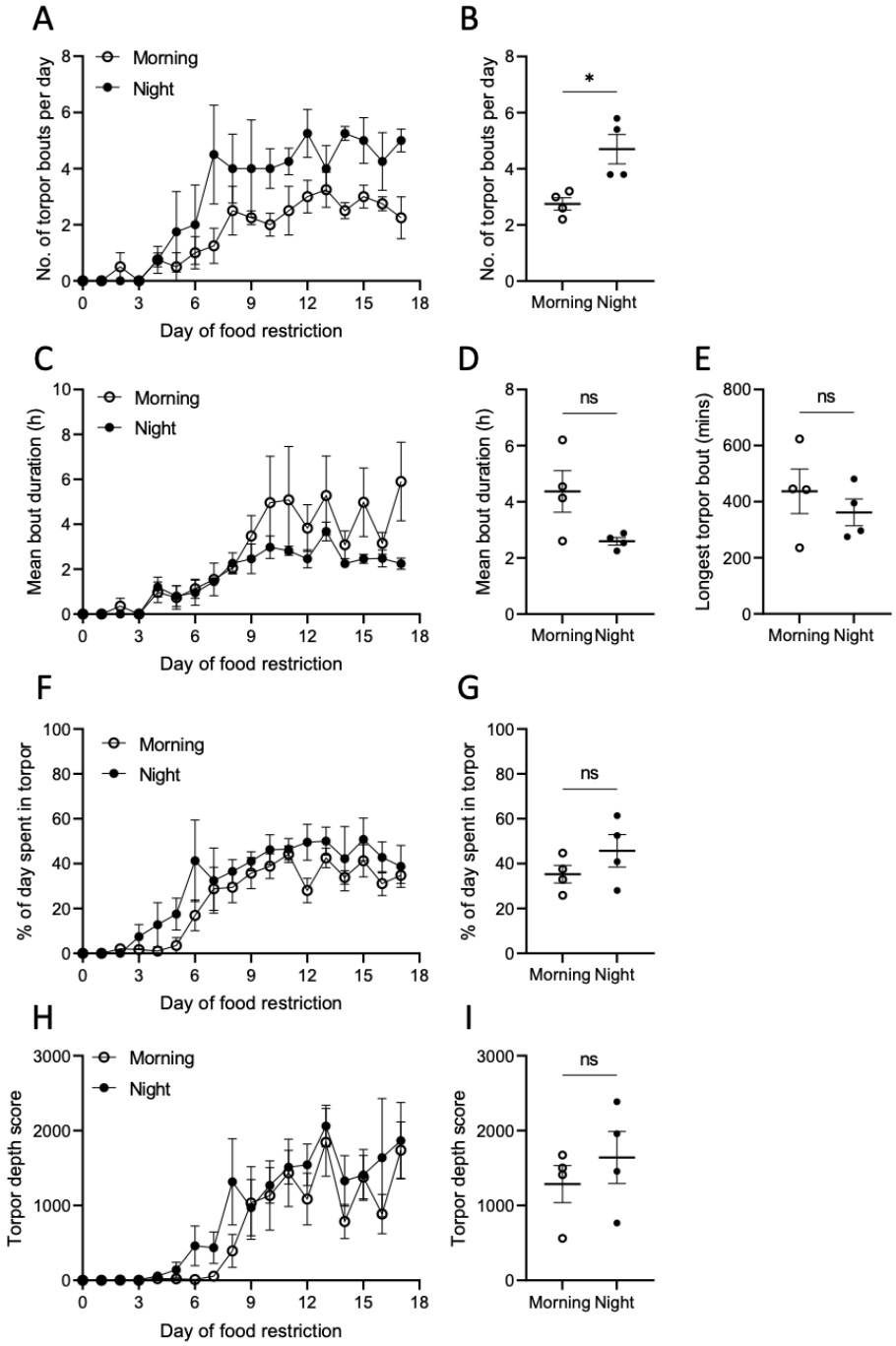

Figure 7: Effect of food restriction and feeding schedule on torpor characteristics. (A). The number of torpor bouts entered each day increased over days of food restriction for both morning and night fed mice. (B) Night fed mice entered significantly more torpor bout per day over the once bodyweight had stabilised ($p=0.0142$, $t(6)=3.42$; unpaired t-test). (C). The mean duration of torpor bouts each day increased during the initial 8-9 days of food restriction. Comparison of mean bout duration (D), and longest bout duration (E) showed no differences between groups (mean length: $p=0.0558$, $t(6)=2.366$; longest bout: $p=0.4473$, $t(6)=0.8130$; unpaired t-tests). (F) Percentage of the 24-hour recordings spent in torpor increased over days of food restriction. (G) No difference in percentage of time spent in torpor was found between groups ($p=0.2525$, $t(6)=1.266$; unpaired t-test). (H) Torpor depth, calculated as the area beneath the torpor threshold increased with time. (I) No difference was found in torpor depth between groups ($p=0.4390$, $t(6)=0.8287$; unpaired t-test). n=4 for each group; data are represented as a mean value ± SEM. ns: non-significant ($p>0.05$), *$p<0.05$.

One of the main aims of this chapter was to determine whether feeding schedule would shift the circadian timing of torpor bouts, in line with previous findings in hamsters (Paul et al., 2004). To this end, the time at which animals entered torpor, as determined by the time at which Tskin dropped below the threshold for torpor detection of >3SD below the median euthermic Tskin for each individual mouse, was calculated and compared between groups. Due to some animals not entering torpor on some days towards the beginning of food restriction, a mixed-effects model with repeated measures was used to investigate the effect of time and group on torpor timing, instead of a 2-way ANOVA which cannot account for missing values, although a mixed-effects model cannot determine interaction.

This method of analysis revealed a main effect of group but no significant main effect of day of food restriction, suggesting that the time of fasting-induced torpor under circadian control rather than being influenced by the increasing metabolic challenge (Figure 8A; Group: $F_{(1,6)}$=17.0, p=0.0062; Day of FR: $F_{(1.47,8.20)}$=0.8942, p=0.4137; mixed-effects model with repeated measures). Building on these findings, torpor timing once all mice were reliably entering torpor was compared between groups. No difference in torpor timing was found between groups towards the end of food restriction, as torpor timing began to converge between the two groups, possibly reflecting a balance between the competing circadian drives of light and food timing being reached (Figure 8B; Morning: 14.2 ± 0.353 ZTh; Night: 12.2 ± 0.931 ZTh; p=0.0933, t(6)=1.99; unpaired t-test). It is possible that a difference may have been observed with a larger sample size as both groups, but especially the night group, showed high levels on variation in torpor timing.

Previously, it has been suggested that provision of food is sufficient to transiently delay the timing of torpor onset in mice with brain-specific circadian gene mutations (van der Vinne et

al., 2018). As such, the latency to the first torpor bout following feeding was investigated to do determine whether the same effect is observed in wild-type mice, which may help to inform the development of an optimal feeding schedule to provide a window of opportunity in which behavioural or physiological measurements can be taken whilst mitigating the potential effects of torpor. Due to not all mice entering torpor at the beginning of food restriction, a mixed-effect model was used to analyse the effect of day of food restriction and group on latency to torpor. This revealed a significant effect of both day of food restriction and of group, although an interaction cannot be determined using this method (Figure 8C; Day of FR: $F_{(2.94, 16.6)}$=3.52, p=0.039; Group: $F_{(1,6)}$=35.9, p=0.001; mixed-effects model with repeated measures). Interestingly, the latency to torpor following feeding decreased over days of food restriction for the morning fed group, whilst the night fed group was more consistent. This may reflect the increasing metabolic challenge as food restriction progresses requiring the latency to torpor to be reduced to conserve energy in the morning fed group. The between group difference may reflect differences in the competing circadian drives of light and food timing. The between group difference disappeared towards the end of food restriction, once all mice were reliably entering torpor and bodyweight had stabilised (Figure 8D; p=0.082, t(6)=2.09; unpaired t-test). However, this analysis appears to be trending towards significance, which may become apparent in a larger sample size.

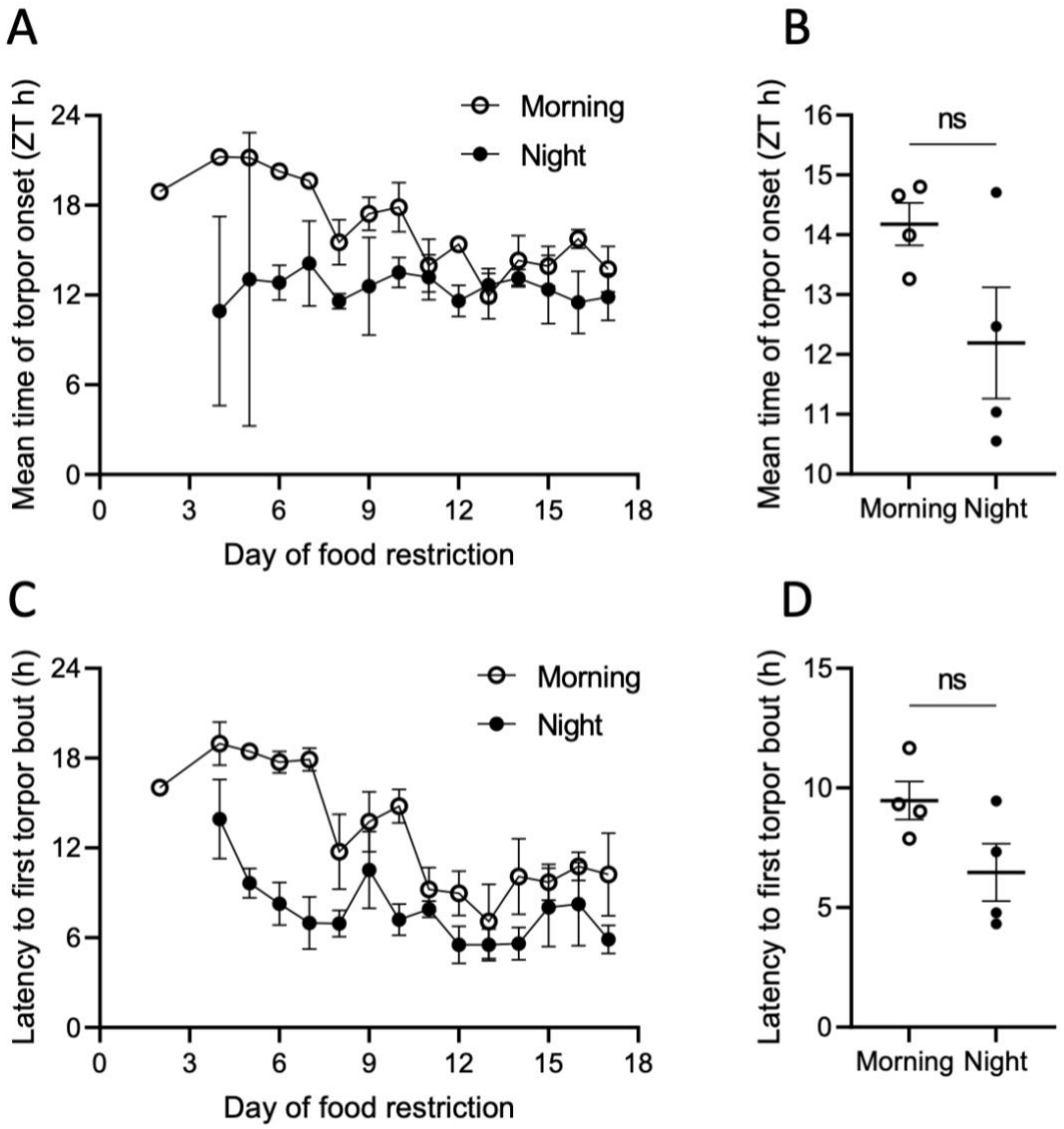

Figure 8: Effect of feeding schedule on the timing of and latency to torpor. (A) The mean time of torpor onset over days of food restriction for morning and night feeding. A main effect of group but not of day of food restriction was found (Group: $F_{(1,6)}$=17.0, p=0.0062; Day of FR: $F_{(1.47,8.20)}$=0.8942, p=0.4137; mixed-effects model with repeated measures). (B) Comparison of the mean time of torpor onset on the final 6 days of food restriction. (Morning: 14.2 ± 0.353 ZTh; Night: 12.2 ± 0.931 ZTh; p=0.0933, t(6)=1.99; unpaired t-test). (C) The latency to the first torpor bout following feeding over days of food restriction. A significant main effect of both day of food restriction and group where found (Day of FR: $F_{(2.94, 16.6)}$=3.52, p=0.039; Group: $F_{(1,6)}$=35.9, p=0.001; mixed-effects model with repeated measures). (D) No significant difference in torpor latency was observed between groups when comparing the final 6 days of food restriction (p=0.082, t(6)=2.09; unpaired t-test). N=4 for each group. Data are represented as a mean value ± SEM. ns: non-significant (p>0.05).

Finally, the effect of feeding schedule on the circadian regulation of body temperature was investigated by comparing Tskin measurements during the light period versus the dark period.

As mice are nocturnal in laboratory conditions, body temperature is typically higher during the dark period due to higher levels of locomotor activity (Castillo et al., 2005). Comparison of mean Tskin during the light phase (ZT 0-12) versus the dark phase (ZT 12-24) under ad libitum fed conditions found that Tskin was significantly higher in the dark phase for night fed mice (Figure 9B; $p=0.0036$, $t(3)=8.38$; paired t-test). Surprisingly, no significant difference was found between phases for the morning fed mice (Figure 9A; $p=0.1126$, $t(3)=2.22$; paired t-test), although this lack of significance may be explained by the small sample size. During food restriction, the distribution of Tskin shifts as mice spend more time in torpor. Morning-fed mice were found to have a significantly higher mean Tskin during the light phase compared to the dark phase when food restricted (Figure 9C; $p=0.0152$, $t(3)=5.03$; paired t-test), likely due to torpor typically occurring during the dark phase in this group, as shown in Figure 5B. Conversely, the night fed group had a higher mean Tskin during the dark phase compared to the light phase which is also likely influenced by the torpor timing (Figure 9D; $p=0.0152$, $t(3)=5.03$; paired t-test). Comparison of Tskin between groups during food restriction revealed that there was no difference in mean Tskin during the light phase (Figure 9E; Morning: $27.1 \pm 0.319^{o}C$; Night: $26.1 \pm 0.273^{o}C$; $p=0.0620$, $t(6)=2.29$; unpaired t-test), whereas the night-fed group has significantly higher Tskin compared to the morning-fed group during the dark phase (Figure 9F; Morning: $24.2 \pm 0.607^{o}C$; Night: $26.9 \pm 0.337^{o}C$; $p=0.0086$, $t(6)=3.83$; unpaired t-test). These data suggest that feeding schedule was able to shift torpor bouts and activity profiles to some extent. More studies with larger sample sizes are required going forward; further, measures of activity patterns such as running wheels or passive infrared sensors may further uncover differences in circadian parameters.

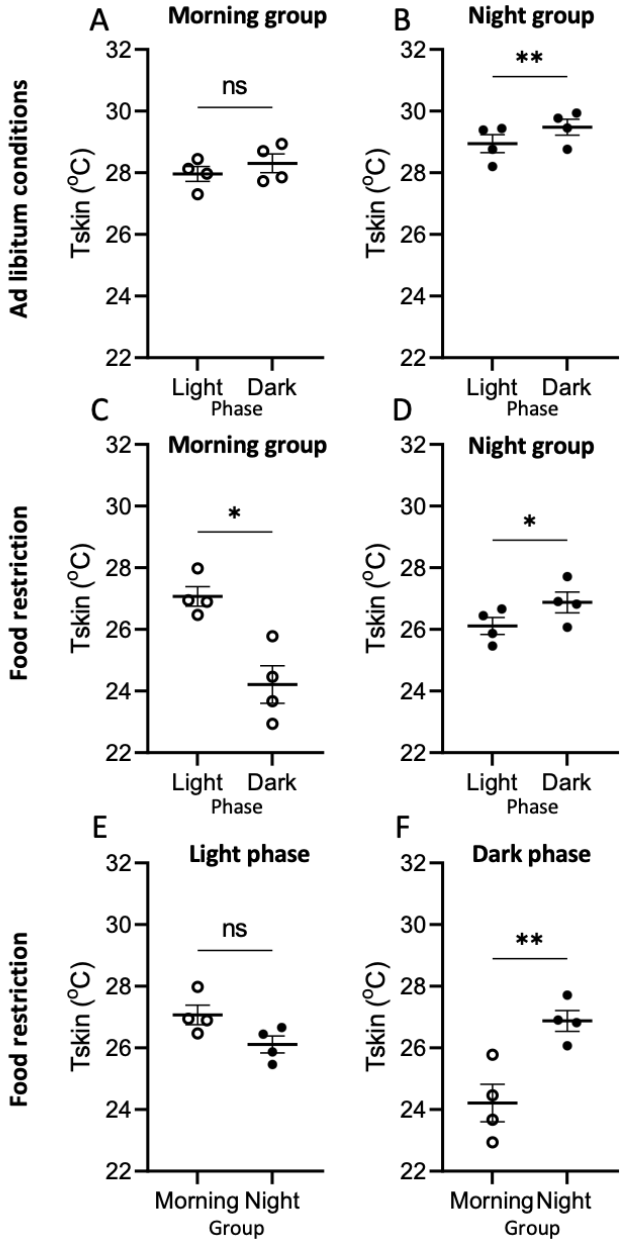

Figure 9: Comparison of circadian fluctuation in body temperature before and during food restriction, between feeding schedules. (A) No significant difference in Tskin in the light versus the dark phase was found during ad libitum feeding for the morning group ($p=0.1126$, $t(3)=2.22$; paired t-test). (B) Tskin was significantly higher during the dark phase during ad libitum feeding for the night group ($p=0.0036$, $t(3)=8.38$; paired t-test). (C) Tskin was significantly lower in the dark phase during food restriction compared to the light phase for the morning fed group ($p=0.0086$, $t(6)=3.83$; unpaired t-test). (D) Tskin was significantly higher in the dark phase during food restriction compared to the light phase for the morning fed group ($p=0.0086$, $t(6)=3.83$; unpaired t-test). (E) Comparison of Tskin during the light phase of food restriction between morning and night feeding ($p=0.0620$, $t(6)=2.29$; unpaired t-test). (F) Comparison of Tskin during the dark phase of food restriction between morning and night feeding ($p=0.0086$, $t(6)=3.83$; unpaired t-test). N=4 for each group. Data are represented as a mean value ± SEM. ns: non-significant ($p>0.05$), *$p<0.05$, **$p<0.01$.

3.4 Discussion

This chapter aimed to determine whether a common food restriction protocol would be sufficient to induce torpor in mice. In addition, the effect of feeding schedule on torpor propensity and characteristics was investigated.

The data presented here demonstrate that torpor is readily induced in response to this food restriction protocol regardless of feeding time. Daily torpor bouts were observed reliably in all mice from day 8 of food restriction onwards, coinciding with a decrease in bodyweight to ~85-90% of free-feeding bodyweight. Notably, a bodyweight of 85-90% is a common target bodyweight for behavioural testing and would be the point at which testing would ordinarily commence (Pioli et al., 2014), during which mice are spending 35-45% of the 24-hour day in torpor (Figure 7F, G), indicating that torpor is likely being inadvertently induced over the course of behavioural testing. Multiple animals started entering torpor much sooner than day 8, however, with one mouse in the morning group entering torpor on the second day of food restriction. By day 4 of food restriction, just over 60% of mice were entering torpor, demonstrating that torpor can also be induced at higher bodyweights, especially under an increasing metabolic challenge with prolonged food restriction.

Whilst experienced researchers may not take behavioural or physiological measurements from torpid mice, it is possible that inexperienced researchers may be unaware of the signs to looks for. As such, based on the findings presented in this chapter demonstrating the high propensity of torpor induction during food restriction, it is possible that any such measurements would be confounded by the presence of torpor. Moreover, the long term effects of torpor are largely unknown, so it is possible that torpor will still be confounding measurements even if taken when the mouse is not torpid. For example, there is evidence that torpor is associated with sleep

disruption and altered brain activity (Huang et al., 2021; Northeast et al., 2019; Vyazovskiy et al., 2017), and altered levels of circulating hormones such as thyroid hormones (Bank et al., 2015), leptin (Gavrilova et al., 1999), and ghrelin (Gluck et al., 2006), all of which could have long lasting effects on physiology. Moreover, there are some examples of torpor altering performance in behavioural tasks, an area in which food restriction is common. For example, torpor was found to improve memory retention of the location of a hidden platform in a Morris water maze in mice, compared to non-torpor controls (Nowakowski et al., 2009). Further, a study in ground squirrels that had learned to complete a Barnes maze before entering hibernation reported a high degree of variability in spatial memory between individuals when the task was conducted after hibernation (Hensleigh et al., 2022).

Here, mice were singly housed throughout the study due to thermal cameras only being able to record the hottest pixel in the cage. Single housing is common practice during torpor studies in mice due to the use of thermal cameras (Hitrec et al., 2019), or due to surgery being required to implant temperature-sensitive telemeters (Oelkrug et al., 2011). However, group housing is often used during behavioural testing to reduce cage costs and for welfare benefits. There is some evidence in other species that torpor still occurs in group settings, although social thermoregulation via huddling can modulate torpor characteristics (Geiser, 2010). For example, torpor in group housed Siberian hamsters (*Phodopus sungorus*) was shallower but longer in comparison to solitary hamsters (Jefimow et al., 2011). Furthermore, a study in group housed sugar gliders (*Petaurus breviceps*), a species of marsupial possum, found that food restriction resulted in synchronisation of torpor bouts, possibly to maximise energy savings (Nowack & Geiser, 2016). There is no record of social torpor being investigated in laboratory mice in the published literature, although evidence from other species suggests that the data presented in this chapter would be applicable to group housed mice also.

Feeding schedule did affect some aspects of torpor propensity and characteristics, including the number of torpor bouts and the circadian rhythm of body temperature. During the study, it was determined that it would not be appropriate to swap the feeding schedules between groups as initially planned, as the physiology of the mice had been so profoundly altered by the initial food restriction. As a result, the group sample sizes may have been too small and underpowered to detect some differences. It is possible that a larger sample size may have reported additional between group differences, such as torpor timing and duration, in line with previous studies (van der Vinne et al., 2018). Furthermore, food timing is not the primary zeitgeber for the circadian clock, therefore competing circadian forces (i.e., food timing, light-dark cycle) may explain why a clear effect was not observed. Future work may be able to expand on these findings by food restricting mice in constant darkness to mitigate the influence of light timing.

Interestingly, the number of torpor bouts did significantly differ between groups, although there was no difference in the overall percentage of time spent in torpor. There was also no significant difference in the duration of torpor bouts between groups, but bouts were generally shorter in the night fed group which may explain why there was no difference in the overall time spent in torpor, despite the number of bouts differing. It is possible that the feeding schedules result in differing degrees of conflict between food and light timing, with the light zeitgeber driving torpor bouts to the end of the dark phase whilst the feeding time delays and disrupts this drive. This is in line with a previous study that reported that feeding delayed or prevented torpor from occurring at the typical circadian time (van der Vinne et al., 2018). As such, the increased number of bouts may be compensating for the reduced duration, therefore suggesting that torpor is adaptive to guarantee a certain degree of energy savings to be achieved. The idea that torpor may be adaptive is supported by previous studies that show that

torpor bouts are extended when ambient temperature is reduced due to increased energy required for thermoregulation (Buck & Barnes, 2000).

3.4.1 Conclusions

Overall, the data presented in this chapter demonstrate that torpor induction and feeding schedule are important considerations when designing experiments involving food restriction. One approach would be to control for the presence of torpor by conducting tasks or taking physiological samples when torpor history, including number and depth of bouts, is comparable between individuals and days to minimise within and between individual variation. In addition, feeding schedule should be consistent between animals and between days, and should be detailed in the methods when results are reported to facilitate replication. Finally, it may be possible depending on the study to provide food at a time that will minimise the impact of torpor on subsequent experimental interventions. For example, food could be provided in the morning to shift torpor bouts to the dark phase, thus providing a window of opportunity to perform experimental manipulations.

Chapter 4: Investigating the effect of torpor on activity, behaviour, and learning

4.1 Introduction

Behaviour is often regarded as the optimal functional readout of underlying neurology and physiology, therefore making behavioural tasks a useful tool for phenotyping genetic

mutations, pharmacological agents, and altered behavioural states, such as sleep. As such, many tasks have been developed and validated to investigate the many modalities of behaviour, including cognitive processes such as learning and memory.

Some tasks rely on spontaneous behaviours but many of the most frequently used tasks employ food restriction and food reward to motivate task engagement (Barkus et al., 2022), and to assist with learning of more complex tasks (Makowiecki et al., 2012). In the previous chapter, it was demonstrated that torpor is readily induced in mice when maintained on a food restriction protocol that is common in behavioural research. Moreover, these data showed that torpor bouts began to reliably occur at the target bodyweight for most appetitive behavioural tasks of ~85% of ad libitum bodyweight. Torpor itself is associated with profound changes in central and peripheral physiology (Melvin & Andrews, 2009), which then may be reflected in subsequent behaviours. Previous studies have shown that the behavioural state of mice prior to undertaking a task can significantly alter their performance (Milinski et al., 2021). For example, performance in a touchscreen task and a novel object recognition task were significantly altered by the degree of voluntary wheel running, and the amount of time spent asleep prior to conducting the tasks. As such, it is possible that the variation in torpor bout timing, length, and depth may also differentially effect behaviour due to differing effects on underlying physiology.

The potential for torpor to influence behavioural data has been previously investigated but the results are largely inconclusive. For example, there are multiple studies that report that torpor enhances (McNamara & Riedesel, 1973; Nowakowski et al., 2009), impairs (Bullmann et al., 2019; Palchykova et al., 2006), or has no effect on memory (Bullmann et al., 2016; Hensleigh et al., 2022; Ruczynski & Siemers, 2011). These conflicting results can be attributed to differences in experimental paradigm, behavioural task, model species being used, and form of

torpor being investigated. As a result, the impact of torpor on behaviour remains largely undetermined.

Much of the current literature has been conducted in hibernators that enter multi-day torpor, and so may not be applicable to studies using food restricted laboratory mice. As mice are a much more widely used model species within biomedical research, it is important that this gap is addressed. However, torpor induction in mice is dependent on food restriction which may also influence behaviour and brain function. For example, food restriction was found to impair memory in a novel object recognition task (Carlini et al., 2008), and spatial recognition in a Y-maze (Fu et al., 2017). In addition, a recent study found an impairment during a visual discrimination task due to a reduction in coding precision during food restriction (Padamsey et al., 2021). Conversely, food restriction enhanced memory during a maze task in which mice had to remember the location of a hidden food reward (Hashimoto & Watanabe, 2005), and in a Morris water maze (Kuhla et al., 2013). It should be noted that these experiments were performed using food restricted mice and did not account for torpor. As such, the discrepancy in these findings may be as a result of torpor induction, or due to torpor differentially impacting different aspects of cognition.

Studies investigating torpor at a neuronal level have reported significant remodelling of neuronal and dendritic morphology that is restored upon arousal. Notably, a transient reduction in the number of dendritic spines and synapses has been observed in the hippocampus and in the cortex, that is reversed within 2-3 hours following arousal from torpor (Popov et al., 1992; Popov & Bocharova, 1992; Roelandse & Matus, 2004; Von Der Ohe et al., 2006). However, it should be noted that these data have been gathered from hibernators that enter much deeper

torpor bouts than can be achieved by mice, although data from mouse brain slices stored at cold temperatures also show a loss of dendritic spines (Roelandse & Matus, 2004).

These studies suggest that torpor represents a period of significant neural plasticity due to this transient remodelling of neurones and dendrites. As a result, this has led to recent interest in the capabilities for torpor mechanisms to inform treatments for neurodegenerative disease. For example, there have been reports of torpid hamsters showing increased accumulation of hyperphosphorylated tau protein during torpor, which is then reversed upon arousal (Arendt & Bullmann, 2013), with similar findings reported in pharmacologically induced 'synthetic torpor' in rats (Hitrec et al., 2021; Luppi et al., 2019). Moreover, a recent study in a mouse model for Alzheimer's disease found that a single bout of torpor restored contextual fear memory, indicating the potential for clinical translation of these findings, and supports the enhancement of cognitive processes following torpor (de Veij Mestdagh et al., 2021).

This chapters aims to provide insight into the effects of fasting-induced torpor on behavioural performance in mice. It is hoped that the results presented here will help to inform the experimental design of studies uses food restricted behavioural tasks, and to identify whether torpor is a confounding factor. Moreover, these data may also have relevance to clinical data into the investigation of neurodegenerative disease.

4.1.1 Experimental aims

i) Investigate the effects of torpor induction on locomotor activity, exploratory behaviour, and emotionality, using an open field task

ii) Investigate the effects of torpor on short-term recognition memory using a novel object recognition task

4.2 Methods

4.2.1 Animals and recording conditions

Mice were acquired from an in-house colony at the University of Oxford's Biomedical Services Building for this study (C57BL/6J; males; aged ~8 weeks; n=12), and individually housed in wire top cages (M3 cage, 48 x 15 x 13 cm) on a 12:12 h light-dark cycle (12:12 LD; lights on at 7 am) for the duration of the experiment. Cages were housed in custom made light-tight chambers (LTCs) with four cages per chamber (Fisher et al., 2012; Tam et al., 2021). LTCs were illuminated by warm white LED strips during the light phase of the 12:12 LD cycle. Ambient room temperature and relative humidity were maintained at 21 ± 1°C and 60 ± 1%, respectively. Water was provided ad libitum throughout.

4.2.2 Experimental protocol

To investigate the effect of torpor induction on subsequent behavioural performance, a subset of mice (n=6) was placed on a chronic food restriction protocol, as previously described in Chapter 2. In brief, mice received a single food ration (~2.0-2.5g) per day at zeitgeber time 10 (ZT 10; 5pm) and were weighed ~1.5 hours after food was provided. Food restricted mice were maintained at ~85% of their free feeding bodyweight throughout. The remaining mice (n=6) were fed ad libitum throughout as a non-food restricted control group; mice were assigned to each group in a pseudo random manner. A non-food restricted control was used due to mice readily entering torpor even when under mild food restriction, meaning that there would have been the potential influence of prior torpor history.

Tskin was continuously monitored in all mice using non-invasive thermal imaging cameras to detect torpor, as previously described in Chapter 2. Torpor was determined as when Tskin

decreased by >3 SD below the euthermic median Tskin for >1 hour. Behavioural testing commenced once all food restricted mice were reliably entering torpor daily (after ~1 week of food restriction).

4.2.3 Open field task

An open field task was conducted to measure the effect of torpor on locomotor activity, exploratory behaviour, and anxiety associated behaviours (e.g., thigmotaxis (avoidance of the arena centre), freezing, faecal boli etc). Open top square arenas were used to run the task (Retail Acrylics, Cardiff UK; 25.5cm x 25cm x 25cm), with one food restricted animal and one control mouse being run simultaneously in separate arenas. Arena walls were made from clear acrylic which were covered with black paper to prevent external visual stimuli from influencing performance. The arena base was made from white opaque acrylic to contrast with the mouse, allowing for easier detection of behaviours by the tracking software. Light levels were adjusted so that the light intensity at the bottom of the arena was ~150 lux.

Cameras were mounted above each arena and connected to a laptop running the tracking software, ANY-Maze (Stoelting Co. Illinois; version 4.5). The software was programmed to divide the arenas into 4 x 4 quadrants and was used track the nose, centre of the mouse's body, and the mouse's tail throughout each trial. Entry into and exit from a quadrant was defined as when the base of the tail crossed the quadrant boundary line. Each trial lasted 10 minutes.

All mice were tested in the open field at four time points to determine the effects of prior torpor history on performance. The first timepoint was conducted immediately following arousal from torpor (time 0), as determined using the thermal imaging cameras. Subsequent trials were performed at 20 minute intervals relative to arousal from torpor (+20 mins, +40 mins, and +60

mins). Behavioural testing commenced at ~ZT 1 due to torpor bouts typically occurring during the dark period (Figure 10).

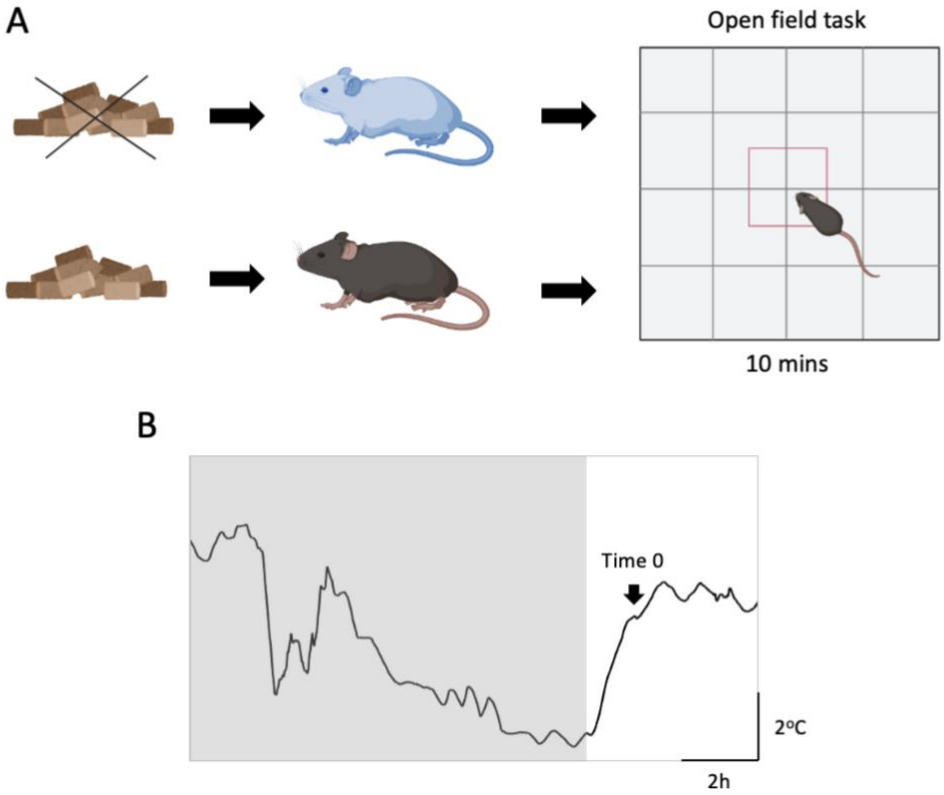

Figure 10: Experimental paradigm for open field task. (A) Mice were either food restricted to induce torpor or remained on ad libitum feeding (n=6 for each group). Following reliable induction of daily torpor, one mouse from each group performed an open field task in parallel. The task lasted for 10 minutes and behaviours were tracked using ANY-Maze software. (B) Peripheral body temperature was monitored to determine when mice were in torpor. Mice were first tested immediately following arousal from torpor (Time 0) and then in 20 minute intervals from this point (Time +20, Time +40, Time +60).

4.2.4 Novel object recognition task

Approximately 1 week following completion of the open field task, mice completed a novel object recognition task (NORT) to assess the effects of torpor on object recognition memory. The torpor group were maintained on food restriction throughout. The same arenas as used for the OFT were used here to mitigate any potential confounding effect of neophobia and competition for attentional resources that can occur when mice are placed in a novel environment.

The NORT was split into three phases: sample phase, inter-trial interval (ITI), and testing phase. During the sample phase, two identical objects (A1 and A2) were placed on predefined markers in the arena. Each mouse was then placed in the chamber, as far away from the objects as possible, and allowed to explore for 10 minutes. Mice were then returned to their home cages for an inter-trial interval (ITI) of 5 minutes. Following the ITI, mice were placed in the testing arena with a new identical object (A3), and one novel object (B1) for the test phase which lasted 3 minutes (Figure 11). The position of the novel and familiar object (i.e., left or right), and which of the objects was novel (A vs. B), was counterbalanced across animals.

All mice completed two NORT trials. The first trial was conducted ~20 minutes after arousal from torpor, whilst the second was conducted ~4 hours later when food restricted mice had been euthermic for a number of hours. A different batch of objects were used in each of the trials; objects were counterbalanced across the two trials. Objects were chosen so that they were visually different in size and shape, but similar in texture and appeal. Initial pilot experiments in a separate cohort confirmed that there was no innate preference for any of the objects used in this experiment.

ANY-Maze software was used to track the nose and body of each mouse during the NORT. The mouse was determined to be investigating an object when its nose was close to the object; climbing on an object was not deemed as exploration of that object and thus did not contribute towards total exploration time.

Exploration of objects during the sample and test phase were used to calculate absolute discrimination, and a discrimination index as described by Lueptow (Lueptow, 2017). The

discrimination index (DI) was calculated by subtracting the time spent exploring the familiar object from the time spent exploring the novel object, divided by total exploration time. As such, the DI accounts for differences in total exploration time and provides a measurement between -1 and +1, where -1 is preference for the familiar object, 0 indicates no preference and indicates chance, and +1 is preference for the novel object.

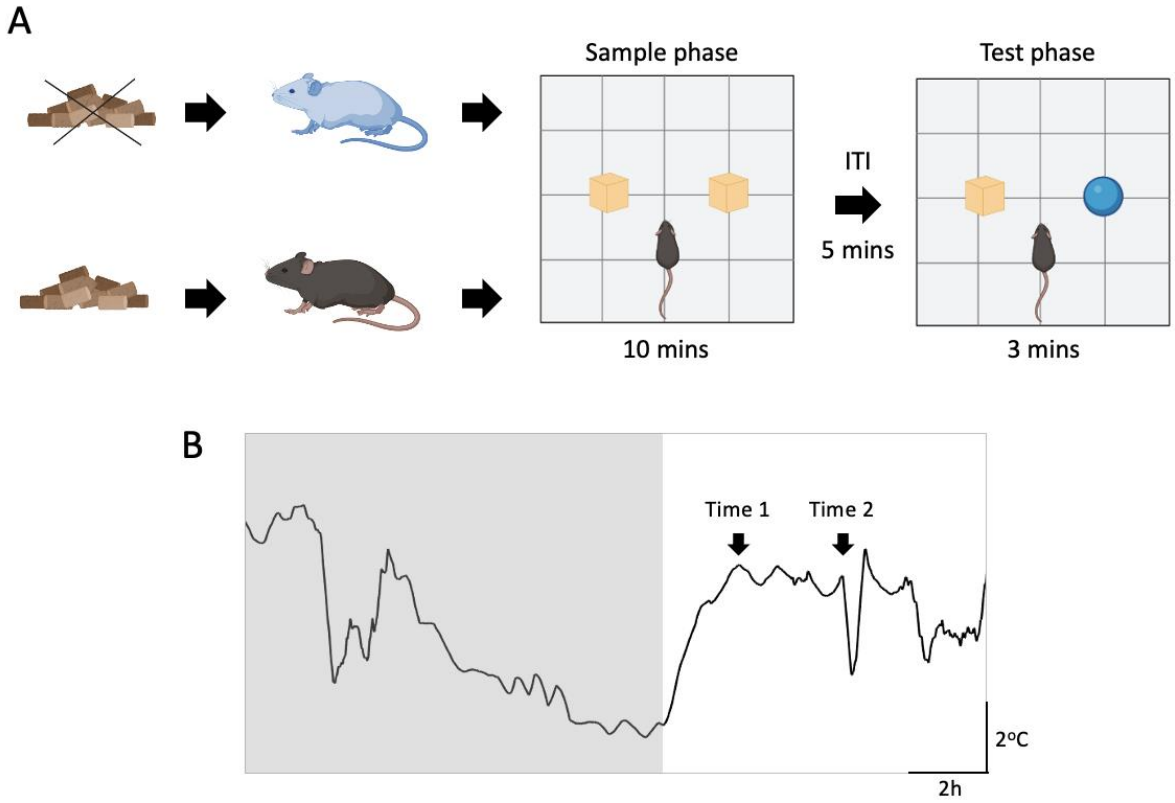

Figure 11: Novel object recognition task paradigm. (A) Mice were either food restricted to induce torpor or remained on ad libitum feeding (n=6 for each group). Following reliable induction of daily torpor, one mouse from each group performed the novel object recognition task, consisting of a sample phase (10 mins) using two identical objects, an inter-trial interval (5 mins), and a test phase (3 mins) with one familiar and one novel object. Behaviours were tracked using ANY-Maze software. (B) Peripheral body temperature was monitored to determine when mice were in torpor. Mice were first tested 20 minutes following arousal from torpor (Time 1) and again ~4 hours later when food restricted mice had been euthermic for a number of hours (Time 2).

4.2.5 Task optimisation: Ethanol versus Anistel

Behavioural testing is notorious for being heavily influenced by environmental factors that can contribute to variability and inconclusive results. The behavioural tasks described in this chapter were no exception, and many of the mice appeared to be disengaged with the task resulting in low overall exploration times. Indeed, many of the mice did not reach the inclusion criteria of >20s of total object exploration time during the sample phase of the NORT. As such, the possibility for external environmental factors to have been influencing exploratory behaviour were investigated.

The cleaning substance Anistel™, formerly Trigene™, a common disinfectant used in laboratory settings, was used to clean the arena between trials. Often, 70% ethanol is the preferred substance for cleaning equipment between trials due to it being odourless and quick to evaporate. However, this may not always be the case or common practice across laboratories. Moreover, it has been suggested previously that Anistel™ may influence mouse behaviour due to its strong smell (López-Salesansky et al., 2016); mice rely heavily on olfactory cues and exposure to certain odours can induce anxiety and aversive behaviours (Hacquemand et al., 2013; Janitzky et al., 2015). As such, the effect of cleaning substance on behaviour was investigated.

To this end, a small pilot study was conducted in a new batch of mice from the same in-house colony (N=6; C57BL/6J; male; ~8 weeks old) to investigate whether cleaning substance would influence behavioural performance. Food and water were provided ad libitum to all mice throughout this study. Mice underwent behavioural testing in the open field task and then the novel object recognition task as previously described. A crossover design was used for this study to allow for within and between subject analysis to be conducted, thus increasing the statistical power. As such, half the mice (n=3) first completed the tasks when the arena was

cleaned with ethanol, whilst the arenas for the remaining mice were cleaned with Anistel™ (n=3). Approximately one week later, the tasks were re-run using the other cleaning substance (Figure 12).

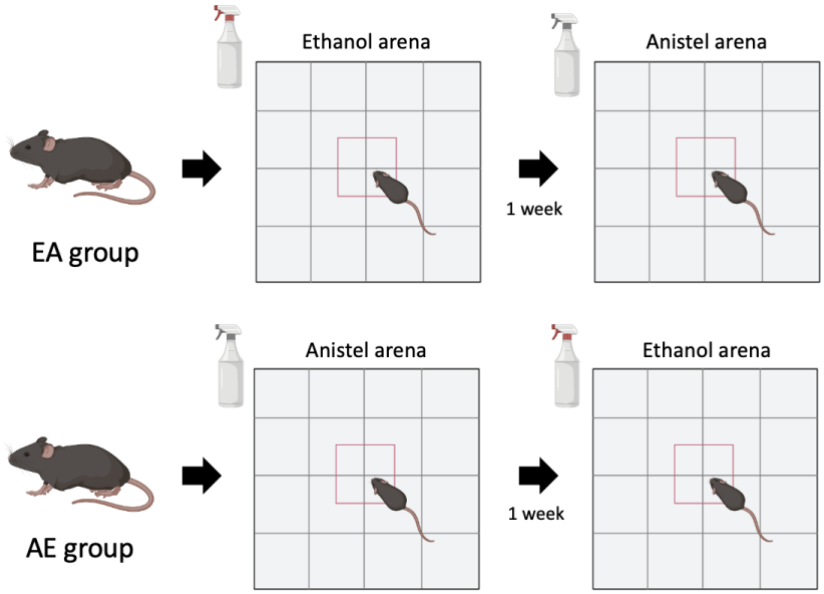

Figure 12: Paradigm for investigating the effects of cleaning substance on behavioural performance. Mice (N=6) completed an open field task and a novel object recognition task that had been cleaned with either 70% ethanol or Anistel™. After one week, the tasks were re-run, but the arenas were cleaned with the substance to which the mice had not previously been exposed.

4.2.6 Statistical analysis

Data were exported from the ANY-Maze software to Microsoft Excel and analysed using GraphPad Prism (version 9). All data were tested for normality using a Shapiro-Wilk test prior to analysis. Parametric data are presented as mean values ± SEM whilst non-parametric data are presented as median values and 95% confidence intervals. Data from the open field task and novel object recognition task were analysed using a 2-way ANOVA with repeated measures. Where the ANOVAs found an interaction to be present, post-hoc analysis was performed using a multiple comparisons test. A Geisser-Greenhouse correction was used following ANOVAs on all occasions. Data presented in Figures 7 and 8 were analysed using either student t-tests or Mann-Whitney U tests, depending on the data distribution. One-sample

t-tests were used to compare the discrimination index during the NORT with chance, where chance is equal to zero.

4.3 Results

4.3.1 Open field task

The open field task revealed a stark difference in locomotor activity and exploratory behaviour in the fasted mice compared to the non-fasted mice at time 0. Representative examples of trajectory plots from a mouse in each group, shown in Figure 13, indicate that fasted mice do not engage with, or explore, the novel environment when testing occurred immediately following torpor. However, subsequent time points indicate that locomotor activity and exploratory behaviour were restored in fasted mice.

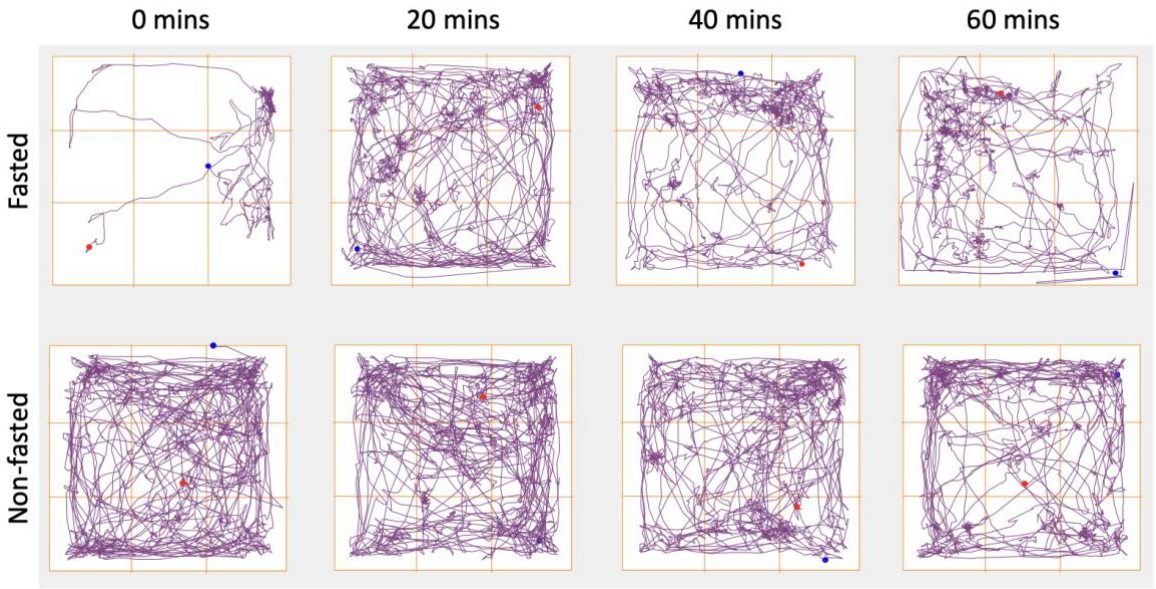

Figure 13: Trajectory plots during an open field task. Plots showing locomotor activity from a representative fasted (top panel) and non-fasted mouse (bottom panel) during testing at different time points relative to a torpor bout. Each test lasted 10 minutes. Plots were generated using ANY-maze tracking software. Red circles indicate the position of the mouse at the start of the trial, whilst the blue circles indicate the ending position.

Quantification of the distance travelled, and time spent immobile during the task confirmed these initial observations. Analysis using a 2-way ANOVA found reported a significant interaction between time point*group, a significant main effect of individual, but no significant main effect of time or group (Figure 14A; interaction between timepoint*group: $F_{(3,30)}=14.4$, $p<0.0001$; main effect of individual: $F_{(10,30)}=2.34$, $p=0.0349$; main effect of time point: $F_{(2.16,21.6)}=3.02$, $p=0.0664$; main effect of group: $F_{(1,10)}=2.15$, $p=0.1736$; 2-way ANOVA with repeated measures). Due to the significant interaction between time point*group, a post-hoc analysis was performed which revealed that fasted mice travelled significantly less distance at Time 0 compared to non-fasted controls (fasted: 2.02 ± 0.533 m; non-fasted: 22.5 ± 1.65 m; $p<0.0001$, Šidák's multiple comparisons test). No significant differences between groups were reported at subsequent time points. Distance travelled reduced across trials for the non-fasted group, likely due to the environment becoming more familiar. No significant main effect of trial was reported, although it does appear to be trending towards significance.

The initial difference in distance travelled between groups may be explained by differences in the time spent immobile as a significant main effect of time point, group, and individual were found, in addition to a significant interaction between timepoint*group (Figure 14B; interaction between timepoint*group: $F_{(3,30)}=22.5$, $p<0.0001$; main effect of individual: $F_{(10,30)}=2.51$, $p=0.0251$; main effect of time point: $F_{(2.14,21.4)}=6.65$, $p=0.005$; main effect of group: $F_{(1,10)}=9.04$, $p=0.0132$; 2-way ANOVA with repeated measures). Post-hoc analysis revealed that a significant difference between groups was found at Time 0 (fasted: 519.6 ± 18.3 s; non-fasted: 113.9 ± 13.5 s; $p<0.0001$, Šidák's multiple comparisons test), therefore indicating that increased immobility in the fasted group was the reason for less distance travelled.

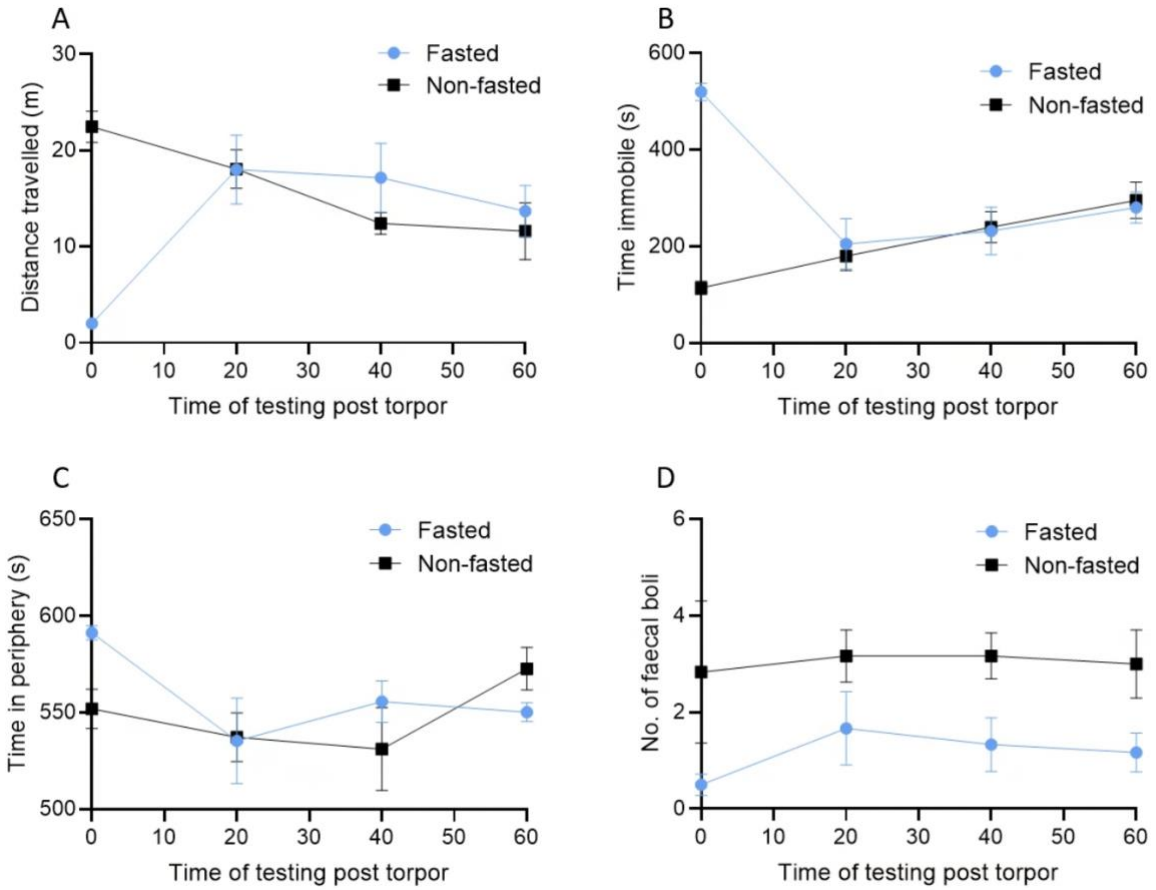

Figure 14: Testing immediately following torpor reveals reduced locomotor activity, whilst effect on putative measures of anxiety were mixed in an open field task. (A) Fasted mice travelled significantly less distance in at Time 0 compared to non-fasted mice. Distance travelled returned to levels comparable with non-fasted controls at later time point. (B) Fasted mice spent significantly more time immobile compared to non-fasted mice at Time 0, with levels becoming comparable at later time points. (C) Total amount of time spent in the arena periphery during each task (D) Number of faecal boli present in the arena at the end of each trial. Each trial lasted 10 mins. Data are represented as a mean value ± SEM (n=6 for both groups).

Next, putative measures of anxiety (thigmotaxis and faecal boli) were investigated and compared between fasted and non-fasted mice over each trial. Due to fasted mice showing reduced exploratory behaviour during the initial trial at Time 0, it was hypothesised that they may show heightened neophobia and anxiety during subsequent testing compared to non-fasted mice. To this end, the amount of time spent by each mouse in the periphery of the arena, and the number of faecal boli present in the arena at the end of each test, as both metrics are

associated with higher levels of anxiety (Hall 1932, Hall 1934, Lister 1990). It is important to note that the increased levels of immobility in fasted mice during the first trial may impact the amount of time spend in the centre vs periphery at this timepoint, and so may not be reflective of anxiety levels. As such, care must be taken when interpreting these data.

A significant effect of testing time point was found when comparing the time spent in the periphery over each trial a mice became more habituated to the arena (Figure 14C; main effect of time: $F_{(2.12, 21.3)}$= 5.60, p=0.0101; 2-way ANOVA with repeated measures). A significant interaction between testing timepoint*group and a significant main effect of individual were also found, but no main effect of group was observed (interaction between timepoint*group: $F_{(3, 30)}$= 4.01, p=0.0164; main effect of individual: $F_{(10, 30)}$= 4.88, p=0.0003; main effect of group: $F_{(1,10)}$= 0.4269, p=0.5282; 2-way ANOVA with repeated measures). Post-hoc analysis revealed a significant difference in time spent in periphery was only observed at Time 0 (fasted: 591.4 ± 3.72 s; non-fasted: 552.0 ± 10.2 s; p=0.039, Šidák's multiple comparisons test). However, as previously mentioned this time point may be affected by the increased levels of immobility observed in fasted mice at this timepoint.

Fasted mice produced consistently fewer faecal boli across trials than non-fasted mice (Figure 14D). Analysis of these data found a significant main effect of group, but no effect of timepoint or individual. Moreover, no significant interaction between timepoint*group was observed (interaction between timepoint*group: $F_{(3, 30)}$= 0.1865, p=0.9047; main effect of individual: $F_{(10, 30)}$= 1.188, p=0.3370; main effect of group: $F_{(1,10)}$=10.3, p=0.0094; main effect of timepoint: $F_{(1.73,17.3)}$=0.4707, p=0.6056; 2-way ANOVA with repeated measures).

4.3.2 Novel object recognition task

Following the open field task, a novel object recognition task was used to assess the effect of prior torpor history on short term recognition memory. To this end, both fasted and non-fasted mice completed two NORTs the first being conducted ~20 minutes following arousal from torpor whilst the second NORT was conducted after fasted animals had been euthermic for a number of hours. First, the amount of time each mouse spent exploring the objects in the sample phases (Figure 15 A&E) and during the first minute of the test phases (Figure 15 B&F) were compared. No between group differences were found in the time spent exploring the object except during the test phase of the second NORT in which fasted mice spent significantly longer the exploring objects than non-fasted mice (Fasted: 16.6 ± 3.73s; Non-fasted: 2.77 ± 1.41s; $t(10)=3.48$, $p=0.0059$, unpaired t-test; Figure 15F). Notably, total exploration time during was very low for both groups during both NORTs, with only 25% of animals exploring the objects during the sample phase for >20s, a threshold that is often used an inclusion criteria in NORT studies (Lueptow, 2017). However, due to the relatively small sample size used, all animals were used for subsequent analysis, but care must be taken in the interpretation of these results.

Next, the discrimination index was calculated to account for differences in exploration time between animals with a discrimination index of zero being equal to chance. To determine whether a novelty preference was present in either of the NORTs, the discrimination index for both groups was compared against chance using one sample t-tests. This found no significant difference between the discrimination index and chance for either group in either of the NORTs, indicating that no novelty preference was present (NORT1: fasted vs chance: $t(5)=1.69$, $p=0.153$; non-fasted vs chance: $t(5)=0.671$, $p=0.532$; NORT2: fasted vs chance:

t(5)=1.18, 0.294; non-fasted vs chance: t(5)=0.284, p=0.788). As such, the conclusions that can be drawn from these data are limited. The discrimination index was also compared between fasted and non-fasted animals although no differences were found in either NORT (NORT1: t(10)=0.737, p=0.478; NORT2: t(10)=0.475, p=0.645; unpaired t-tests; Figure 15 C&F).

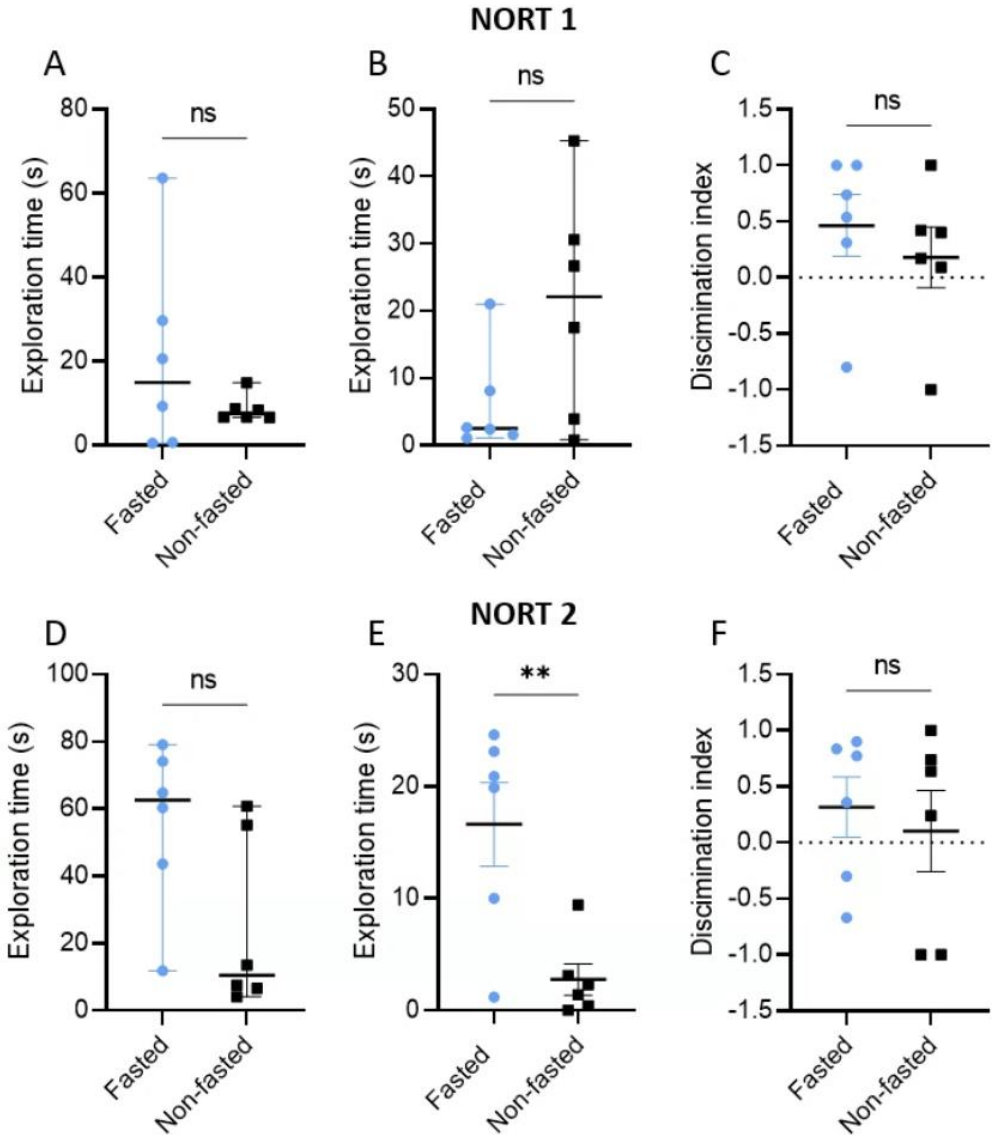

Figure 15: Performance during two novel object recognition tasks (NORT) in fasted and non-fasted mice. (A) Total time spent exploring both objects in the first minute of the test phase of the first NORT conducted shortly following arousal from torpor. (B) Total time spent exploring two identical objects during the test phase of the first NORT. (C) Comparison of discrimination index between groups during the first NORT. (D) Total time spent exploring two identical objects during the sample phase of the second NORT conducted following an extended period or euthermia. (E) Total time spent exploring two identical objects during the test phase of the second NORT. (F) Comparison of discrimination index between groups during the first trial. Data are presented as the mean ± SEM, except B and D which show the median and IQR. N=6 for both groups. ns: non-significant (p>0.05). Dotted lines on C and F indicate chance.

Next, performance during each of the NORTs were compared to determine whether time of testing relative to torpor affects behavioural outcomes and memory. Total exploration time during the sample phase significantly increased from 20.7 ± 9.76 s during NORT1 to 55.7 ± 10.1 s in NORT2 for the fasted group ($t(5)=3.11$, $p=0.0264$, paired t-test; Figure 16A). When accounting for exploration time using the discrimination index, no difference between trials was found ($t(5)=0.320$, $p=0.762$, paired t-test; Figure 8B).

Due to the NORTs being conducted at different time points, non-fasted animals were run in parallel to control for potential circadian influences. Two of the six animals showed an increase in total exploration time during the second NORT; however, comparison of the whole group did not show an overall significant change ($W=9$, $p=0.313$, Wilcoxon test; Figure 16D). Moreover, no difference in the discrimination index was reported between the NORTs for the non-fasted group ($t(5)=0.191$, $p=0.856$, paired t-test; Figure 16E).

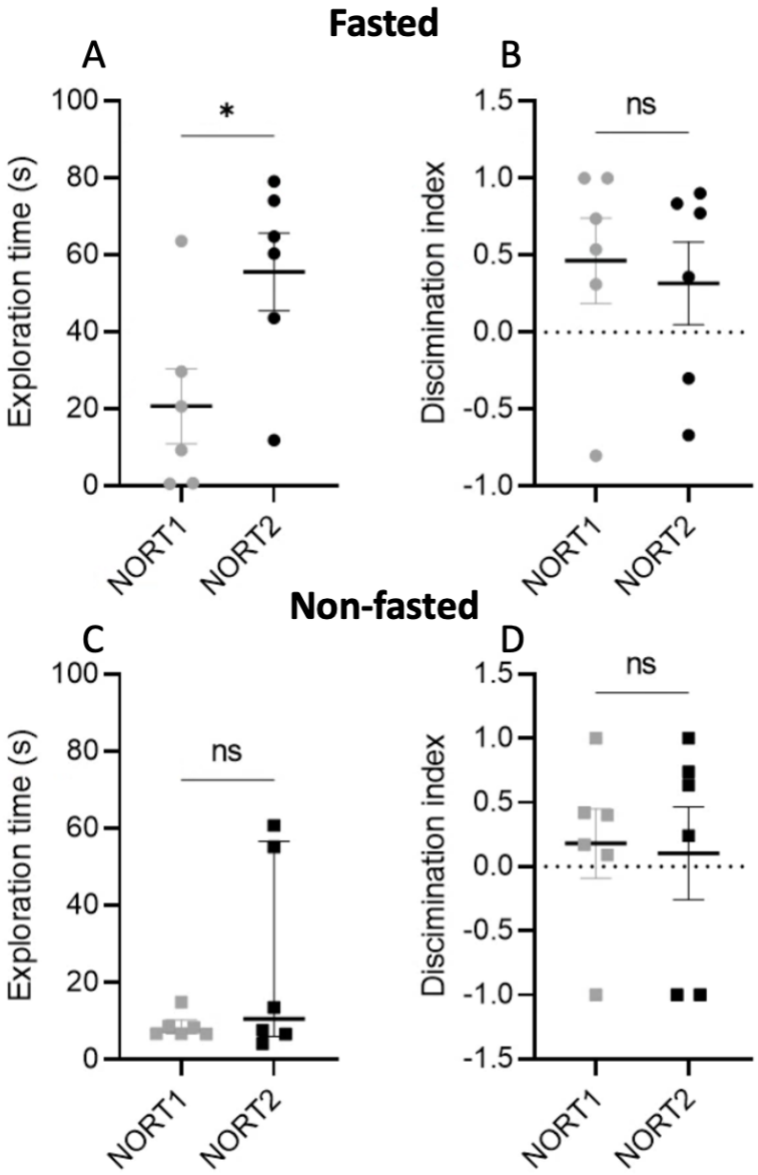

Figure 16: Comparison of performance in a novel object recognition task between trials. (A-C) Difference in total exploration time during the sample phase, absolute discrimination, and discrimination index between Trial 1 and Trial 2 in fasted mice. (D-E) Difference in total exploration time during the sample phase, absolute discrimination, and discrimination index between Trial 1 and Trial 2 in non-fasted mice. Trial 1 was conducted following complete rewarming from torpor (~ZT1), whilst trial 2 was conducted following an extended period of euthermia (~ZT6). Data are presented as the mean ± SEM, except E which shows the median and IQR. N=6 for both groups. *p<0.05, ns: non-significant (p>0.05).

4.3.3 Task optimisation: Ethanol vs. Anistel

Following inspection of the NORT data presented above, it was apparent that mice were not performing better than chance in either group. It is well known that environmental factors can significantly impact the performance of mice during such tasks (Saré et al., 2021), as such potential factors that may have contributed to the lack of task engagement were investigated. One such factor was the choice of cleaning agent used to wipe down the arena and objects between animal as it was noted that Anistel^{TM}, a common disinfectant used in laboratory settings, had been used to clean the arenas between tasks. It has been suggested previously that Anistel^{TM} may influence mouse behaviour due to its strong smell (López-Salesansky et al., 2016) and so may explain why even the control group were not performing better than chance. As a result, a pilot study was conducted to investigate the effect of cleaning with Anistel^{TM} versus 70% ethanol on behavioural performance in a separate cohort of mice using a counterbalanced cross over design.

Open field task

Ad libitum fed mice completed the open field task as previously described with the order of exposure to cleaning agent being counterbalanced so that mice were tested in an arena cleaned with either ethanol first or Anistel first (EA group and AE group, respectively). Quantification of the distance travelled across each trial indicated that mice travelled further in the first trial. Analysis of these data using a 2-way ANOVA revealed a significant interaction between group*cleaning agent with no main effect of group or cleaning agent (group*cleaning agent: $F_{(1,4)}$=32.7, p=0.0046; main effect of group: $F_{(1,4)}$=2.49, p=0.190; main effect of cleaning agent: $F_{(1,4)}$=0.3049, p=0.8384; 2-way ANOVA). Post-hoc analysis using a Šidák multiple comparisons test demonstrated that mice travelled significantly further during the first trial for

both group. This, in addition to the ANOVA results, suggests that there is no effect of cleaning agent on distance travelled and the differences observed are due to the trial order (Figure 17A).

Further, comparisons in putative measures of anxiety when the arena was cleaned with ethanol or Anistel were performed. No main effect of group or cleaning agent were found when comparing the time spent in the periphery (main effect of group: $F_{(1,4)}$=0.264, p=0.6345; main effect of cleaning agent: $F_{(1,4)}$=5.56, p=0.0779; 2-way RM ANOVA), nor were any main effects observed when measuring the number of faecal boli (main effect of group: $F_{(1,4)}$=0.1084, p=0.7585; main effect of cleaning agent: $F_{(1,4)}$=6.818, p=0.1967; 2-way RM ANOVA). Moreover, no significant interaction between group*cleaning agent was observed for either variable (time spent in the periphery: $F_{(1,4)}$=0.3799, p=0.5710; no. of faecal boli: : $F_{(1,4)}$=0.2727, p=0.6291).

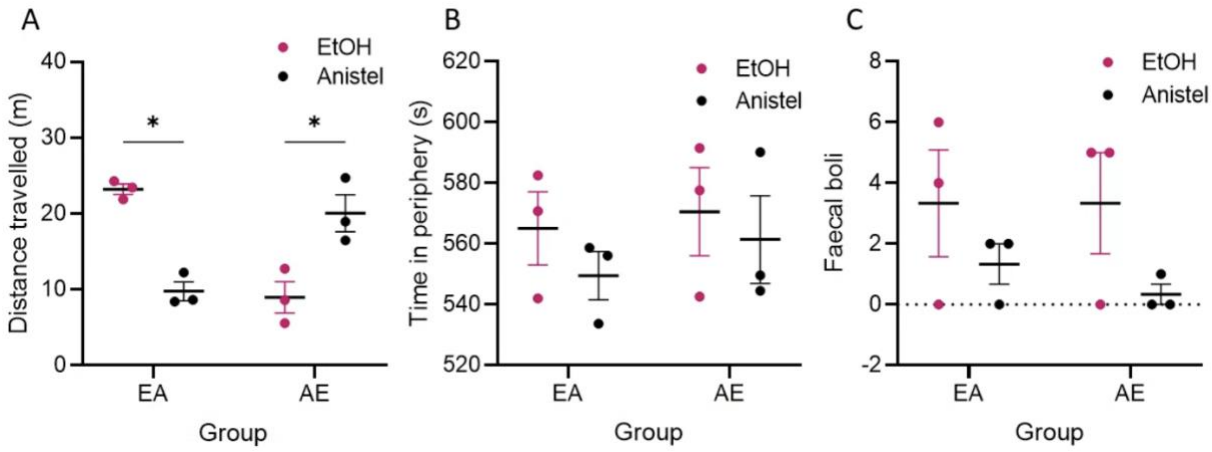

Figure 17: Effect of cleaning agent on behaviour during an open field task (OFT). (A) Distance travelled during the OFT when the arena was cleaned with either ethanol or Anistel. (B) No effect of cleaning agent was found on the time spent in the periphery during the OFT. (C) No effect of cleaning agent was found on the number of faecal boli produced during the OFT. Data are presented as the mean ± SEM. Each group has an N=3, except D and F which have an N=6. *p<0.05.

Novel object recognition task

It was subsequently investigated as to whether any difference in performance during a novel object recognition task would be observed when the arena and objects were cleaned with ethanol or Anistel. Analysis of the total time spent exploring the two identical objects presented during the sample phase found no main effect of cleaning agent or group (Figure 18A; main effect of cleaning agent: $F_{(1,4)}$=0.601, p=0.4825; main effect of group: $F_{(1,4)}$=1.193, p=0.336). Moreover, no significant interaction between cleaning agent*group was found ($F_{(1,4)}$=1.094, p=0.3545; RM 2-way ANOVA). Similarly, no main effect of cleaning agent was observed on the total time spent exploring both objects during the first minute of the test phase of the NORT (Figure 10B; $F_{(1,4)}$=1.158, p=0.3425; RM 2-way ANOVA), nor was a main effect of group reported ($F_{(1,4)}$=0.1546, 0.7142; RM 2-way ANOVA). In addition, there was no significant interaction between group*cleaning agent ($F_{(1,4)}$=0.1531, p=0.7155; RM 2-way ANOVA).

Comparison of the discrimination index during the test phase of the NORT reported a significant interaction between group*treatment ($F_{(1,4)}$= 9.962, p=0.0343; RM 2-way ANOVA), although follow up post hoc analysis did not find a significant difference between groups (Figure 18C). Further, no main effect of cleaning agent or group were observed on the discrimination index (cleaning agent: $F_{(1,4)}$=2.892, p=0.1643; group: $F_{(1,4)}$=0.2187, p=0.6643; RM 2-way ANOVA). Comparison of the discrimination index in the ethanol and Anistel NORTs with chance found that animals where still not performing significantly better than chance, indicating that a separate environmental factor may be contributing to poor task performance (EtOH vs chance: t(5)=0.2129, p=0.8398; Anistel vs. chance: t(5)=0.9022, p=0.4083; one sample t-tests).

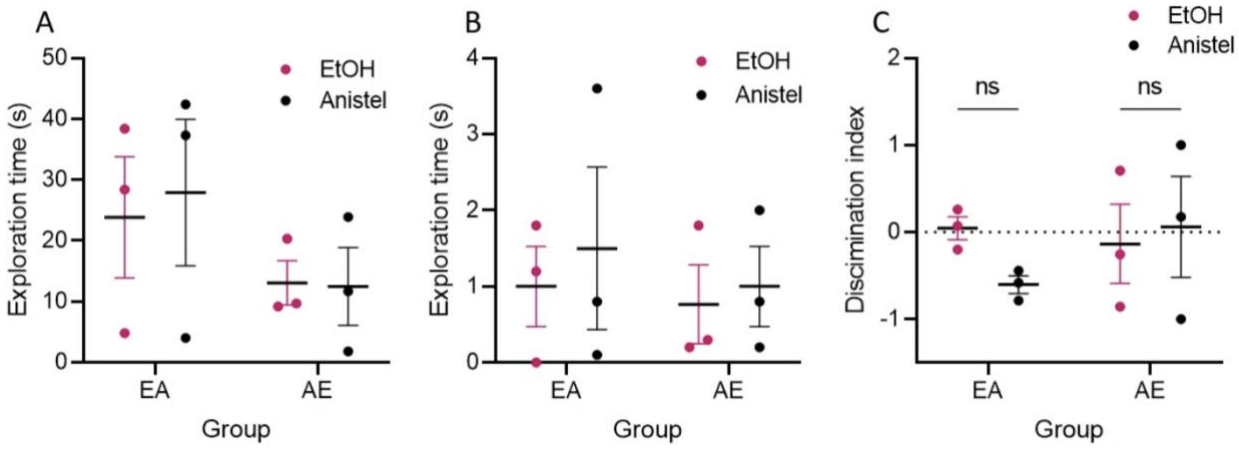

Figure 18: Cleaning substance does not affect performance in a novel object recognition task. (A) Exploration time of two identical objects during the sample phase in an arena cleaned with ethanol or Anistel. (B) Overall exploration time of both the novel and familiar object in the first minute of the test phase. (C) Comparison of the discrimination index during the test phase. The dotted line indicates chance. Group refers to the order in which mice were exposed to each cleaning agent. Data are presented as the mean ± SEM. ns: non-significant ($p>0.05$). EtOH: ethanol; EA group: ethanol then Anistel; AE: Anistel then ethanol. N=3 in each group.

4.4 Discussion

The aim of this chapter was to investigate the effects of fasting and torpor on subsequent behavioural performance, in order to build on Chapter 3's findings which reported that torpor is readily induced in response to a food restriction paradigm that is common for appetitive behavioural tasks. For this chapter, it was hypothesised that the occurrence of torpor may be affecting behavioural outcomes and contributing to variability and confounding data.

Here, it was observed that locomotor activity and exploratory behaviour during an open field task were significantly reduced when testing was conducted immediately following a torpor bout. Testing at subsequent time points in 20 minute intervals following the end of torpor revealed that both locomotion and exploration were restored to levels comparable with non-fasted controls. The impairment during the first trial may reflect a continuation of the lethargy associated with torpor, despite mice having rewarmed to euthermia. It is possible that this time window represents a dynamic period of physiology in which metabolic rate is returning to basal levels, following the rapid increase and overshoot of metabolic rate upon torpor arousal. As such, these data indicate the potential for torpor to confound behavioural outcomes if researchers are unaware of the occurrence of torpor, and therefore conduct experiments during or immediately after this period of lethargy.

Food restricted mice did not show differences in activity levels at time points +20, +40, and +60 minutes post arousal from torpor compared to non-fasted mice tested in parallel, despite having significantly reduced activity levels during the first trial. It was expected that fasted mice would show increased exploratory behaviour during the second trial, due to the lack of engagement during initial exposure. However, this was not the case, indicating that fasted mice may be aware of their environment during the first trial, even though they were not actively

exploring the arena to the same degree as non-fasted mice. This is supported by a study in ground squirrels which reported that hibernation did not alter the level of habituation to an open field arena when tested before and after hibernation (Clemens et al., 2009).

Food restriction has also been shown to induce activity in the hours preceding food timing, termed food anticipatory behaviour (FAA) (Acosta-Rodríguez et al., 2017; Gabloffsky et al., 2022). As such, it is possible that locomotor activity would have been different between groups if testing had been conducted during this period of FAA, therefore highlighting the importance of standardising time of testing due to the altering of circadian activity patterns in response to food restriction (Northeast et al., 2020). Even when food restriction is not being used, many aspects of physiology, including activity, follow circadian patterns across the day and can result in different outcomes depending on when testing is conducted (Richetto et al., 2019; Roedel et al., 2006).

Measures of anxiety associated behaviours, such as thigmotaxis and number of faecal boli, yielded mixed results. Thigmotaxis is thought to provide a reliable indicator of anxiety due to the aversion of rodents as a prey species to be in the centre of exposed, brightly lit, unknown environments, which decreases upon subsequent exposures (Choleris et al., 2001; Seibenhener & Wooten, 2015). During the first trial, fasted mice spent significantly longer in the periphery compared to non-fasted mice which may suggest increased anxiety in this groups. However, when considering the immobility data and distance travelled, this may be explained by the lethargy observed in post-torpid mice. Interestingly, all mice were placed in the centre of the arena at the beginning of the trial, therefore the finding that post-torpid mice spent significantly longer in the periphery also indicates a strong aversion to the centre despite the mice being in a lethargic state.

Another common measurement of anxiety in the OFT is the level of defaecation, with high levels of anxiety being associated with an increased number of faecal boli (Hall, 1934; Lister, 1990; Sestakova et al., 2013). In the data presented in this chapter, non-fasted mice had consistently higher levels of defaecation across trials, which could be used as evidence that fasted mice have lower levels of anxiety across trials. However, this observation is more likely explained by the effect of food restriction itself, with the fasted group having been fed >12 hours prior to testing, whereas the ad libitum fed mice would have likely been eating across the dark phase just prior to testing. Instead, a more appropriate measurement may have been to quantify vertical activity such as rearing behaviour, although this metric is less well established and its ethological relevance to anxiety is debated (Choleris et al., 2001; Lister, 1990) and may also be confounded by the increased levels of immobility observed in post-torpid mice. Unfortunately, rearing behaviour was not tracked during these experiments and so could not be compared.

The second aim of this chapter was to determine whether torpor would alter cognitive performance. To this end, a novel object recognition task (NORT) was used, due to the NORT being a well-established task that is thought to be an ethologically relevant method to assess short term recognition memory. It was initially hypothesised that torpor would impair memory due to torpor being associated with significant synaptic remodelling (Popov et al., 1992; Popov & Bocharova, 1992), and sleep disruption (Huang et al., 2021), and due to previous reports of impaired recognition memory following torpor in hamsters (Palchykova et al., 2006). However, others have reported that torpor protected memory retention in a Morris water maze (Nowakowski et al., 2009).

Here, performance in the NORT was poor overall, with fasted and non-fasted mice failing to explore objects during both the sample and test phases. As such, no clear group differences were observed for any metric of recognition memory. However, it is unclear whether this is due to torpor having no effect on memory, or due to a general lack of task engagement. It is possible that the latter may have been obscuring any effects, especially as many of the mice did not explore objects for >20s during the sample phase, which is often used as an inclusion criteria (Lueptow, 2017), and due to the relatively small sample size used. Moreover, a large degree of variability between animals was observed, especially within the fasted group. It is possible that high variation in this group is a product of torpor induction itself and supports the need to control for the presence of torpor during food restricted studies. For example, a recent study in golden-mantled squirrels, a species of hibernating ground squirrel, reported a high degree of variability in spatial memory retention using a Barnes maze leading to inconclusive results (Hensleigh et al., 2022).

It is possible that food restriction may also influence aspects of cognitive function, including memory. There are conflicting reports in the literature on how food restriction interacts with cognition. Some studies report that food restriction improves memory in a recognition task (Hashimoto & Watanabe, 2005; Hornsby et al., 2016; Kuhla et al., 2013), whereas others report an impairment in memory during food restriction (Carlini et al., 2008; Fu et al., 2017; Padamsey et al., 2021). Many of these studies use mice as their model species for determining the impact of food restriction on memory, but do not measure or account for torpor. As such, food restriction and torpor may be interacting in the effect on memory, thus explaining the discrepancies in reported outcomes. The study design here attempted to disentangle the effects of food restriction and torpor by conducting two trials: one following torpor, and one after an

extended period of euthermia. However, no changes in recognition memory between trials were observed, but may reflect the absence of a robust memory affect in any of the mice.

A previous study in hamsters found that conducting a NORT following a bout of daily torpor resulted in a deficit in recognition memory (Palchykova et al., 2006). Hamsters do not require food restriction in order to enter torpor, instead entering torpor in response to a shortened photoperiod. Consequently, this study will have benefited from not having the potential conflict with the effects of food restriction, therefore explaining why the results presented here do no confirm Palchykova's findings. Moreover, this previous study investigated longer term memory as the test phase was performed >24 h after the sample phase, whereas here short term memory was investigated. A future direction for this work could be to investigate whether an effect of torpor on long term memory is observed in food restricted mice also.

It is also possible that a NORT is not the optimal paradigm that can capture the effect of torpor on behaviour. An issue with behavioural phenotyping is that the tests available are often only able to assess a limited number of behaviours at a time. The NORT is primarily used to assess recognition memory, however, it is possible the torpor impacts a different aspect of learning or memory. For example, a study in food restricted mice reported that a single bout of torpor effected spatial memory in a Morris water maze (Nowakowski et al., 2009). Similarly, a significant effect of hibernation on memory retention was observed in ground squirrels during a visual discrimination task (McNamara & Riedesel, 1973).

Future investigations may wish to investigate a wider range of aspects of learning and memory in relation to fasting-induced torpor in mice. A potential starting point could be to focus on hippocampal-dependent tasks, such as spatial mazes, as there is evidence that torpor induces

synaptic remodelling in the hippocampus (Von Der Ohe et al., 2006). Further, the effect of torpor on learning has largely been neglected in the literature thus far, although there is some evidence that torpor may enhance hippocampal-dependent contextual memory (Weltzin et al., 2006). However, a caveat to this study is that it measured freezing behaviour in response to stimulus which is likely to be confounded by the lethargy and immobility associated with torpor. As food restriction and food rewards are often used during the training phase of complex tasks, this may provide an important area for investigation, and may help to explain differences in learning curves and low numbers of animals reaching the sufficient criteria.

4.4.1 Environmental factors and behaviour

An additional aim that developed following the food restricted behavioural testing was to investigate the influence of environmental factors on performance. It has been well documented that mice are particularly sensitive to their environment and many factors can significantly alter behavioural outcomes. These can range from human related factors such as the use of certain handling techniques (Hurst & West, 2010), level of habituation to the experimenter (Lewejohann et al., 2006), and gender of the experimenter (Georgiou et al., 2022; Nigri et al., 2022), to aspects of the environment such as light intensity, background noise, and olfactory cues (Saré et al., 2021).

Although many aspects of the environment have been investigated, very few reports on cleaning substance as a potential environmental confound exist in the literature, despite olfaction being one of the primary senses used by mice (Olsson et al., 2003). Common cleaning products used in laboratory settings frequently have strong odours which may be unpleasant to mice, thus increasing anxiety responses and aversive behaviour. As a result, 70% ethanol is often used to minimise aberrant olfactory cues due to it being odourless. However, this is not

a standardised procedure, and it is possible that other cleaning products may be used instead due to convention in that laboratory.

A survey of 51 institutions in the UK reported that Anistel™ was the most common cleaning product used, with 7-19% of respondents reporting that they thought cleaning product would influence standardisation and mouse physiology (López-Salesansky et al., 2016). Moreover, this study highlighted the variability in animal husbandry between institutions. However, this study did not go on to determine whether the use of Anistel™ had a meaningful effect on subsequent behaviour.

This chapter found no evidence to suggest that Anistel™ alters behaviour in the open field task or novel object recognition task compared to 70% ethanol. Moreover, the mice during the NORT continued to perform no better than chance regardless of the cleaning agent used, indicating that a different environmental factor that was not investigated may have been contributing to poor performance. Despite no difference being observed here, it is still important to control for confounds where possible and to remain consistent. Moreover, it is possible that an effect would have been observed if the task had relied on odour discrimination such as during a modified novel object recognition task, which uses novel and familiar odours instead of objects (Tam et al., 2017).

4.4.2 Conclusions

In conclusion, this chapter shows that time of testing relative to torpor can affect locomotor activity and exploratory behaviour, therefore highlighting the importance of accounting for and controlling for torpor in response to food restriction. Although no evidence was found of torpor significantly influencing performance in a novel object recognition task, it is possible that

torpor may alter other modalities of cognition. As such, it is likely that the induction of torpor is a significant source of variation in behavioural studies if animals are tested with differences in their torpor history, especially if the task being used relies on locomotor activity. Future work may be able to build on these findings by conducting a larger battery of behavioural tasks, testing longer term memory, and comparing animals with differences in prior torpor history. Finally, this chapter further highlights the importance of implementing appropriate standardisation and controls to reduce levels of variation.

Chapter 5: Investigating the relationship between sleep and fasting-induced torpor

5.1 Introduction

From a behavioural perspective, it can be difficult to distinguish between sleep and torpor. Sleep has been defined as a behavioural state characterised by a species-specific stereotypical posture, quiescence, increased arousal threshold to external stimuli, and rapid reversibility (Piéron, 1913). Although no behavioural criteria for torpor have been outlined, all of Piéron's sleep criteria could apply to some extent. Electrophysiological data provides further evidence for a close link between sleep and torpor. For example, brain activity during shallow torpor closely resembles the NREM stage of sleep (Deboer & Tobler, 1995; Heller & Ruby, 2004), with some describing torpor as "continuous with and homologous to sleep; more specifically, it is primarily an extension of slow wave sleep" (Walker et al., 1979). Some studies have subsequently defined torpid brain activity as 'hypothermic NREM sleep' due to their close resemblance (Huang et al., 2021). Torpor has been shown to be entered via NREM sleep, with brain temperature beginning to decrease only during this state and never during wakefulness (Walker et al., 1977). These findings have been used to suggest that sleep and torpor are evolutionary extensions of one another and exist on a continuum.

Despite this, there are also many differences between sleep and torpor. Notably, a downward shift in EEG frequencies is observed during torpor compared to sleep (Deboer & Tobler, 1995; Huang et al., 2021). Additionally, there is a marked departure from the predictable cycling between NREM and REM states during torpor, due to the near abolition of REM sleep (Deboer & Tobler, 1995). As a result, it has been suggested that torpor is incompatible with sleep which is further supported by an increase in the intensity of slow wave activity (SWA) following a

torpor bout, due to SWA being a hallmark of sleep pressure and usually observed following sleep deprivation (Deboer & Tobler, 2000, 2003). Moreover, species that enter multi-day torpor periodically arouse from torpor; these interbout intervals are predominantly spent asleep and show increased slow wave activity (Daan et al., 1991; Trachsel et al., 1991), and hypothalamic lesions that prevented interbout arousals were found to be fatal in ground squirrels (Satinoff, 1967). Silvani and colleagues have suggested that the lack of physiological slow waves due to the frequency shift during torpor may explain the high intensity SWA following the end of the bout (Silvani et al., 2018).

It is apparent from the literature that there is a relationship between sleep and torpor, and that the two processes interact, however, the exact ways in which this occurs remains unclear. The majority of existing research has been conducted in hibernating species, or in species that enter photoperiod-induced daily torpor, whilst comparatively little is understood about fasting-induced torpor. Investigation of fasting-induced torpor may shed new light on the relationship and interaction between sleep and torpor, therefore furthering our understanding of torpor. In addition, improved understanding of torpor may also help to answer questions about sleep, due to the close link between the two states. Furthermore, this will also help to address gaps in our knowledge about the physiology of mice in general. As the most popular model species for biomedical research, including sleep research, it is necessary to have a thorough understanding of the mouse's physiology in order to interpret data, avoid confounds, and put appropriate controls in place.

5.1.1 Experimental aims

To address these gaps, the experimental aims of this chapter were to:

i) Investigate how torpor induction alters sleep-wake architecture

ii) Investigate brain activity during torpor and sleep using chronic electrophysiological recordings in ad libitum fed and food restricted mice

This chapter is exploratory in nature due to a lack of existing published data in laboratory mice. Throughout this chapter, sleep-wake architecture will be characterised for the first time in food restricted mice in the presence of torpor. In addition, the EEG brain activity across vigilance states and the underlying spectra frequencies will be investigated across euthermic and torpid states. These data will allow us to determine how comparable fasting-induced daily torpor is to seasonal daily torpor, in addition to furthering our understanding of sleep.

5.2 Methods

5.2.1 Experimental animals

Adult male C57BL/6J mice (Charles River Laboratories, UK) were used during this study (n=8; aged ~9 weeks at time of surgery). Mice were singly housed in custom made Plexiglas cages for chronic electrophysiological recordings (20.3 x 32 x 35 cm), placed in sound attenuated Faraday chambers (Campden Instruments, Loughborough, UK), with two cages per chamber. Chambers were illuminated using cool white LED light strips (Maplin, UK) on a 12:12 light-dark schedule (lights on at 10am, light levels 120-180 lux). Ambient temperature was maintained at 22 ± 2^{o}C and relative humidity at 60 ± 1%. Water was provided ad libitum throughout and all animals underwent daily health checks.

5.2.2 Surgical and recording procedures

All animals (n=8) were implanted with EEG (frontal, occipital, and cerebellum) and EMG electrodes as described in detail in section 2.6. Electrophysiological data acquisition, processing, sleep scoring, and analysis were performed as described in section 2.7. As torpor is not defined using EEG data, standard sleep scoring criteria were applied when scoring food restricted days. This included the periodic EMG bursts lasting ≥ 16 s as a result of shivering during torpor bouts being scored as wake.

After a 1-week period of ad libitum food access, all mice were placed on food restriction as described in section 2.2 to induce torpor; food was provided at ZT 10 (8 pm) and animals were weighed just before lights off. Additional food was provided at weighing time if bodyweight was below 85% of free feeding bodyweight.

Torpor bouts were detected using continuous thermal imaging cameras installed ~20 cm above the base of the cage, with one camera recording two cages simultaneously. Thermal data were processed, analysed, and used to detect torpor as described in sections 2.3 and 2.4.

5.2.3 Statistical analysis

Data were processed using MATLAB (The Math Works Inc., USA), and analysed using MATLAB and Prism (GraphPad, v9, USA). All data were tested for normality prior to analysis using a Shapiro-Wilk test. Analysis of sleep-wake architecture was performed using two-tailed paired t-tests or a non-parametric equivalent where appropriate. Comparisons of spectra frequency bands were conducted using a repeated measures two-way ANOVA with a Sidak multiple comparisons post-hoc test; a Geisser-Greenhouse correction was performed if

sphericity was not present. Differences were determined to be statistically significant when $p<0.05$. All data are presented as mean values ± SEM unless otherwise stated. Data analysis was only performed on 7 of the animals, due to the loss of an animal during the recording period, resulting in too few data points across conditions for that mouse to be included.

5.3 Results

Mice weighed 26.8 ± 0.44 g during ad libitum conditions which dropped to 23.9 ±. 0.24 g during food restriction; no mouse lost more than 20% of their free feeding bodyweight. Mean skin body temperature significantly decreased from 29.8 ± 0.28 ^{o}C to 26.6 ± 0.98 ^{o}C during food restriction ($t(6)=15.3$, $p<0.0001$, paired t-test). Mice began reliably entering torpor following ~8 days of food restriction, allowing for four days of EEG recordings during ad libitum conditions, and four days during food restricted conditions.

Good quality electrophysiological recordings were obtained from 7 of the 8 mice originally implanted. As such, data presented in this chapter will be from these 7 animals. Inspection of the raw EEG signals revealed differences in the visual characteristics of vigilance states during torpor compared to euthermic sleep (Figure 19), which will be discussed in more detail later in this chapter.

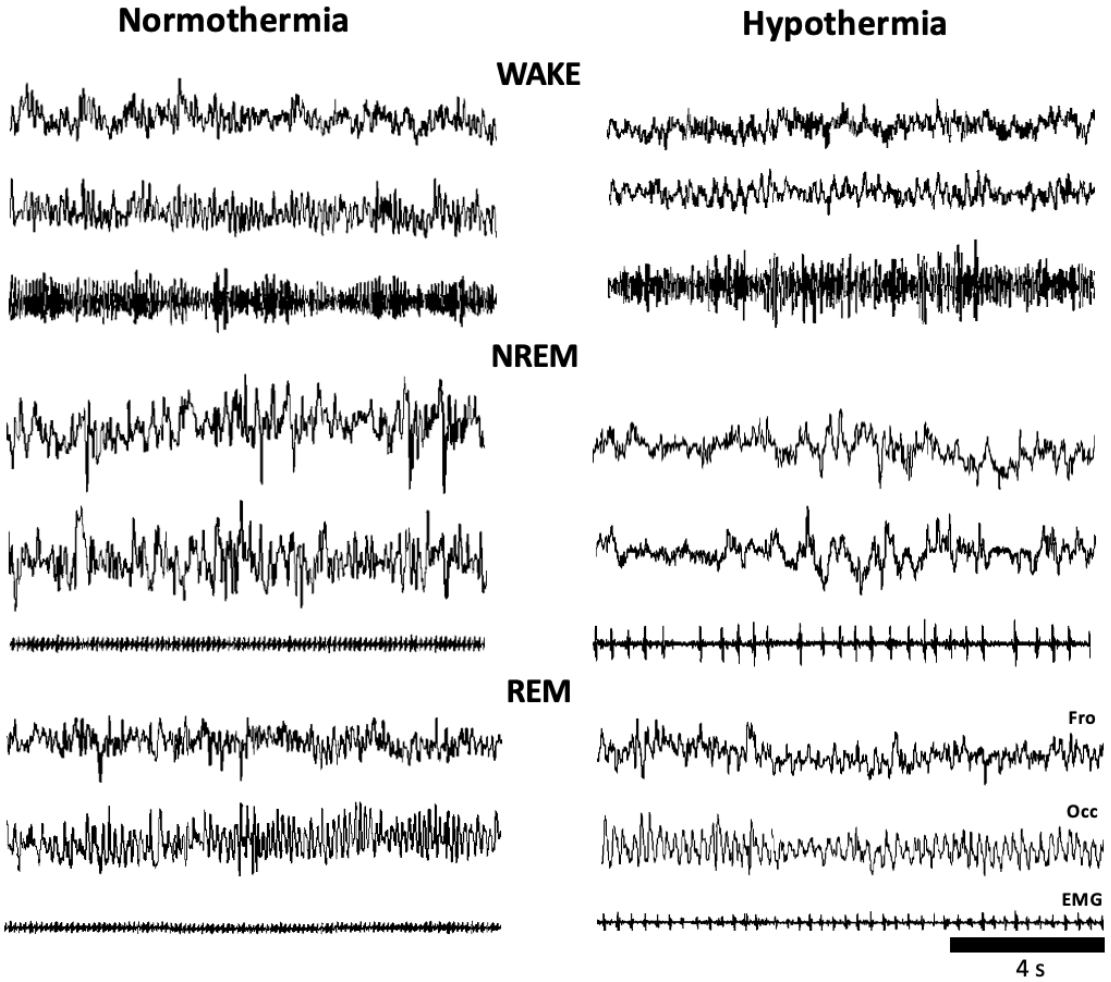

Figure 19: Raw EEG and EMG taken from wakefulness, NREM sleep, and REM sleep in euthermia (body temperature >30°C) and hypothermia (body temperature <24°C), from a representative mouse. EEG traces are shown from recordings taken from the frontal (Fro) and occipital (Occ) regions of the brain. EEG: electroencephalogram; EMG: electromyogram; NREM: non-rapid eye movement; REM: rapid eye movement.

5.3.1 Characterisation of sleep-wake architecture in the presence of torpor

To further understand how sleep and torpor processes interact, it is important to establish how sleep-wake architecture is affected by the induction of torpor. To this end, the time course of EEG slow wave activity was plotted from a representative mouse over 24 hr recordings from an ad libitum fed day and a food restricted day (Figure 20). These reveal a noticeable reorganisation of the global sleep-wake architecture between the two conditions, with the mouse spending much more time awake during the latter half of the light period during food

restriction in comparison to during ad libitum. Moreover, over half of the dark period is spent in a consolidated episode of NREM sleep during food restriction, much of which is attributed to the mouse being in torpor. Subsequently, the mean amount of time spent in each vigilance state per hour (wake, NREM, REM) for all mice was calculated on an undisturbed ad libitum day, and an undisturbed food restricted day in which all mice entered comparable torpor bouts (Figure 21), demonstrating consistent sleep-wake dynamics between animals in both conditions.

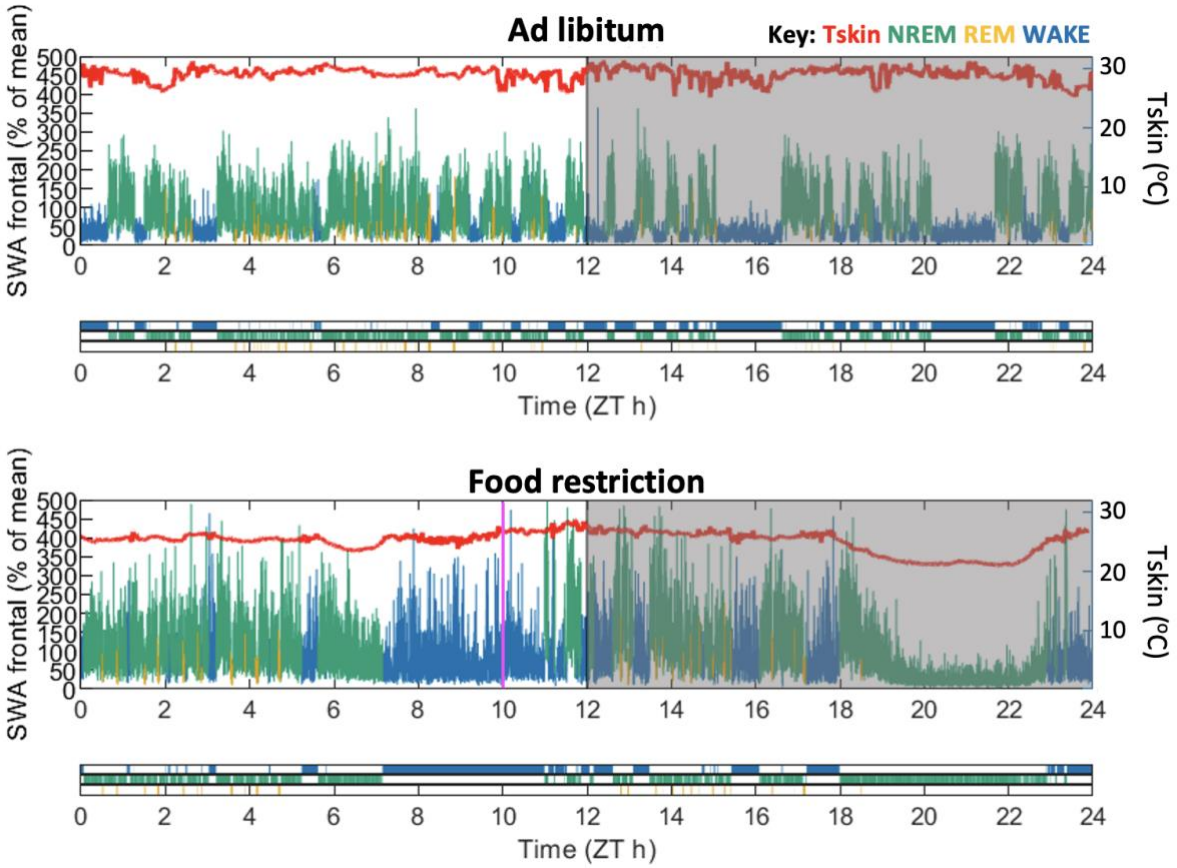

Figure 20: Hypnograms from a representative animal during ad libitum feeding and food restriction. Profiles of EEG slow wave activity (0.5-4 Hz), recorded from the frontal cortex, over 24-hour recordings, represented as a percentage of the mean. Wake, NREM sleep, and REM sleep are represented in blue, green, and amber colour coding, respectively. Peripheral body temperature recordings are presented in red. Grey shading represents the dark phase. The pink line at ZT 10 in the food restricted condition represents when the mice were provided with food.

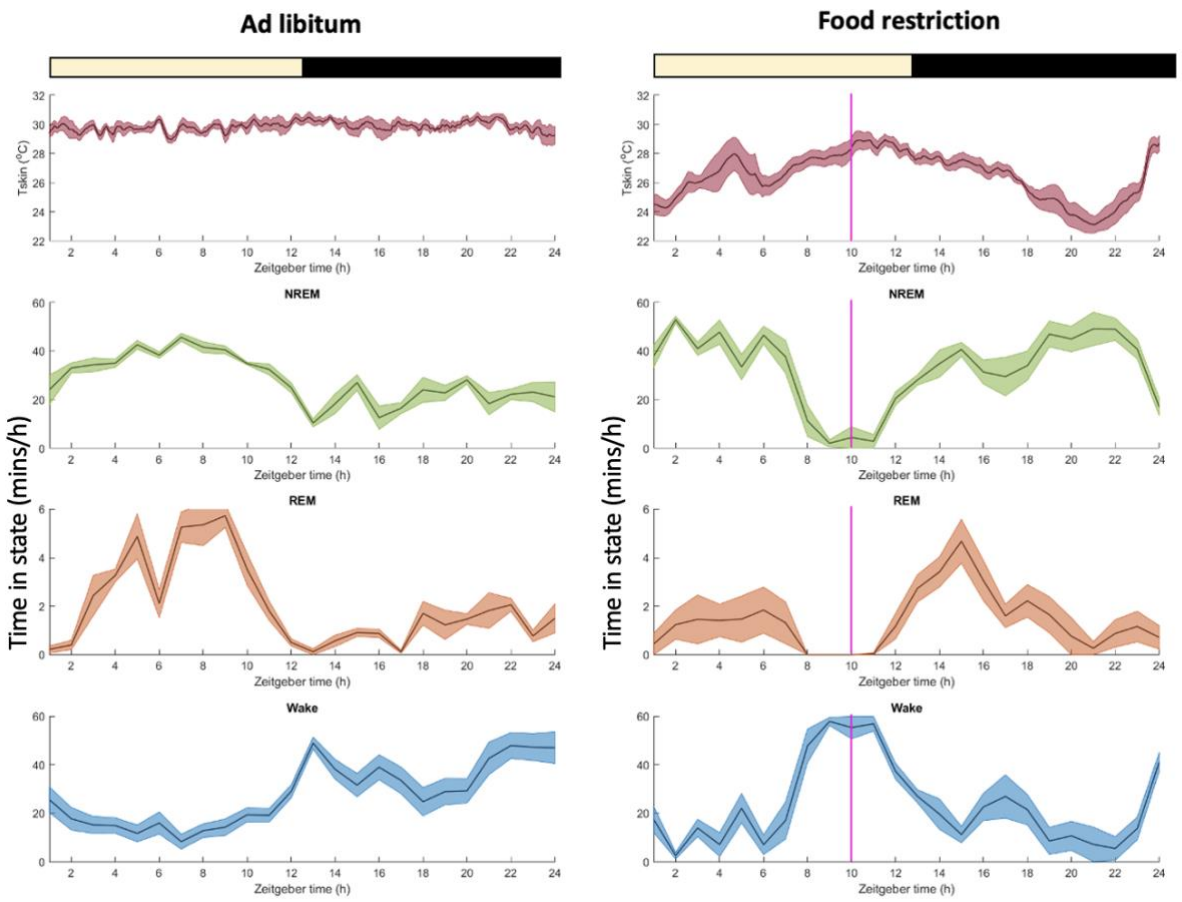

Figure 21: Food restriction and the presence of torpor alters global sleep-wake architecture. Time course of peripheral body temperature, NREM sleep, REM sleep, and wake over 24-hour recordings from an ad libitum day (left) and a food restricted day (right). The pink line indicates time of feeding during food restriction. Data are represented as mean values ± SEM (n=7). NREM: non-rapid eye movement; REM: rapid eye movement; Tskin: peripheral skin temperature.

Quantification of the amount of time spent in each vigilance state for the 12-hour light and dark periods identified significant condition-dependent effects during both periods (Figure 22). During the light period, food restriction resulted in an increase in the amount of time spent in wakefulness (AL: 3.55 ± 0.907 h; FR 5.71 ± 0.178 h; $t(6)=10.5$, $p<0.0001$, paired t-test), at the expense of both NREM sleep (AL: 7.12 ± 0.110 h; FR: 5.63 ± 0.161 h; $t(6)=7.09$, $p=0.0004$, paired t-test) and REM sleep (AL: 0.591 ± 0.0587 h; FR: 0.173 ± 0.0570 h; $t(6)=5.60$, $p=0.0014$, paired t-test). Conversely, during the dark period the opposite is true: the amount of wakefulness (AL: 7.32 ± 0.212 h; FR: 3.60 ± 0.253 h; $t(6)=9.44$, $p<0.0001$, paired t-test) was

significantly decreased during food restriction, with NREM being the predominant vigilance state (AL: 4.07 ± 0.200 h; FR: 7.34 ± 0.220 h; W=28, p=0.0156, Wilcoxon test). Due to food restricted mice spending more time asleep during the dark phase, there was also an increase in REM sleep as mice under ad libitum conditions are typically nocturnal (AL: 2.19 ± 0.00612 h; FR: 0.385 ± 0.105 h; W=26, p=0.0313, Wilcoxon test).

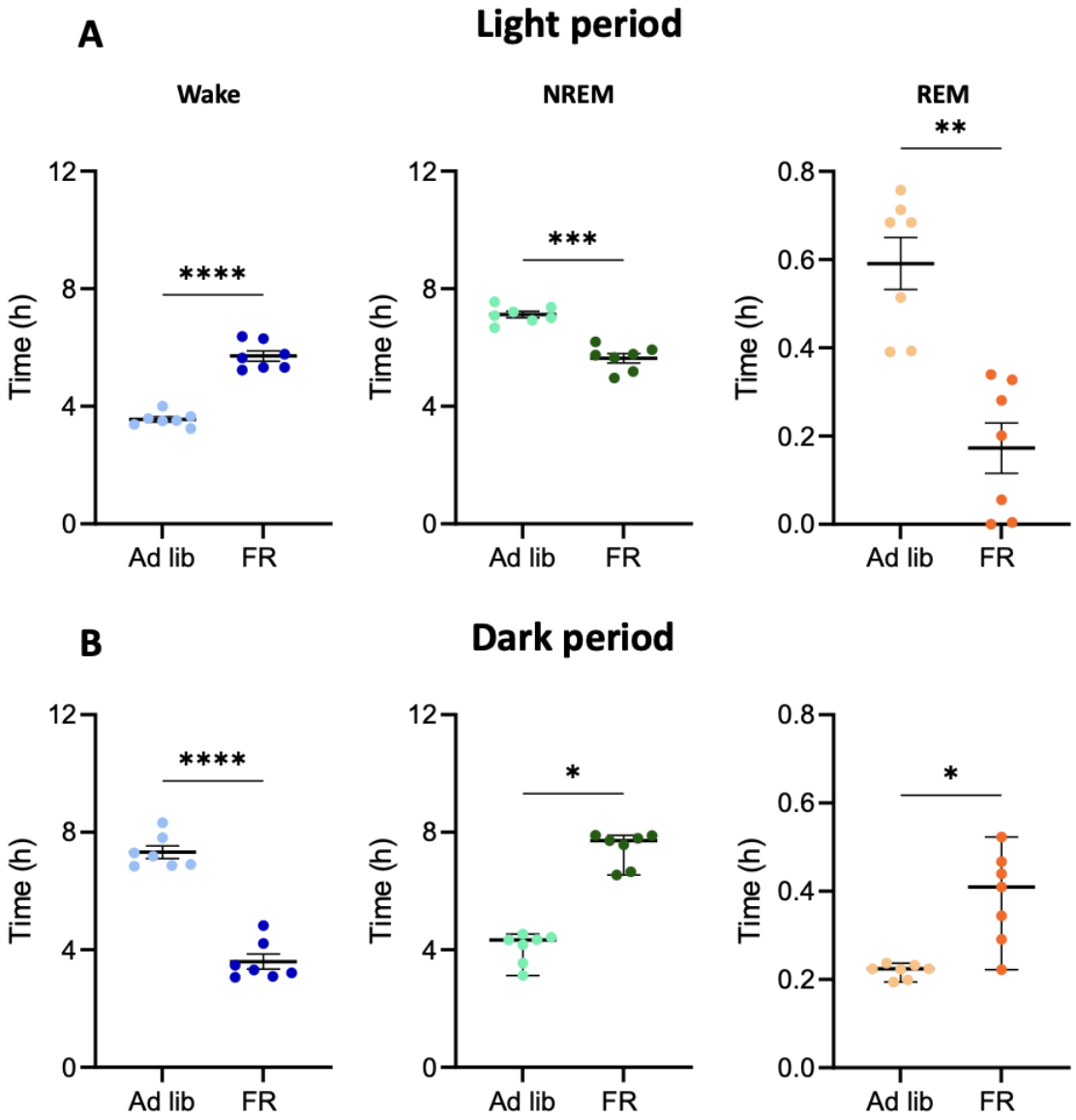

Figure 22: Distribution of vigilance states across the light-dark cycle during an undisturbed ad libitum fed and food restricted day. (A) Amount of time spent in wake (left), NREM sleep (middle), and REM sleep (right) across the 12 h light period during ad libitum fed and food restricted conditions. (B) Amount of time spent in wake (left), NREM sleep (middle), and REM sleep (right) across the 12 h dark period during ad libitum fed and food restricted conditions. N=7, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (calculated using paired t-tests or a Wilcoxon test). NREM: non-rapid eye movement; REM: rapid eye movement.

To determine whether the induction of torpor altered sleep-wake architecture, the number sleep of episodes, the average episode duration, and the maximum episode length of each vigilance state in ad libitum and food restricted conditions were calculated. Only episodes lasting at least 16 s (4 consecutive epochs) were considered for analysis, as brief awakenings (<16 s) are not typically considered as changes of state. Food restriction significantly decreased the number of episodes for wake (AL: 73.0 ± 5.07; FR: 47.9 ± 3.61; $t(6)=3.71$, $p=0.01$, paired t-test), NREM sleep (AL: 111.7 ± 8.09; FR: 64.6 ± 3.92; $t(6)=7.86$, $p=0.0002$, paired t-test), and REM sleep (AL: 56.3 ± 5.10; FR: 20.7 ± 3.19; $t(6)=12.1$, $p<0.0001$, paired t-test), indicating an increased level of vigilance state consolidation. This is further supported by the finding that the average episode duration during food restriction was significantly increased for wake (AL: 6.94 ± 0.424 mins; FR: 11.5 ± 1.15 mins; $t(6)=4.35$, $p=0.0048$, paired t-test), NREM sleep (AL: 5.88 ± 0.499 mins; FR: 13.4 ± 0.889 mins; $t(6)=9.83$, $p<0.0001$, paired t-test), and REM sleep (AL: 1.20 ± 0.0494 mins; FR: 1.66 ± 0.0893 mins; $t(6)=5.41$, $p=0.0017$, paired t-test).

Finally, the longest consolidated bout for each vigilance state was calculated and compared between ad libitum and food restricted conditions. The maximum wake episode for each mouse was much longer in food restricted conditions compared to ad libitum, with the longest episodes of wakefulness occurring in the 1-2 hours before feeding time (AL: 78.2 ± 8.73 mins; FR: 197.5 ± 13.7 mins; $t(6)=9.90$, $p<0.0001$, paired t-test). Mice also showed a greater maximum NREM episode duration during food restriction compared to ad libitum, although much of these episodes coincide with torpor bouts occurring at the end of the dark phase (AL: 20.4 ± 1.68 mins; FR: 130.6 ± 20.9 mins; $t(6)=5.44$, $p=0.0016$, paired t-test). It should also be noted that maximum NREM episode duration in food restricted conditions showed much greater variability than during ad libitum conditions which may be explained by variation in the length of torpor bouts between animals, as bouts can range from 1 to 6 hours long. The maximum

duration of REM sleep episodes did not differ between conditions, despite REM episodes being numerically longer on average during food restriction (AL: 3.10 ± 0.160 mins; FR: 3.50 ± 0.285 mins; t(6)=1.268, p=0.2517, paired t-test) (Figure 23).

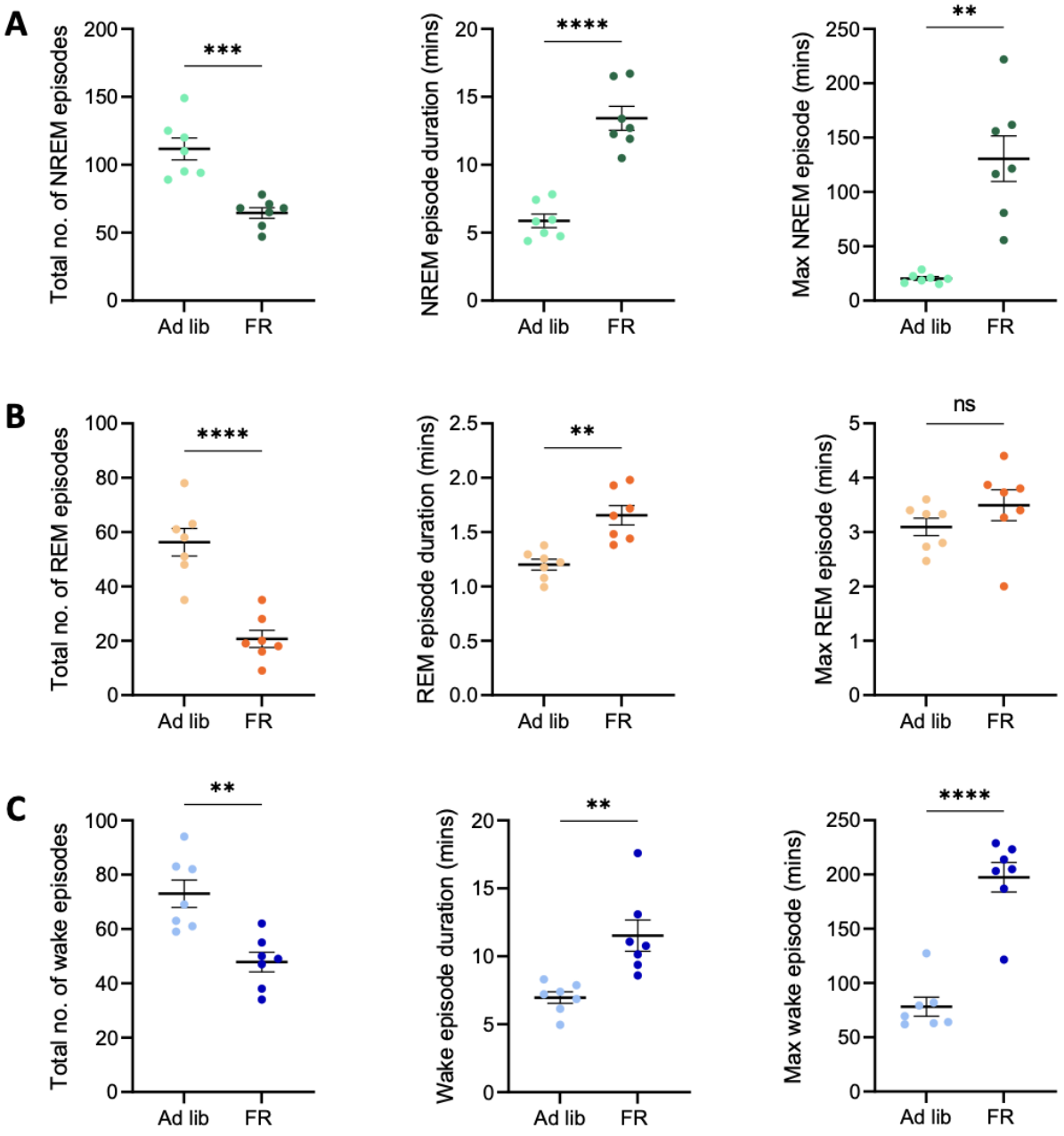

Figure 23: Changes to the sleep-wake cycle during food restriction in the presence of torpor. Total number of consolidated episodes of vigilance state (left), average episode duration (middle), and maximum episode length (right) for NREM sleep (A), REM sleep (B), and wake (C) over 24 h recordings during an ad libitum fed day and a food restricted day. N=7, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (calculated using paired t-tests). NREM: non-rapid eye movement; REM: rapid eye movement. Data are from an undisturbed day of recording during ad libitum and food restricted conditions.

Throughout this study, torpor was defined using peripheral body temperature recordings, as such was relationship between body temperature and states of vigilance were investigated. To this end, the body temperature and the percentage of time spent in each vigilance state in hourly bins for each animal were calculated. The temperature and vigilance state data were then aligned, and the average amount of time spent in the vigilance state for each degree of body temperature was calculated (e.g., 25.0-25.9^{o}C etc.). This process was repeated across four days of recording during ad libitum conditions, and four days during food restricted conditions.

Simple linear regression analysis revealed a modest, but significant, positive relationship between the percentage of time spent in wake and body temperature ($r=0.3288$, $p<0.001$). The amount of REM sleep also showed a significant positive relationship with body temperature ($r=0.1539$, $p<0.0001$), which is unsurprising as REM sleep has been shown to be dependent on body temperature with less REM sleep occurring at lower body temperatures (Deboer & Tobler, 1995). In contrast, the amount of NREM sleep showed a negative relationship with body temperature, which may be explained by the expression of long torpor bouts in which the EEG is predominantly NREM-like sleep ($r=0.5271$, $p<0.0001$) (Figure 24).

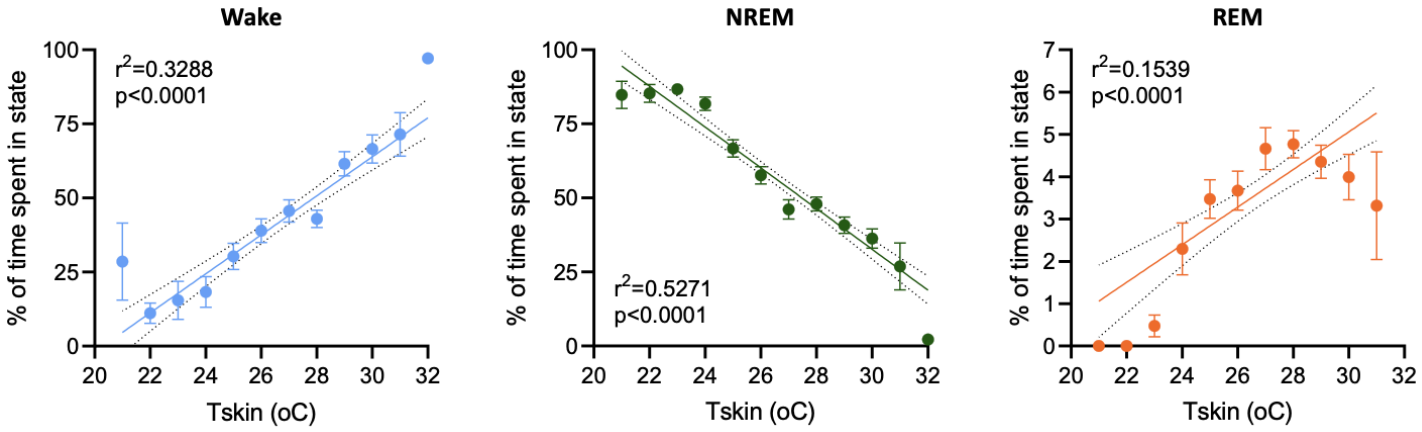

Figure 24: Relationship between the amount of time spent in each vigilance state and body temperature. Simple linear regression analysis of mean peripheral body temperature (Tskin) and the mean percentage of the recording spent in wake (left), NREM sleep (middle), and REM sleep (right). Data were taken from eight 24 h recording files (four ad libitum days, four food restricted days in the presence of torpor); each data point represents an average across animals (n=7). NREM: non-rapid eye movement; REM: rapid eye movement.

5.3.2 Electrophysiological correlates of torpor

The second aim for this chapter is to investigate brain activity during torpor. The raw EEG signals presented in Figure 19 show a noticeable visual change in the signals during hypothermia, which may be explained be the decrease in cortical brain temperature (Deboer & Tobler, 1995). This is more apparent when plotting spectrograms, which show the power of the frequencies present during ad libitum and food restricted conditions (Figure 25). Note that torpor bouts are dominated by slow frequencies (<4 Hz) with very low power in the high frequencies range.

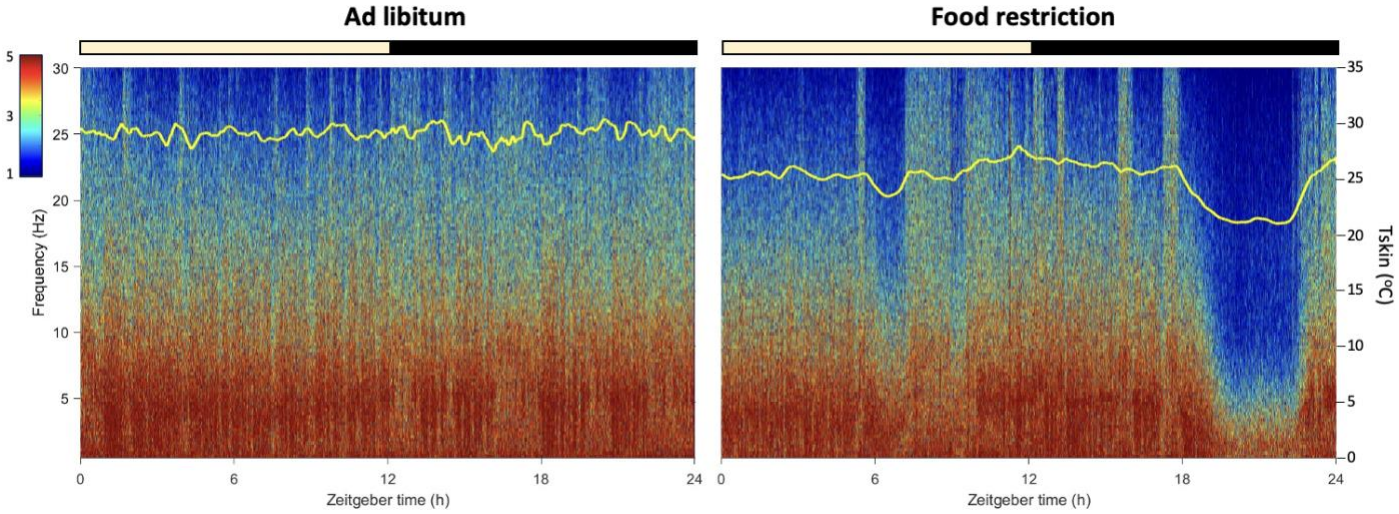

Figure 25: EEG power density spectrograms from a representative mouse during an undisturbed 24 h recording during ad libitum and food restricted conditions. The yellow trace indicates peripheral body temperature (Tskin). EEG power density spectra are colour-coded on a logarithmic scale (μV2/0.25 Hz), with blue representing the lowest frequency density and red representing the highest frequency density.

To investigate the effects of torpor on EEG spectra more closely, the frontal and occipital EEG spectra across vigilance state during ad libitum, euthermic food restricted, and hypothermic food restricted conditions were compared (Figure 26). Frequencies during hypothermia showed a relative reduction in EEG power density during wakefulness and NREM sleep in both the frontal and occipital derivations. The theta peak during REM sleep was also shifted to slower

frequencies during hypothermia, which was most apparent in the occipital derivation, in line with previous reports (Deboer, 2002; Huang et al., 2021).

Very few differences were found between ad libitum and euthermic food restricted spectra with only a few frequency bins reaching statistical significance during NREM and REM sleep, exclusively in the frontal derivation. In contrast, hypothermic and ad libitum spectra showed differences across a wide range of frequencies in all vigilance states. Significant differences in the EEG power during euthermic food restriction compared to hypothermic food restriction were also observed across vigilance states, suggesting that body temperature, rather food restriction itself is driving spectral changes. These observations were supported by statistical analysis using 2-way ANOVAs on log-transformed values, which consistently found a significant effect of condition across vigilance states (NREM: $F_{(1.91,11.46)}$=46.14, p<0.0001; REM: $F_{(1.381, 8.284)}$=13.07, p=0.0044; Wake: $F_{(1.38,8.30)}$=9.99, p=0.0092). Moreover, a significant interaction between condition and frequency was found for each vigilance state (NREM: $F_{(2.35,14.11)}$=32.35, p<0.0001; REM: F(3.40,20.40)=7.49, p=0.0011); Wake: $F_{(2.46,14.7)}$=8.55, p=0.0023).

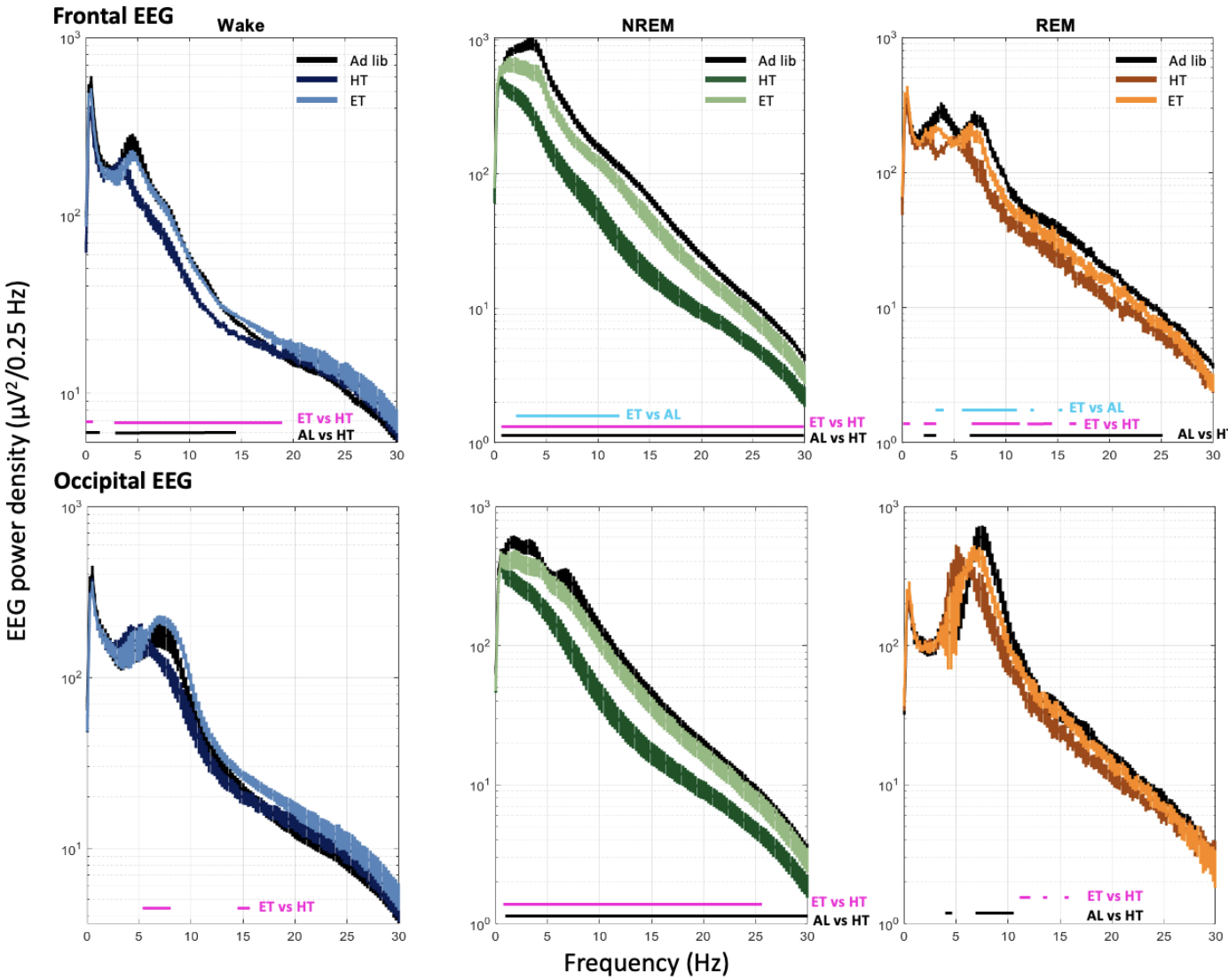

Figure 26: EEG power spectra during wakefulness (left), NREM sleep (middle), and REM sleep (right), calculated for the frontal (top) and occipital (bottom) derivation. Power densities are presented on a logarithmic scale from 0-30 Hz, binned in 0.25 Hz intervals. Spectra are presented from an ad libitum fed day and from a food restricted day. Tskin recordings were used to differentiate the food restricted spectra into hypothermic (HT) spectra and euthermic (ET) spectra. Hypothermia was determined as when Tskin was more that 3 standard deviations lower than the ad libitum median temperature for >1h, for that mouse. Spectral data are presented as mean values ± SEM (n=7). Significant differences between conditions for each frequency bin are indicated by the bars ($p<0.05$, 2-way ANOVA with Tukey's post hoc multiple comparisons test). NREM: non-rapid eye movement; REM: rapid eye movement.

Next, it was investigated whether the observed decrease in spectral power was state specific and whether this shift was due to changes in body temperature, or due to changes in sleep intensity during food restriction. To this end, the mean EEG power spectra for epochs scored as waking, NREM sleep, and REM sleep for each degree of body temperature (e.g., 25.0-25.9°C etc.) was calculated during four days of ad libitum fed recordings, and four days of food restricted recordings. An overall reduction in mean EEG power was found as a function of decreasing body temperature for all vigilance states but was most pronounced for NREM sleep (r^2=0.3958, p<0.0001; Figure 27), consistent with previous reports in mice (Huang et al., 2021) and Djungarian hamsters (Deboer & Tobler, 1996). This reduction in EEG power may be explained by a temperature dependent decrease in EEG amplitude, as seen in the raw signals, thus contributing to a reduction in overall power.

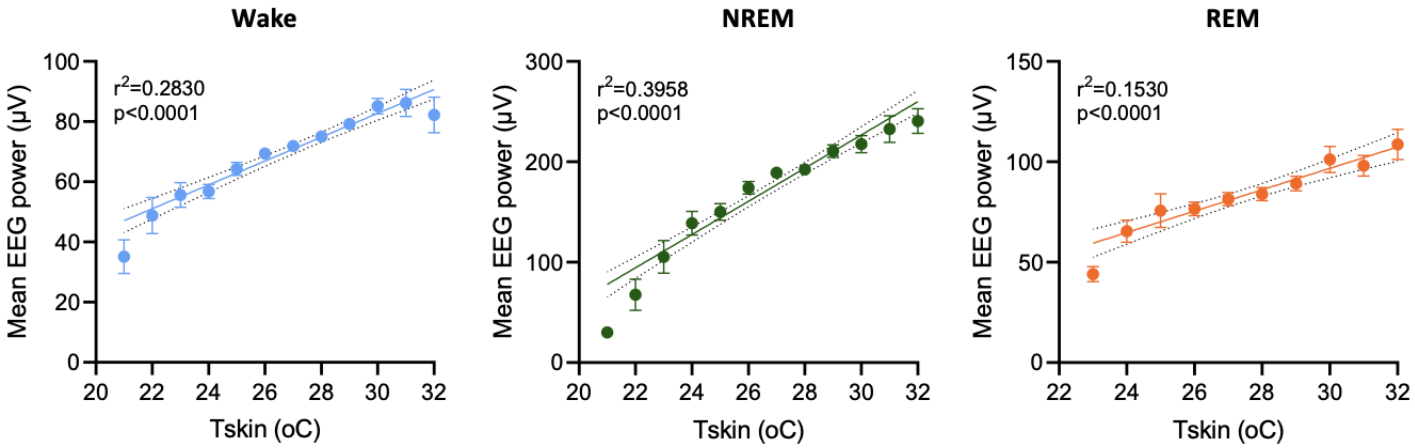

Figure 27: Relationship between body temperature and mean EEG power during different vigilance states. Simple linear regression analysis of the mean EEG power for epochs scored as wake (left), NREM sleep (middle), and REM sleep (right), for each 1°C of body temperature (Tskin). Data were taken from eight 24 h recording files (four ad libitum days, four food restricted days in the presence of torpor); each data point represents the mean value ± SEM (n=7). NREM: non-rapid eye movement; REM: rapid eye movement.

Finally, the course of EEG slow wave activity across a 24-hour recording period was investigated on an undisturbed ad libitum fed day and food restricted day (Figure 10a). Slow wave activity was consistently lower during the food restricted days compared to the ad libitum

recording, possibly as a result of the reduction in EEG power density in food restricted spectra as shown in Figure 9. Analysis using a mixed effects model found a significant effect of time and feeding condition on slow wave activity (time: $F_{(23,138)}=12.39$, $p<0.0001$; condition: $F_{(1,6)}=76.79$, $p=0.0001$), in addition to a significant interaction between time and condition ($F_{(23,113)}=11.64$, $p<0.0001$).

Slow wave activity (SWA) increased from ZT11 to ZT12 in food restricted mice to levels comparable with SWA at these time points during ad libitum conditions. However, SWA dropped steadily from this point until the end of the recording with SWA during food restriction becoming significantly lower than during ad libitum conditions (Figure 28, left). The peak in SWA at ZT11-12 during food restriction occurs shortly after feeding at ZT 10 and is predominately composed of euthermic recovery sleep, indicating increased slow wave intensity following satiation. Food restricted SWA returns to being significantly lower than during ad libitum conditions from ZT 14 to ZT 24. Notably, a large increase in SWA is observed at ZT 24 during ad libitum conditions, which is due to mice beginning to sleep as their active period comes to an end and sleep pressure is high; this trend is much less apparent during food restriction, possibly due to the restructuring of sleep-wake architecture.

Slow wave activity can sometimes provide a skewed perspective when there are differences in the total sleep time (Plante et al., 2016). An alternative method is to use slow wave energy (SWE), which is the cumulative sum of delta power across the sleep period. Due to the findings that food restriction, and the presence of torpor, shift global sleep-wake architecture, SWE was compared in addition to SWA (Figure 28, right). To this end, the cumulative sum of delta power across an undisturbed 24 hr recording during food restriction was calculated and expressed as a percentage of the cumulative total SWE of ad libitum condition. Similar to the SWA, SWE

was consistently lower during the food restricted condition across 24 hours compared to ad libitum. A two-way ANOVA with repeated measures found a significant effect of time of recording ($F_{(23,138)}$=112.9, p<0.0001) and feeding condition ($F_{(1,6)}$=18.11 p=0.0053). A significant interaction between time and treatment group was also found ($F_{(23,138)}$=8.875, p<0.0001). A Šidák's multiple comparisons test found that the differences between groups started from ZT 8 to ZT 24.

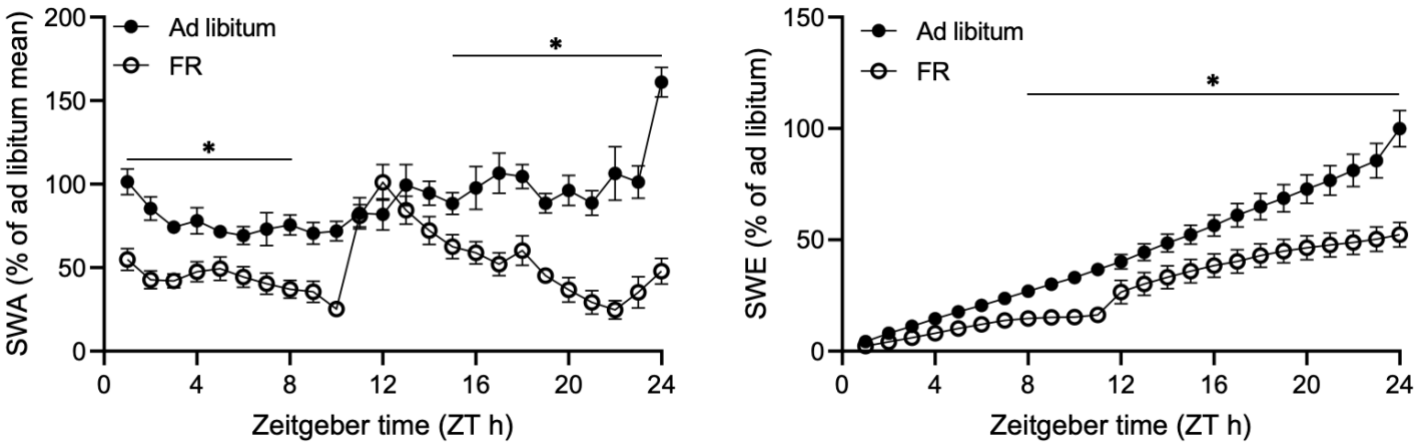

Figure 28: Effect of food restriction and torpor on EEG slow wave activity during NREM sleep. (A) Time course of EEG slow wave activity in 1 h intervals over 24 h recordings during food restriction and ad libitum feeding, expressed as a percentage of the ad libitum mean. (B) Cumulative sum of EEG delta power during all artifact free epochs scored as NREM sleep (slow wave energy) in 1 h intervals over 24 h recordings during food restriction and ad libitum feeding. N=7, *p<0.05 (2-way ANOVA with repeated measures and Šidák's multiple comparisons test).

5.4 Discussion

This chapter aimed to investigate changes to sleep-wake architecture in the presence of fasting-induced torpor, and to investigate changes in brain activity during torpor bouts. Analysis of daily sleep-wake architecture revealed a dramatic reorganisation of vigilance states across a 24 hour recording during food restriction. Mice are a naturally nocturnal species in laboratory conditions which during the ad libitum feeding is reflected in the majority of the light phase spent asleep, whilst the dark phase is mostly spent in wakefulness with short 'naps' interspersed. In contrast, food restriction results in a shift towards diurnality with sleep mostly occurring during the dark phase and the proportion of time spent wake during the light phase being significantly higher than compared to ad libitum conditions. This is consistent with previous reports which demonstrate a promotion of diurnal activity patterns in response to low food availability in order to maximise survival so that arousal and activity correspond to when food is anticipated to be available (Northeast et al., 2019; van der Vinne et al., 2019). It is likely that the shift towards diurnality is, at least in part, being driven by mice entering torpor predominantly in the dark phase during this study and due to food anticipation occurring during the day. It is possible that if food had been presented at a different time, then the sleep-wake architecture during food restriction would look different due to the competing circadian factors of food and light, although this would need further investigation (Northeast et al., 2020).

The mice in this study were provided with food at the end of the light phase (ZT 10) which coincided with a consolidated bout of wakefulness starting ~2 hours before feeding, further suggesting a shift towards maximising feeding opportunity and therefore survival. The consolidated wake bouts observed here in the 1-2 hours before feeding also suggest the presence of food anticipatory activity (FAA), in which food restricted mice anticipate food availability (Northeast et al., 2019). This may also represent a shift in circadian entrainment

around food availability due to feeding being a strong circadian zeitgeber (Northeast et al., 2020), and therefore contributing to diurnal activity patterns.

Consistent with previous findings, an increase in NREM sleep was observed as body temperature decreased during food restriction (Daan et al., 1991; Huang et al., 2021; Walker et al., 1977, 1979). Further, reduction of body temperature into a hypothermic torpor bout was initiated during a period of NREM sleep, accompanied by a gradual decline in NREM power, so that it was impossible to make a clear distinction of where euthermic NREM sleep ended and hypothermic NREM began using EEG recordings alone. This confirms work conducted in non-fasting induced models of torpor (Harris et al., 1984; Vyazovskiy et al., 2017; Walker et al., 1977), suggesting that initiation via NREM sleep may be a key component of torpor.
All vigilance states showed an increased level of bout consolidation during food restriction with NREM, REM, and wake significantly increasing in average episode duration, although the maximum REM episode length did not change between conditions. Sleep fragmentation is associated with increased energy expenditure, hyperphagia, and a reduction in sleep quality (Baud et al., 2013); therefore, increasing state consolidation during food restriction may be maximising the quality of euthermic sleep to compensate for hypothermic sleep, in addition to contributing to energy savings whilst still providing sufficient foraging opportunities.

A marked reduction in the amount of REM sleep was observed as body temperature decreased, with REM sleep being close to abolished at Tskin measurements of <24^{o}C. This is unsurprising as previous work has demonstrated a strong temperature dependence of REM sleep. For example, REM sleep was strongly depressed by cold exposure in rats, a strictly homeothermic species (Cerri et al., 2005). Even small changes in ambient temperature within the thermoneutral zone have been shown to significantly alter the amount of REM sleep, with the

maximal amount of REM sleep occurring toward the upper bound of the thermoneutral zone (Szymusiak et al., 1981). In heterothermic species, REM sleep has previously been shown to disappear at low body temperatures (Deboer & Tobler, 1995; Huang et al., 2021), and that there is a strong correlation between brain temperature and the amount of REM sleep (Berger, 1984). Near abolition of REM sleep may be a necessary component for deep torpor, as regulation of body temperature is impaired during REM sleep with thermogenesis being suspended due to muscle atonia (Szymusiak, 2018), increased cortical temperature (Komagata et al., 2019), and increased peripheral blood flow (Harding et al., 2020). As such, the absence of REM sleep during torpor may allow for tighter control of body temperature, and thus maximising energy savings at the expense of REM sleep.

The second aim of this chapter was to investigate brain activity and EEG spectra during torpor. A number of studies have investigated this previously in species that undergo hibernation or spontaneous torpor in response to changes in photoperiod that formed the basis of this work (Daan et al., 1991; Deboer & Tobler, 1995; Vyazovskiy et al., 2017; Walker et al., 1979). However, relatively little research has been conducted in models for fasting-induced torpor, leaving a gap in our understanding of torpor electrophysiology.

Work in hibernators has previously struggled to record electrophysiological signals during deep torpor due to the very low body temperatures these species can maintain (Krilowicz et al., 1989). Peripheral body temperature never reached below 21^{o}C in this study, so although marked changes to the EEG activity were observed, vigilance states were still able to be scored. Hypothermia was still found to significantly alter EEG spectra, however, most notably by shifting overall spectra to slower frequencies as has previously been described (Deboer, 2002; Deboer & Tobler, 1995). Moreover, EEG power was decreased as a function of body

temperature which was most apparent in NREM sleep, in line with published literature (Deboer & Tobler, 1996; Huang et al., 2021; Vyazovskiy et al., 2017). This reduction in EEG power may be occurring as a result of changes to underlying mechanisms in response to temperature changes. For example, it is well established that enzymatic activity slows at lower temperatures, which can contribute to the inhibition of synaptic vesicle release, signalling cascades, and ionic currents (Sonntag & Arendt, 2019). Torpor is also associated with significant changes in dendritic and neuronal morphology, including a reduction in dendritic branching and spine density (Von Der Ohe et al., 2006). These changes in the central nervous system may be contributing to a breakdown of network synchronisation, or a reduction in the number of synapses able to contribute to electrical signalling, resulting in the observations described here. This might reflect the underlying processes reported to lead to changes in EEG activity during other altered states of consciousness such as anaesthesia (Sheroziya & Timofeev, 2015).

Earlier studies in hibernators and photoperiod-induced torpor report that sleep following torpor bouts is dominated by deep sleep, with increased intensity of slow wave activity (Daan et al., 1991; Deboer & Tobler, 1994). Slow wave activity is widely accepted as a marker of deep restorative sleep and an indicator of sleep pressure, which has led to the hypothesis that torpor is a sleep depriving state (Borbély, 2013). In contrast, the findings presented here show elevated levels of wake following emergence from torpor in laboratory mice, possibly to increase the number of foraging opportunities. Analysis of EEG slow wave activity and slow wave energy showed that slow wave activity is generally lower when mice are in torpor, and that the cumulative delta power does not reach the same levels as during ad libitum conditions. This may be interpreted as in support of torpor as a sleep depriving state, as it could be argued that

the lack of physiological slow waves prevents or reduces the ability for restorative processes that are typically associated with slow waves from occurring (Silvani et al., 2018).

Interestingly, a spike in slow wave activity occurs shortly following feeding and reaches levels comparable with ad libitum euthermic sleep. This may reflect an increase in sleep pressure as a result of the long waking bout prior to feeding; it may also suggest that the presence of torpor has been limiting the ability to dissipate sleep pressure, which is supported by the finding of slow wave activity and slow wave energy being significantly lower during food restricted conditions compared to ad libitum conditions. The large increase in EEG slow wave activity shortly following feeding may reflect a compensatory rebound effect that the mouse is only able to achieve when in a less metabolically challenged state and is able to prioritise sleep over energy conservation. Work in Djungarian hamsters which enter shortening photoperiod induced daily torpor has shown an increase in slow wave activity during sleep following torpor bouts, suggesting a sleep depriving effect of torpor (Deboer & Tobler, 2000, 2003; Palchykova et al., 2002; Vyazovskiy et al., 2017). The data presented here may also reflect torpor as a sleep depriving effect but due to the fasting-induced nature of torpor in mice the effects of fasting must be alleviated in order for sleep rebound to be enabled. An alternative explanation may be that body temperature's effect on EEG is masking slow wave activity.

5.4.1 Conclusions

This study provides evidence to support the hypothesis that sleep and torpor are closely related states, that interact to facilitate a careful balance between energy conservation and sleep-related restorative processes. The electrophysiological recordings presented here closely resemble those reported in seasonal and photoperiod-induced torpor, with the reduction in EEG power and entrance into fasting-induced torpor via NREM sleep being the main similarities. However,

the additional facet of being in an actual energy deficit, opposed to a perceived energy deficit, results in the restructuring of sleep-wake architecture in a way that is distinct from non-fasting induced torpor and ad libitum conditions. The finding that EEG slow wave activity and slow wave energy are reduced when mice are entering torpor compared to ad libitum conditions may provide some evidence to support work in hibernators and Djungarian hamsters that suggest that torpor is a sleep depriving state, however, further work is required to investigate the effects of fasting-induced torpor on sleep homeostasis. This is the aim of the following chapter.

Chapter 6: Investigating the effect of fasting-induced torpor on sleep homeostasis

6.1 Introduction

The data presented in Chapter 5 revealed a strong relationship and interaction between sleep and fasting-induced torpor that were reflective of published work in hibernating species and species that undergo photoperiod-induced daily torpor. A question that was not addressed in Chapter 5 was the effects of torpor on sleep homeostasis, which will be expanded on in this chapter.

Sleep homeostasis is a central component of sleep regulation; it posits that sleep need accumulates during wakefulness, resulting in increased feelings of 'sleepiness', which is then alleviated during subsequent sleep (Borbély, 2022). Slow wave activity (SWA), defined by high amplitude and a frequency of EEG signal between ~0.5-4.5 Hz, is thought to be a reliable marker of sleep homeostasis in mammalian species (Greene & Frank, 2010). Under normal conditions, SWA is greatest at the beginning of the rest phase and dissipates with subsequent sleep (Franken et al., 1991; Trachsel et al., 1989). In addition, SWA is enhanced by sleep deprivation and reduced by naps (Dijk, 2009). The exact role of SWA is unknown, however, it is thought to be associated with restorative processes, memory consolidation, and synaptic plasticity (Bjorness et al., 2009; Greene & Frank, 2010; McCoy & Strecker, 2011; Vyazovskiy et al., 2009).

Several previous studies in hibernators have reported an increase in SWA during sleep following emergence from hibernation in multiple species, similar to that observed following sleep deprivation (Daan et al., 1991; Trachsel et al., 1991). Moreover, hibernators periodically

rewarm to euthermia; these interbout intervals are primarily spent asleep and are dominated by high intensity SWA, suggesting a possibility that the animals were deprived of restorative sleep during hibernation (Heller & Ruby, 2004). Prevention of these rewarming intervals via hypothalamic lesions can be fatal, suggesting that restorative sleep following hibernation is a necessary process (Satinoff, 1967).

Similar findings have been reported following daily torpor. For example, SWA intensity is increased during sleep following daily torpor in Djungarian hamsters (Deboer & Tobler, 2000), which was found to be qualitatively similar to that observed following sleep deprivation (Deboer & Tobler, 2003). Moreover, there is evidence to support that the SWA following torpor is homeostatically regulated (Deboer & Tobler, 2000; Palchykova et al., 2002), however, this may not be the case for deep hibernation (Larkin & Heller, 1999).

These findings have resulted in the hypothesis that daily torpor is a sleep depriving state, although this has not been established for fasting-induced torpor. Due to the similarities between fasting-induced torpor and daily torpor (Huang et al., 2021), it is reasonable to suggest that fasting-induced torpor may also be sleep depriving. Sleep deprivation is associated with a variety of negative health effects and cognitive deficits in both humans and rodents (Killgore, 2010; Knutson et al., 2007); as such, it is possible that sleep deprivation due to torpor induction may be associated with these effects also. Food restriction paradigms in biomedical research are commonly performed over long periods of time, therefore the torpor induction may also be resulting in chronic sleep deprivation, further confounding the data being produced.

To address this, this chapter investigated whether sleep following fasting-induced torpor in mice is homeostatically regulated by comparing EEG following torpor to sleep deprived

conditions. Doing so will further our understanding of whether torpor is a sleep depriving state and provide greater insights into the regulation and modulation of sleep and torpor. Moreover, this will help to inform the design of experiments using mice in which fasting is required, in order to account for torpor induction and consequential sleep deprivation.

6.1.1 Experimental aims

i) To investigate whether sleep following fasting-induced torpor increases EEG slow wave activity

ii) Determine whether the SWA following fasting-induced torpor is comparable to conventional sleep deprivation

6.2 Methods

6.2.1 Experimental animals

Adult male C57BL/6J mice, aged ~9 weeks at the start of the experiment, were used for this study. Animals (n=8) were obtained from Charles River Laboratories, UK. In order to reduce the number of animals used across experiments, the data presented in this chapter were collected from the same animals as presented in Chapter 5.

6.2.2 Experimental and recording procedures

All animals (n=8) were implanted with EEG and EMG electrodes as described in Section 2.6 of Chapter 2. Mice were given at least one week to recover from surgery and 3 days to habituate to the recording chambers. Food restriction, as described in Section 2.2, was used to induce torpor in these mice to allow for comparison between sleep deprivation and torpor.

All mice underwent three experimental conditions: i) ad libitum fed sleep deprivation; ii) food restricted sleep deprivation; and iii) undisturbed torpor. Sleep deprivation was performed between ZT 20 and ZT 0 (6 am – 10 am). Although sleep deprivation is typically performed at the beginning of the light phase (ZT 0) as this is the circadian period of sleep for nocturnal rodents, such as mice (Krone et al., 2021), ZT 20 – ZT 0 was chosen to allow for more direct comparison with homeostatic sleep pressure after sleep deprivation and following torpor, as torpor typically occurs at the latter end of the dark period. Moreover, it had previously been observed during this thesis that torpid mice frequently rewarm at lights on and then may re-enter a shallower torpor bout. Lights on (ZT 0) was chosen as the time to end sleep deprivation to allow for more direct comparison with the deeper initial torpor bout. The food restricted sleep deprivation condition was performed following >1 week of food restriction when all mice were reliably entering torpor daily.

Sleep deprivation was achieved by providing the mice with novel objects in their home cage environment, such as cardboard, plastic objects, and tissue paper as previously described in Chapter 2 (Fisher et al., 2016; Krone et al., 2021). Exposure to novel objects utilises the innate exploratory behaviour of mice and is therefore considered less stressful than other techniques such as electric shock or presentation of unpleasant stimuli (Blumberg et al., 2004; Todd et al., 2010). At the end of sleep deprivation, all objects were removed, and the mice left undisturbed for the remainder of the 24-hour recording period. During food restricted conditions, mice were provided with a small ration of food of ~2 g shortly before the end of sleep deprivation to mitigate the acute wake-promoting effects of hunger, allowing for subsequent sleep to be recorded.

6.2.3 Statistical analysis

Data were processed using MATLAB (The Math Works Inc., USA), and analysed using MATLAB and Prism (GraphPad, v9, USA). All data were tested for normality prior to analysis using a Shapiro-Wilk test; in instances where normality was not present, an equivalent non-parametric test was used. Analysis of groups over time and between group interactions were conducted using repeated measures two-way ANOVA, with an appropriate post-hoc test. Other comparisons between the 3 groups were performed using one-way ANOVAs with repeated measures and an appropriate post-hoc multiple comparisons test. Sphericity was not assumed when performing ANOVAs, therefore Geisser-Greenhouse corrections were also used. Paired two-tailed t-test were used to compare total EEG slow wave activity on the baseline day versus post manipulation. Differences were determined to be statistically significant when $p<0.05$. All data are presented as mean values ± SEM unless otherwise stated. Data analysis was only performed on 7 of the animals, due to the loss of an animal during the recording period.

6.3 Results

6.3.1 Body temperature

Analysis of Tskin in 1-hour intervals over the 24-hour recordings following the end of each condition found a significant effect of zeitgeber time ($F_{(3.77,22.1)}=6.058$, $p=0.0021$), condition ($F_{(1.66,9.96)}=14.55$, $p=0.0015$), and a significant interaction between time and condition ($F_{(4.50,27.0)}=3.66$, $p=0.0139$; 2-way ANOVA with repeated measures). Post hoc analysis revealed that there was no difference in Tskin between groups at ZT 7-11, corresponding to when food restricted animals display food anticipatory behaviour (Figure 29A).

Additional analysis of Tskin was performed on the first 6 hours following the end of each manipulation, as this is when the effects of sleep deprivation are most apparent (Borbély et al., 1984). Food restricted sleep deprivation was found to transiently inhibit entry into torpor, with no torpors occurring following this condition in the first 6 hours. Despite the absence of torpor, euthermic Tskin was significantly lower in the food restricted condition sleep deprivation condition compared to the ad libitum sleep deprivation condition (effect of condition: $F_{(1.325,7949)}$=12.59, p=0.0057, repeated measures one-way ANOVA). No additional torpor bouts were observed in the first 6 hours following the end of the undisturbed torpor bouts, resulting in no difference in Tskin between the post-torpor condition and the food restricted sleep deprivation condition (p=0.9096; Tukey's multiple comparisons test). Comparison of the whole 24 hour recording found that Tskin following food restricted sleep deprivation was lower than during ad libitum conditions, although this may be explained by the induction of torpor ($F_{(1.66,9.96)}$=14.55, p=0.0015, repeated measures one-way ANOVA

Next, Tskin following each condition was compared to Tskin on their respective baseline day (Tskin across the 24 hours prior to the manipulation) to determine whether Tskin was altered following each manipulation. This revealed a significant increase in body temperature in the food restricted sleep deprivation and torpor condition (FR baseline: 26.6 ± 0.3827ºC; FR post-SD: 28.0 ± 0.3809ºC; t(6), p=0.0005, paired t-test) (torpor baseline: 26.5 ± 0.2881ºC; post-torpor: 28.0 ± 0.5569ºC; t(6), p=0.00024, paired t-test), whereas there was no difference between baseline and post-sleep deprivation body temperature during ad libitum feeding (baseline: 29.6 ± 0.2684ºC; post-SD: 29.4 ± 0.2873ºC; t(6), p=0.1457, paired t-test).

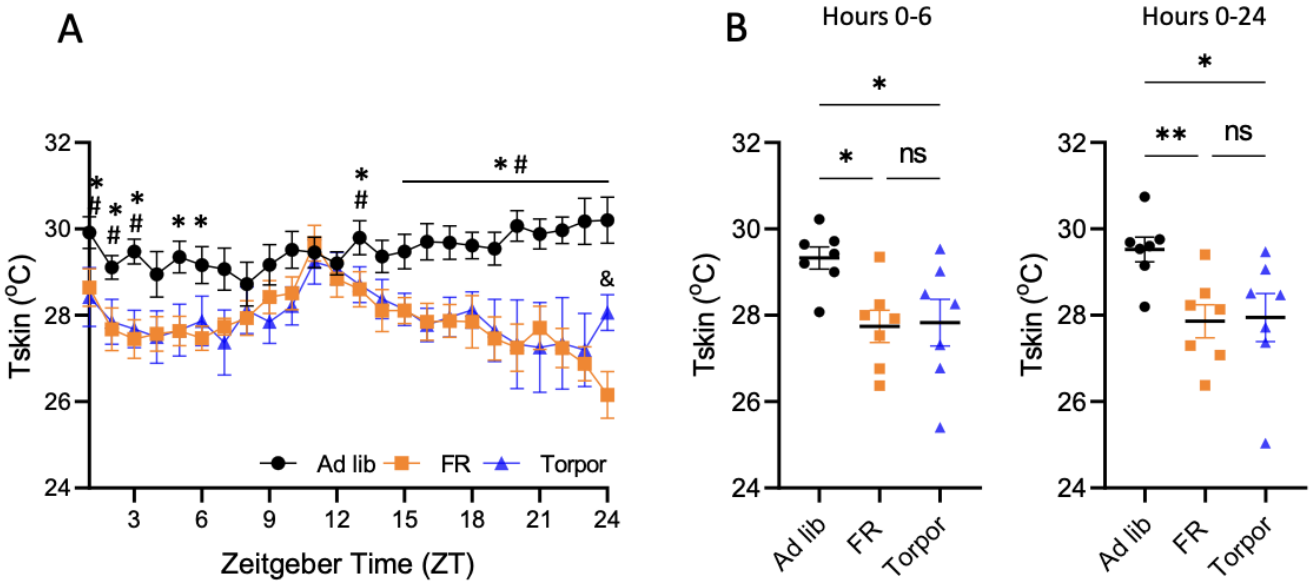

Figure 29: Peripheral body temperature (Tskin) recordings following ad libitum sleep deprivation, food restricted (FR) sleep deprivation, and torpor. (A) Time course of mean (± SEM) Tskin in 1-hour bins (n=7). *p<0.05 ad libitum vs FR, #p<0.05 ad lib vs torpor, &p<0.05 FR vs torpor; 2-way RM ANOVA with Tukey's post hoc. (B) Comparison of mean Tskin between hours 0-6 (left) and hours 0-24 (right) between the three conditions. Data are presented as a mean value ± SEM (n=7); data points represent an individual mouse. *p<0.05, **p<0.01, ns: not-significant (p>0.05); one way RM ANOVA with Tukey's post hoc.

6.3.2 Vigilance states

Next, the effect of each condition on global sleep-wake architecture was investigated over 6 hours immediately following sleep deprivation or torpor (Figure 30). A significant main effect of time (wake: $F_{(2.28,13.7)}$=39.5, p<0.0001; NREM: $F_{(2.19,13.1)}$=38.6, p<0.0001; REM: $F_{(2.26,13.6)}$=18.9, p<0.0001) and condition (wake: $F_{(1.45,8.72)}$=11.0, p=0.0061; NREM: $F_{(1.49,8.96)}$=8.085, p=0.0133; REM: $F_{(1.70,10.2)}$=6.67, p=0.0166) were found for all vigilance states, in addition to a significant interaction between time*condition for wake and NREM but not REM (wake: $F_{(3.98,23.9)}$=5.65, p=0.0024; NREM: $F_{(3.92,23.6)}$=6.58, p=0.0011; REM: $F_{(3.68,22.1)}$=1.03, p=0.4080; 2-way ANOVA with repeated measures).

The acute effects of sleep deprivation or torpor were then investigated in the first 0-2 hours, and 0-6 hours, following the end of the manipulation. A significant effect of condition was found for all vigilance states in the first 2 hours (NREM: $F(1.92,11.5)=46.2$, $p<0.0001$; REM: $F(1.83,11.0)=11.2$, $p=0.0026$; wake: $F(1.92,11.5)=45.2$, $p<0.0001$; RM one-way ANOVAs). Post-hoc analysis using a Tukey's multiple comparisons test revealed that mice spent significantly longer in NREM and REM sleep following ad libitum sleep deprivation compared to following food restricted sleep deprivation or torpor. Unsurprisingly, mice also spent significantly less time awake following ad libitum sleep deprivation compared to the other conditions (Figure 2).

When the analysis window was extended to 0-6 hours following the end of each manipulation, a significant effect of treatment remained for all vigilance states (NREM: $F(1.49,8.96)=8.09$, $p=0.013$; REM: $F(1.70,10.2)=6.67$, $p=0.017$; wake: $F(1.43,8.72)=11.0$, $p=0.0061$; REM one-way ANOVAs). However, post-hoc analysis revealed that some of the differences had changed with mice spending more time awake and less time in NREM sleep post-torpor than following ad libitum sleep deprivation, but showed difference compared to mice following food restricted sleep deprivation. Mice spent significantly less times in NREM sleep and REM sleep following food restricted sleep deprivation compared to following ad libitum sleep deprivation, but no difference was found in the amount of time spent awake.

Differences in the amount of time spent in NREM sleep and wake disappeared when analysing over 24 hours (NREM: $F_{(1.90,11.4)}=3.30$, $p=0.0754$; Wake: $F_{(1.73,10.4)}=2.64$, $p=0.238$; RM one-way ANOVA); however, the amount of time spent in REM sleep over 24 hours was significantly higher following ad libitum sleep deprivation compared to food restricted sleep deprivation (ad lib vs FR: $p=0.0467$), but not compared to torpor (ad lib vs torpor: $p=0.0883$).

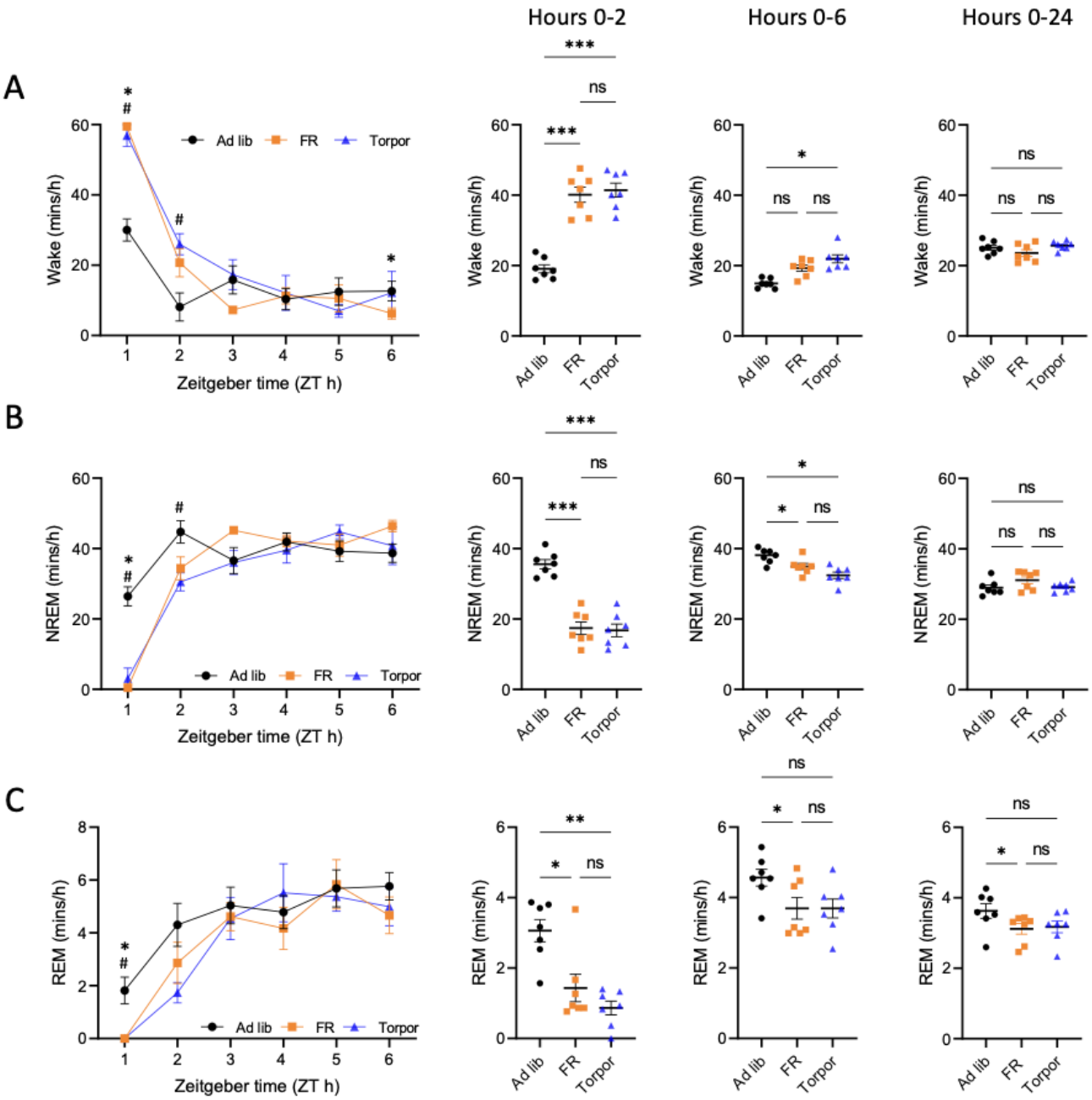

Figure 30: Amount of time spent in each vigilance state following the end of ad libitum sleep deprivation, food restricted sleep deprivation, and torpor. (A-C, left) Amount of time spent in wake, NREM, and REM, in 1 h bins. Data are shown as mean values ± SEM (n=7); *p<0.05 ad libitum vs FR, #p<0.05 ad lib vs torpor; RM 2-way ANOVA with Tukey's post hoc. (A-C, right) Comparison of mean time spent in state in hours 0-2, 0-6, and 0-24. Data are presented as a mean value ± SEM (n=7); data points represent an individual mouse. *p<0.05, **p<0.01, ***p<0.001; RM-one way ANOVA with Tukey's post hoc. NREM: non-rapid eye movement; REM: rapid eye movement; FR: food restriction; ns: not-significant (p>0.05).

Subsequent sleep-wake architecture was further investigated by assessing the number of sleep-wake episodes and duration (Figure 31). Due to the limited differences observed over 24 hours, analysis was focused on the acute effects observed in the 6 hours following the end of sleep deprivation or torpor. Despite finding differences in the amount of time spent in NREM sleep, no differences were found in the number of NREM episodes (Friedman statistic=4.85, p=0.0937; Friedman test), average wake bout duration ($F_{(1.28,7.68)}$=1.79, p=0.2254; RM one-way ANOVA), or maximum duration ($F_{(1.85,11.1)}$=2.67, p=0.1156; RM one-way ANOVA) between the three conditions. There was also no difference in the number of wake episodes ($F_{(1.76,10.6)}$=0.332, p=0.6988 RM one-way ANOVA) or average duration ($F_{(1.92,11.5)}$=1.83, p=0.2052; RM one-way ANOVA), but the longest wake episode was increased following food restricted sleep deprivation and torpor compared to ad libitum sleep deprivation (ad lib vs FR: p=0.0013; ad lib vs torpor: p=0.0177; ($F_{(1.85,11.1)}$=2.67, p=0.1156; RM one-way ANOVA, Tukey post hoc test). This may be explained by the latency to the first NREM episode being significantly longer, likely due to the mice eating the food provided towards the end of torpor/sleep deprivation.

There were fewer REM sleep episodes following food restricted sleep deprivation and torpor compared to ad libitum sleep deprivation (ad lib vs FR: p=0.0064; ad lib vs torpor: p=0.0048; FR vs torpor: p=0.8884; RM one-way ANOVA, Tukey post hoc), but average REM episode length was greater following food restricted sleep deprivation compared to ad libitum (ad lib vs FR: p=0.0163; ad lib vs torpor: p=0.0941; FR vs torpor: p=0.7519). There was no difference in the length of the longest REM episode between conditions (Friedman statistic: 0.5185, p=0.8560; Friedman test). A significant main effect of condition was found when comparing latency to first REM episode ($F_{(1.50,9.02)}$=20.1, p=0.0008; RM one-way ANOVA); a Tukey's post hoc test revealed that latency to the first REM episode was significantly increased

following food restricted sleep deprivation and torpor compared to ad libitum sleep deprivation (ad lib vs FR: p=0.0006; ad lib vs torpor: p=0.0037; FR vs torpor: p=0.9972). This again may be explained by increased latency to fall asleep in these conditions, and due to lower body temperatures.

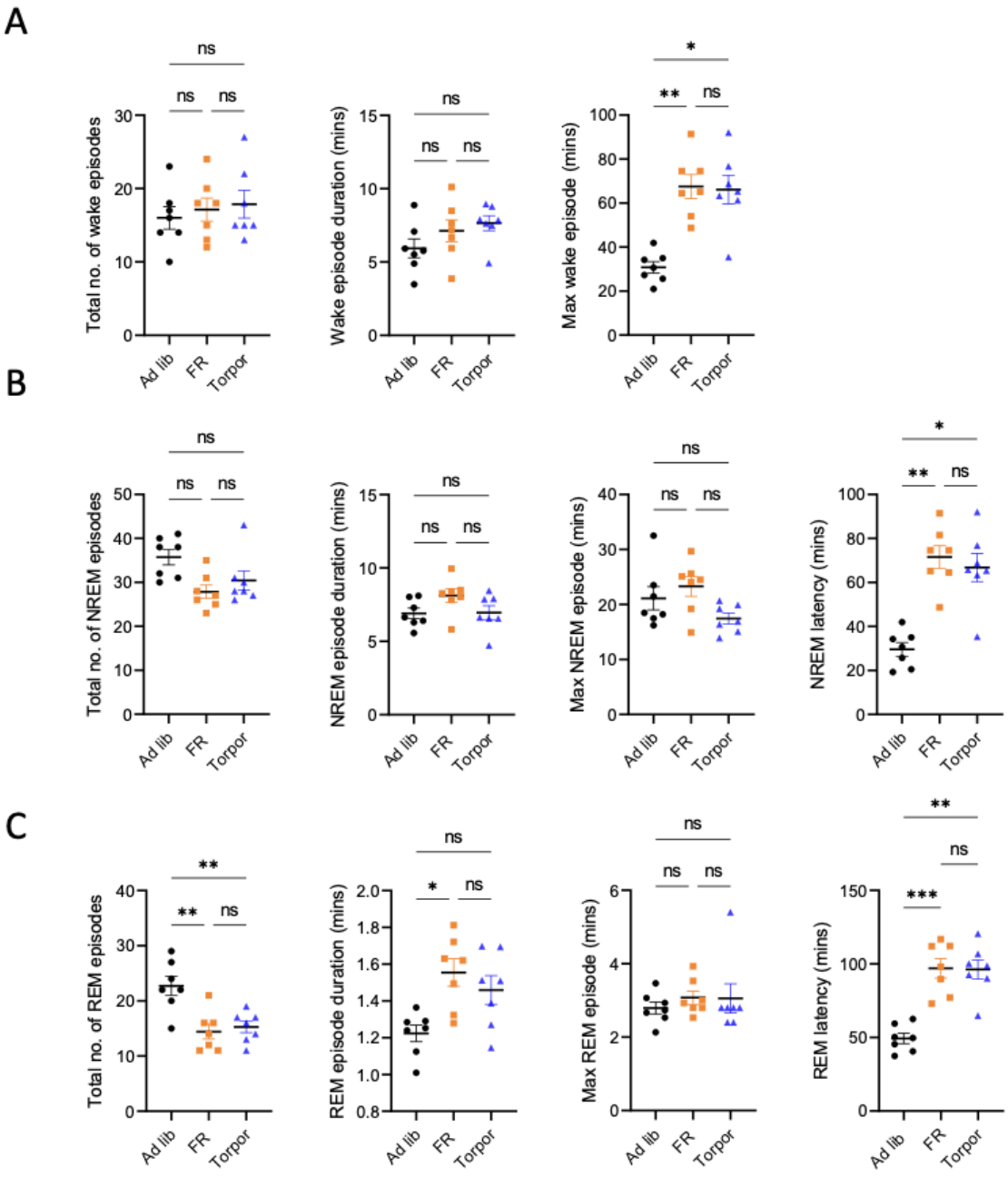

Figure 31: Sleep-wake architecture in the first 6 hours following ad libitum sleep deprivation, food restricted sleep deprivation, and torpor. Total number of wake (A), NREM (B), and REM (C) episodes, average episode duration, and duration of longest episode. Latency to first NREM (B) and REM (C) episode also shown. Data are presented as mean values ± SEM (n=7); data points represent an individual mouse. *p<0.05, **p<0.01, ***p<0.001, RM-one way ANOVA with Tukey's post hoc. NREM: non-rapid eye movement; REM: rapid eye movement; FR: food restriction; ns: not-significant (p>0.05).

6.3.3 EEG slow wave activity

Sleep following daily torpor in Djungarian hamsters has been suggested to be homeostatically regulated (Deboer & Tobler, 2003; Palchykova et al., 2002), although this may not be the case for deep hibernation (Larkin & Heller, 1999) and has not been explored in fasting-induced daily torpor. To investigate where fasting-induced torpor falls on this scale, the intensity of slow wave activity (0.5-4 Hz) during NREM sleep was compared following torpor, food restricted sleep deprivation, and ad libitum sleep deprivation. Initial visualisation of slow wave activity indicated that there was an increase in slow wave intensity following all three conditions compared to the 24 hour recording taken the day before each condition was performed (see Figure 32 for representative example).

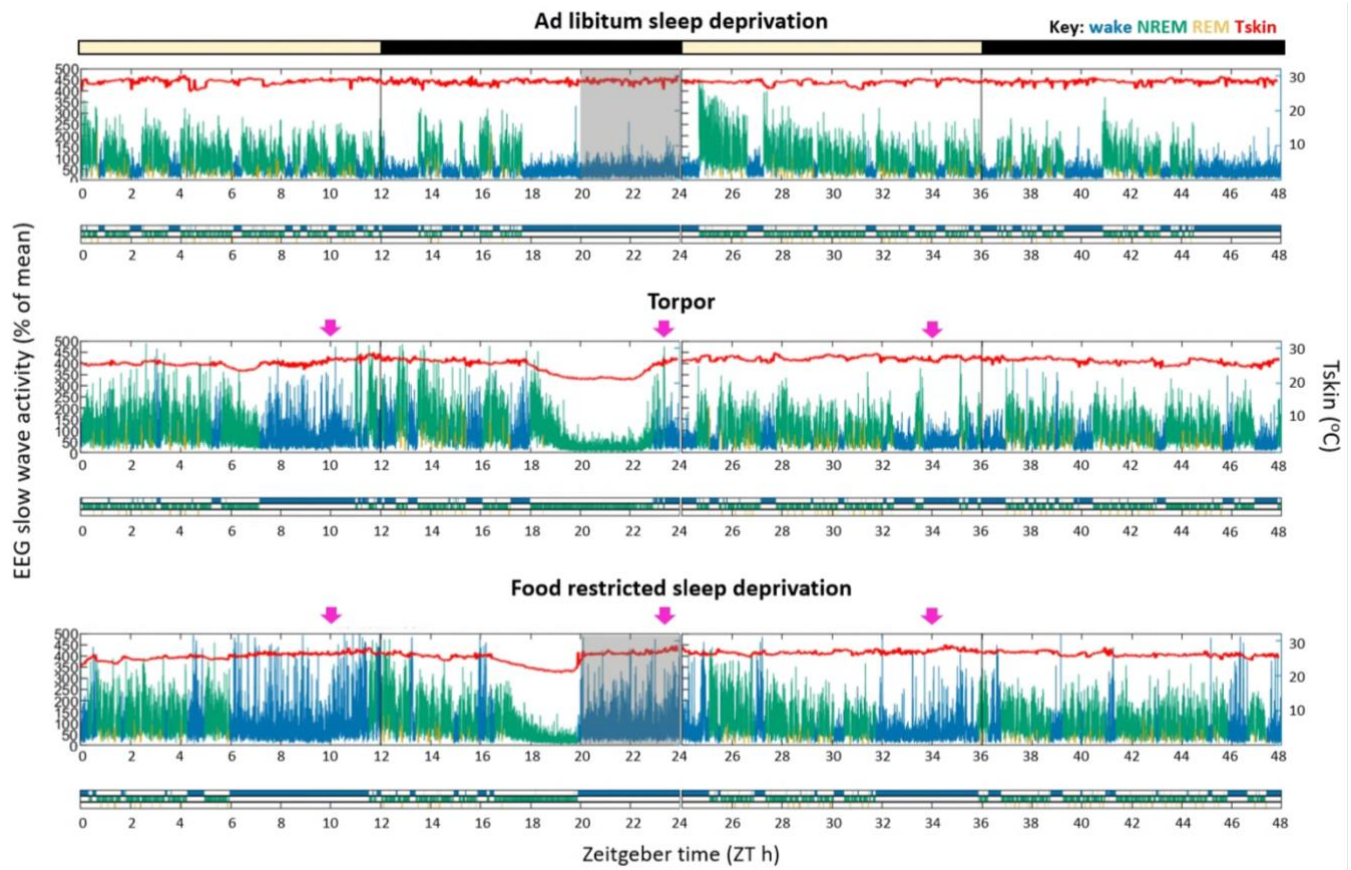

Figure 32: Hypnograms from a representative mouse following ad libitum sleep deprivation (top), torpor (middle), and food restricted sleep deprivation (bottom). Profiles of EEG slow wave activity (0.5-4 Hz), recorded from the frontal cortex, over 48-hour recordings, represented as a percentage of the mean. Wake, NREM sleep, and REM sleep are represented in blue, green, and amber colour coding, respectively. Peripheral body temperature recordings are presented in red. The pink arrows represent when mice were fed during the torpor and food restricted sleep deprivation conditions. Grey shading represents when sleep deprivation was performed. Black and yellow bars represent the light-dark cycle.

Quantification of EEG slow wave activity (SWA) during NREM sleep found a significant increase in intensity following each condition compared to the baseline day, indicating homeostatic rebound of sleep (Figure 33; Ad lib: $t(6)=15.4$, $p<0.0001$; FR: $t(6)=6.58$, $p=0.0006$; torpor: $t(6)=3.11$, $p=0.0210$; paired t-test). Typically, SWA data are normalised by calculating the data as a percentage of the baseline mean. However, due to the positive relationship between EEG power and body temperature observed in the previous chapter (see Chapter 5, Figure 27), it is possible that the lower Tskin reported during food restricted conditions would be influencing SWA. As such, SWA may appear to be suppressed during the

food restricted sleep deprivation and torpor conditions. Moreover, Tskin was found to be significantly increased after the torpor and food restricted sleep deprivation due to the additional food provided. This increase in body temperature compared to the baseline day may then skew the percentage increase in SWA in favour of appearing much greater than it is in reality.

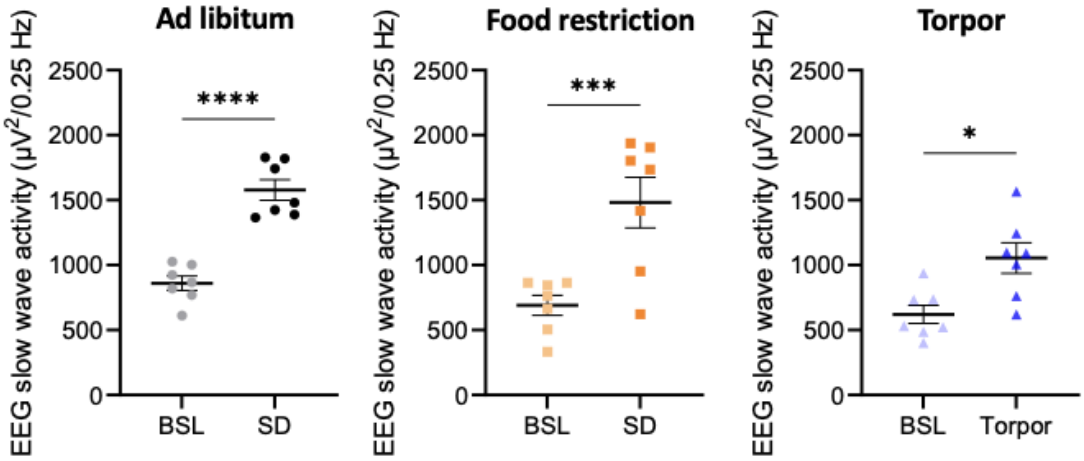

Figure 33: Total EEG slow wave activity in baseline conditions compared to after ad libitum sleep deprivation (left), food restricted sleep deprivation (middle), and torpor (right). *p<0.05, ***p<0.001, ****p<0.0001; paired t-tests. All data are presented as mean values ± SEM (n=7).

To determine whether this may be the case, absolute spectra on the baseline days during wake, NREM sleep, and REM sleep were compared (Figure 34). Analysis using a RM 2-way ANOVA on log-transformed values found a significant main effect of condition across all vigilance states (NREM: $F_{(1.76,10.6)}$=44.2, p<0.0001; REM: $F_{(1.29,6.43)}$=7.68, p=0.026; wake: $F_{(1.37,8.22)}$=7.11, p=0.022); Tukey's multiple comparisons post-hoc tests revealed that the majority of the differences were between ad libitum vs food restricted sleep deprivation baseline days, and ad libitum vs torpor baseline. In addition, simple linear regression analysis between Tskin and EEG SWA reported a significant positive relationship (r^2=0.2610, p<0.0001), indicating that the change in body temperature will affect SWA (Figure 35).

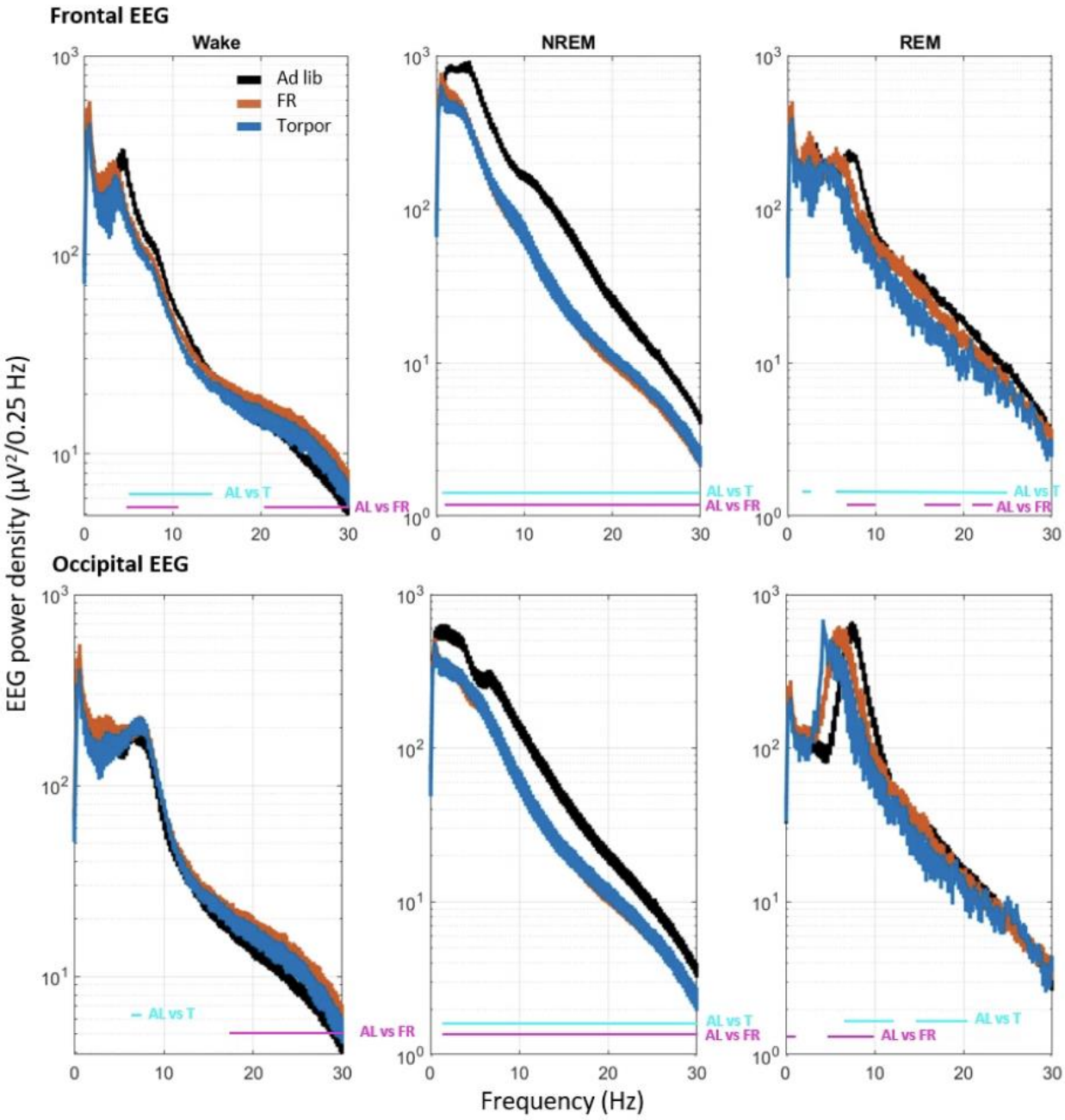

Figure 34: Comparison of EEG power spectra on the baseline days of food restricted sleep deprivation, ad libitum sleep deprivation, and torpor. EEG power values are plotted on a logarithmic scale from 0-30 Hz, binned in 0.25 Hz intervals. Spectra are presented for epochs scored as wake (left), NREM sleep (middle), and REM sleep (right), for the frontal (top) and occipital (bottom) derivations. Spectral data are presented as mean values ± SEM (n=7). Significant differences between conditions for each frequency bin are indicated by the bars ($p<0.05$, 2-way ANOVA with repeated measures). NREM: non-rapid eye movement; REM: rapid eye movement

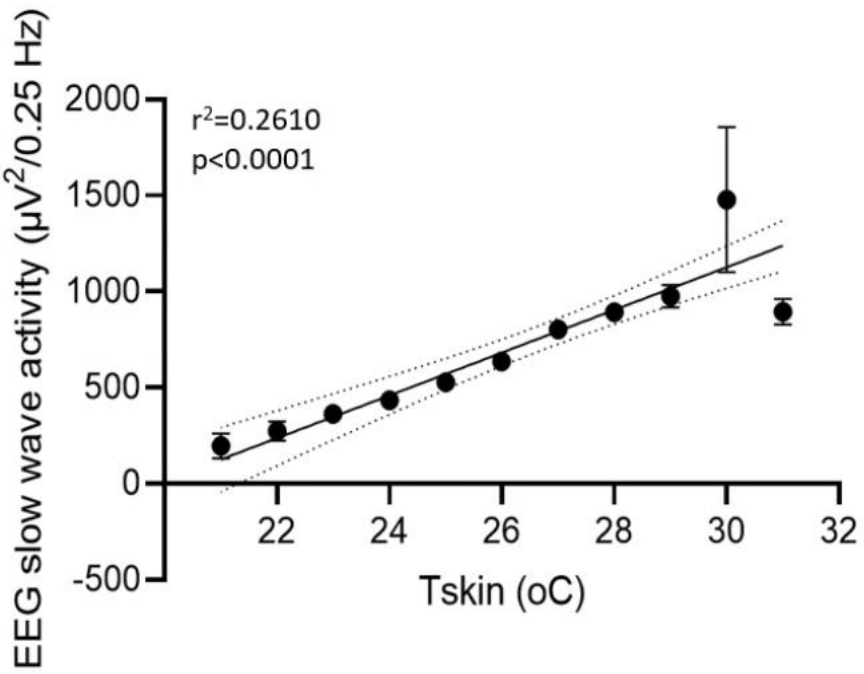

Figure 35: Effect of peripheral body temperature on EEG slow wave activity during NREM sleep. Simple linear regression analysis of mean EEG slow wave activity (0.5-4 Hz) during epochs scored as NREM sleep for every 1°C of peripheral body temperature (Tskin). Data were taken from eight 24 h recording files (four ad libitum days, four food restricted days in the presence of torpor); each data point represents the mean value ± SEM (n=7). NREM: non-rapid eye movement.

To overcome these challenges, SWA was calculated as a percentage of the mean for the baseline day of each condition (Figure 36 A-C), and then also as a percentage of the mean on the ad libitum baseline day (Figure 36 D-F). When normalising to individual baseline days, SWA was significantly greater in the first 6 hours following food restricted sleep deprivation and torpor compared to ad libitum sleep deprivation (ad lib vs FR: p=0.0001; ad lib vs torpor; p=0.0066; FR vs torpor: p=0.8878; RM mixed-effects model, Tukey post hoc). Comparison of the time course of SWA revealed that SWA peaked later at ZT 2 for food restriction and torpor, whereas the peak occurred at ZT 1 during ad libitum. This is likely due to the increased latency to sleep as a consequence of providing food (Figure 31 B&C). Analysis of peak SWA in the first 6 hours found that there was no difference between conditions, despite total ad libitum SWA being lower (F(1.28,7.65)=1.04, p=0.3604; RM one-way ANOVA). SWA dissipated in

all conditions following the peak until ZT 12 at which point there is a second peak in SWA for food restriction and torpor. Analysis of sleep-architecture in Chapter 5 indicates that this secondary peak may be explained by a period of euthermic sleep following feeding.

When data were normalised to the ad libitum baseline day, a slightly different picture emerges (Figure 36 D-F). There is little difference in SWA in the 24 hours following the end of each manipulation, with statistical differences only being observed at ZT 2, 12, and 13. A delay in SWA rebound following the end of food restricted sleep deprivation and torpor is still observed when normalising in this way. Moreover, a secondary peak in SWA following feeding during the food restricted conditions was still present. Comparison of SWA in between hours 0-6 found no difference between ad libitum and food restricted sleep deprivation, and ad libitum sleep deprivation and torpor, whereas food restricted sleep deprivation SWA was significantly greater than following torpor ($p=0.039$; RM one-way ANOVA, Tukey's multiple comparisons test). Interestingly, peak SWA following torpor was significantly lower than both food restricted and ad libitum sleep deprivation (Figure 36F; Torpor vs FR: $p=0.027$; Torpor vs Ad lib: $p=0.017$; RM one-way ANOVA, Tukey's multiple comparisons test), whereas no differences between groups were found when normalised to individual baseline days (Figure 36C).

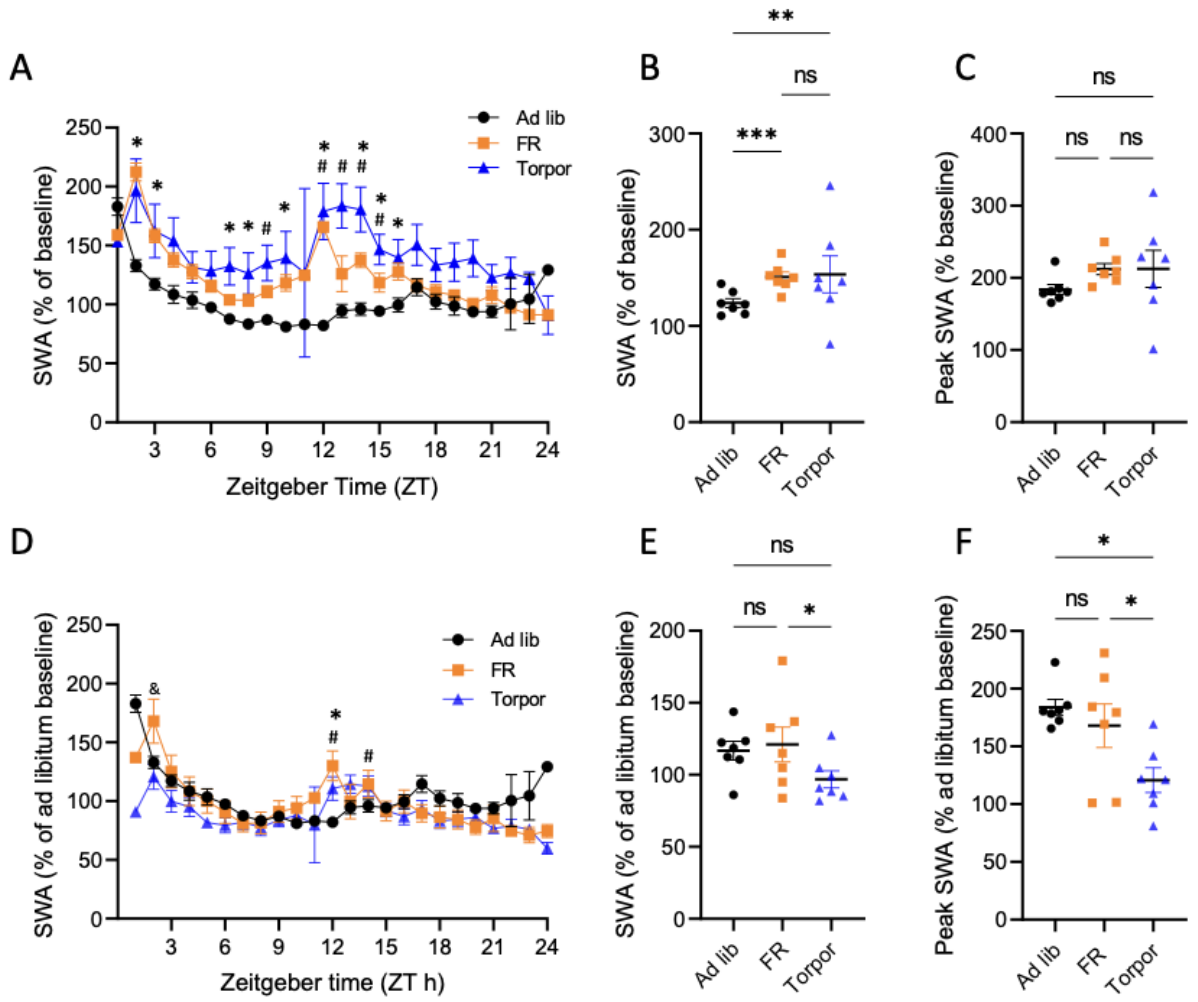

Figure 36: Effect of ad libitum sleep deprivation, food restricted sleep deprivation, and torpor on subsequent EEG slow wave activity. (A) Time course of EEG slow wave activity (SWA), as a percentage of baseline, in the 24-hrs following the end of each condition. *p<0.05 ad lib vs food restriction, #p<0.05 ad lib vs torpor, &p<0.05 food restriction vs torpor; RM 2-way ANOVA with Tukey's post hoc. (B) Comparison of mean SWA in the 6 hours following the end of each condition. **p<0.01, ***p<0.001; RM one-way ANOVA with Tukey's post hoc test. (C) Comparison of peak SWA in the first 6 hours following the end of each condition. ns: not-significant (p>0.05). (D-F) As a percentage of ad libitum baseline. *p<0.05, ***p<0.001, ****p<0.0001; paired t-tests. All data are presented as mean values ± SEM (n=7).

To account for the difference in the amount of sleep in the torpor and food restricted sleep deprivation conditions, slow wave energy (SWE) across the subsequent 24 hours was calculated and compared between conditions. To this end, the cumulative sum of NREM sleep delta power (0.25-4 Hz) across the recordings were calculated and normalised by taking the SWE as a percentage of the maximal cumulative sum on the baseline day (Figure 37A). Due

to the potential effect of body temperature on EEG power, SWE as a percentage of maximal delta power on the ad libitum baseline day for all conditions was also calculated (Figure 37B).

When SWE was normalised to each condition's respective baseline, post-torpor and post food restricted sleep deprivation SWE were consistently higher across the 24 h recording than for following ad libitum sleep deprivation (Figure 37A). A two-way ANOVA found a significant main effect of time ($F_{(1.26,7.35)}$=516.7, p<0.0001), but not condition ($F_{(1.32,7.89)}$=3.72, p=0.084), although there was a significant interaction between time and condition ($F_{(1.64,9,86)}$=5.98, p=0.024). A Tukey's post hoc test found that food restricted SWE was significantly higher than ad libitum SWE between ZT 6-24. No differences in SWE were found between the torpor and food restricted sleep deprivation conditions.

Normalisation of all conditions to the ad libitum baseline day reported opposite findings with SWE following ad libitum sleep deprivation being higher than the other two conditions (Figure 37B). Analysis of these data reported a significant main effect of time but effect of condition and no significant interaction between time*condition (main effect of time: $F_{(1.26,7.52)}$=516.7, p<0.0001; main effect of condition: $F_{(1.32,7.89)}$=3.72, p=0.0839; interaction between time*condition: $F_{(1.64,9.86)}$=5.98, p=0.0238).

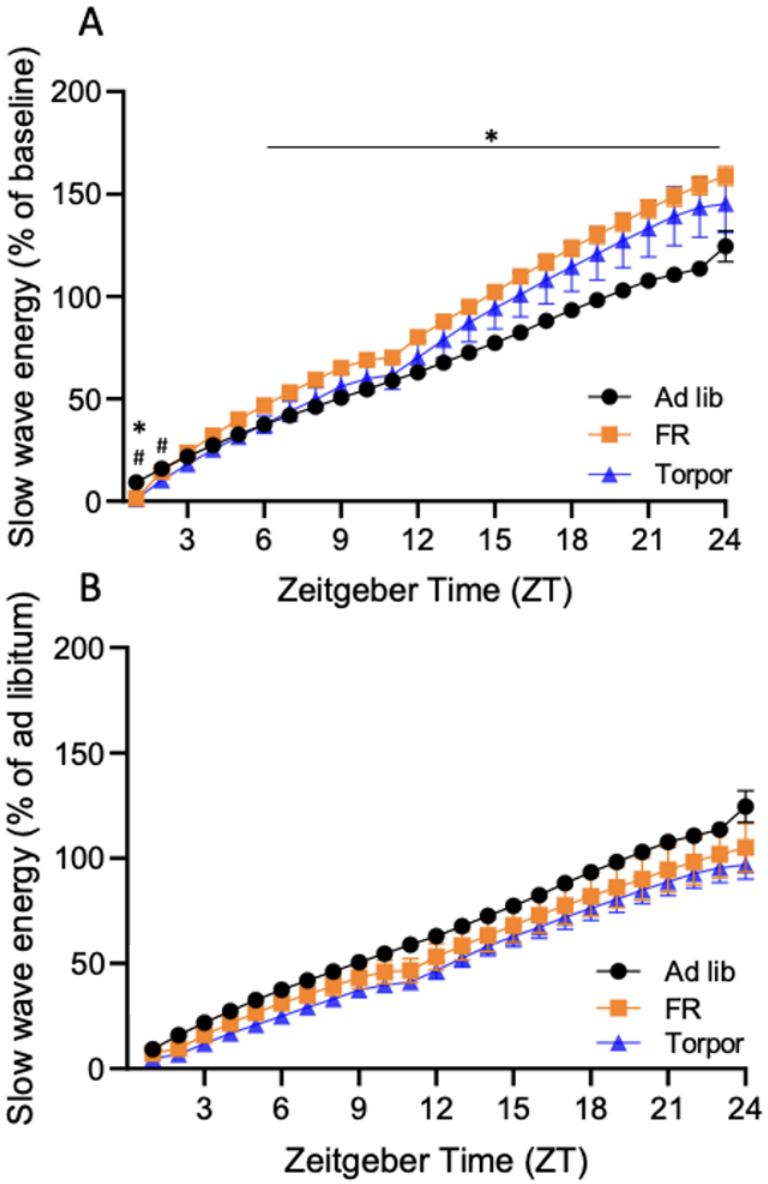

Figure 37: Slow wave energy following the end of ad libitum sleep deprivation, food restricted sleep deprivation, and torpor. Cumulative sum of NREM sleep EEG delta power (slow wave energy) over 24 hrs, expressed as a percentage of the slow wave energy on the baseline day (A), and as a percentage of slow-wave energy on the ad libitum baseline day (B). *p<0.05 ad lib vs food restriction, #p<0.05 ad lib vs torpor, $^{\&}$p<0.05 torpor vs food restriction; RM 2-way ANOVA with Tukey's post hoc. Data are expressed as mean values ± SEM (n=7).

6.3.4 Spectral power

The effect of each conditions on EEG spectra was also compared for each vigilance state in the first 6 hours following the end of each condition. To allow for more direct comparison between conditions, spectra were normalised by calculating the spectra power as a percentage of baseline spectra in the same time window (Figure 38A). Due to the potential effect of body temperature on spectra, all conditions were also normalised to the ad libitum baseline spectra (Figure 38B).

When normalising to each condition's baseline, a main effect of condition on the NREM spectra but no significant interaction between frequency bin and condition was found (Figure 38A; main effect of condition: $F_{(120.0,720.0)}$=6.78, p<0.0001; frequency*condition: $F_{(2.29,13.8)}$=3.21, p=0.067; RM 2-way ANOVA). No differences were found between torpor and food restricted spectra. Differences were found in nearly in all frequency bins between 1.5 and 30 Hz between ad libitum sleep deprivation and torpor conditions, whilst differences between ad libitum and food restricted sleep deprivation were found in frequency bins 13.25-26.25 Hz. Similarly to NREM spectra, a significant main effect of condition was found for REM sleep spectra ($F_{(1.14, 6.86)}$=8.32, p=0.022; RM 2-way ANOVA) but there was no significant interaction between frequency and condition ($F_{(1.63, 9.80)}$=1.14, p=0.347; RM 2-way ANOVA). A post hoc multiple comparisons test revealed differences between food restricted sleep deprivation spectra and torpor spectra between 8.75-24 Hz. Differences between ad libitum and torpor conditions were found in frequencies between 7.25-23.25 Hz. Analysis of wake EEG spectra did not find a main effect of condition ($F_{(1.24, 7.45)}$=3.12, p=0.1148; RM 2-way ANOVA) or a significant interaction between condition and frequency ($F_{(1.14, 6.82)}$=1.61, p=0.2516; RM 2-way ANOVA), but post hoc analysis revealed some differences in frequency bins between groups.

Normalising all conditions to ad libitum baseline spectra resulted in several differences in the relative change in frequencies following food restricted sleep deprivation and torpor (Figure 10B). A significant main effect of condition and significant interaction between condition and frequency were found for epochs scored as NREM sleep (condition: $F_{(1.51, 9.08)}$=26.1, p=0.0003; condition*frequency: $F_{(1.57,9.44)}$=9.13, p=0.0083; RM 2-way ANOVA), and wake (condition: $F_{(1.94,11.6)}$=14.9, p=0.0007; condition*frequency: $F_{(4.02,24.1)}$=6.04, p=0.0016; 2-way RM ANOVA). A multiple comparisons post hoc test found multiple differences between food restricted sleep deprivation and torpor during NREM sleep which had not been found when normalising the data to each condition's individual baseline. For REM sleep, a significant main effect of condition was found but no significant interaction between condition and frequency bin (condition: $F_{(1.95,11.7)}$=6.90, p=0.0109; condition*frequency: $F_{(4.87,29.2)}$=2.22, p=0.0806; 2-way RM ANOVA).

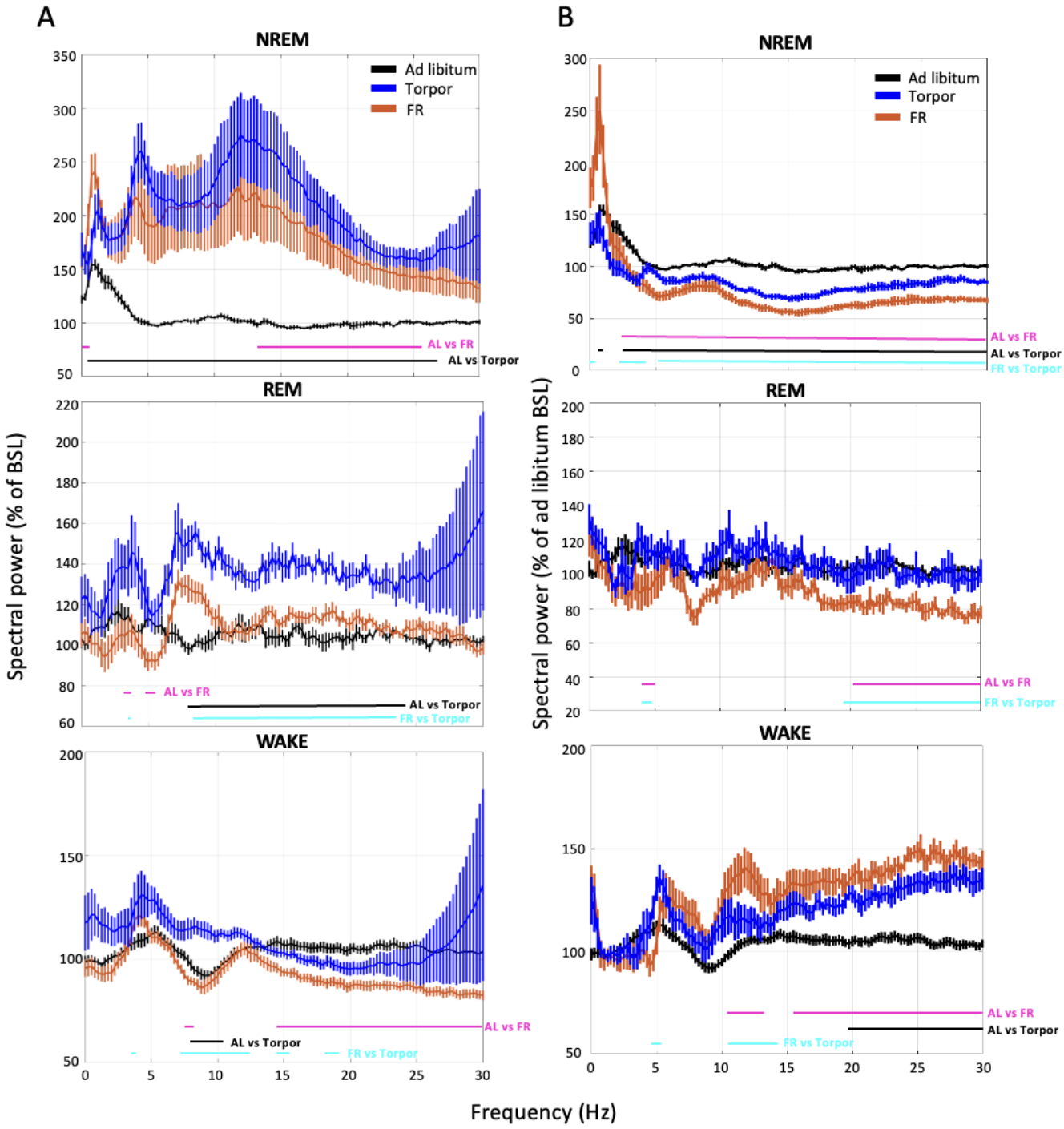

Figure 38: Effect of sleep deprivation and torpor on subsequent spectral power. EEG spectral power, expressed as a percentage of baseline, during NREM sleep (top), REM sleep (middle), and wake (bottom), in the first 6 hours following the end of ad libitum sleep deprivation, food restricted sleep deprivation, and torpor. Spectral power is presented as a percentage of each condition's baseline day (A), and as a percentage of the ad libitum baseline (B). Significant differences ($p<0.05$) between groups for each frequency bins of 0.25 Hz are indicated by the pink (ad libitum vs food restricted sleep deprivation), black (ad libitum vs torpor), and blue (food restricted sleep deprivation vs torpor) colour. Differences were estimated statistically using 2-way ANOVAs with repeated measures, followed by a Tukey's post hoc multiple comparisons test. Data are expressed as a mean value ± SEM (n=7). NREM: non-rapid eye movement; REM: rapid eye movement; AL: ad libitum; FR: food restriction.

6.4 Discussion

The aim of this study was to investigate sleep homeostasis in relation to fasting-induced torpor in mice. Previous work has indicated that sleep following daily torpor in hamsters is homeostatically regulated (Deboer & Tobler, 1994), and the intensity of slow waves is increased when sleep deprivation is performed following arousal from torpor (Palchykova et al., 2002). No record in the published literature could be found regarding sleep homeostasis following fasting-induced torpor in mice.

Unlike previous data presented from daily torpor in hamsters, being in a fasted state may be a potential confound as fasting itself can profoundly alter physiology, including downregulation of cellular processes such as suppression of protein synthesis, utilisation of alternate endogenous fuel stores, and changes in plasma glucose, lipids, and urea (Jensen et al., 2013; Secor & Carey, 2016). As such, it was necessary to include a food restricted sleep deprivation condition to control for the potentially confounding effect of being in a chronically food restricted state. To mitigate the effects of fasting, additional food was provided at the end of food restricted sleep deprivation and torpor. This was also due in part to pilot experiments which found that fasted mice did not sleep following the end of sleep deprivation due to the wake-promoting effects of hunger. However, the provision of additional food had the secondary effect of increasing body temperature, which can alter EEG power, due to the slight alleviation of the metabolic challenge. Consequently, increased SWA intensity may reflect increased body temperature rather than sleep homeostasis; as such, caution should be taken when interpreting the results presented in this chapter.

Analysis of measures of sleep-wake architecture revealed that there was increased wakefulness following both the food restricted and torpor conditions, which was most apparent in hours 0-

2 and to a lesser extent in hours 0-6. This is likely because of mice taking time to eat the food provided, however, it does mean that the resultant reduction in overall sleep time can affect interpretation of SWA. Measures of sleep consolidation did not find differences in the number of episodes of NREM or wake, of the duration of the longest episode, which may indicate similar sleep-wake architecture following each condition. The number of REM sleep episodes was significantly higher in the ad libitum condition, but this may be due to differences in body temperature. Body temperature was significantly lower in torpor and food restricted sleep deprivation conditions which has been shown to positively correlate with the amount of REM sleep as shown in Chapter 5 of this thesis, and elsewhere (Deboer & Tobler, 1995; Huang et al., 2021; Szymusiak et al., 1981).

Sleep homeostasis is predominately assessed by measuring levels of EEG slow wave activity due to the enhancement of slow waves following sleep deprivation (Borbély et al., 1984). Here, an increase in absolute EEG slow wave activity was observed following each condition compared to baseline which may indicate a homeostatic rebound. As no actual sleep deprivation was conducted for the torpor condition, this may support fasting-induced torpor as a sleep depriving state, similar to daily torpor (Palchykova et al., 2002). Interestingly, when SWA was normalised to individual baseline days, SWA following both torpor and food restricted sleep deprivation were significantly higher than SWA following ad libitum sleep deprivation, although peak SWA was similar between conditions. One interpretation would be that this may reflect higher sleep pressure following torpor and food restricted sleep deprivation compared to ad libitum sleep deprivation. However, body temperature significantly increased in the food restricted sleep deprivation and torpor condition compared to baseline, but not in the ad libitum condition. As EEG power is affected by body temperature (Deboer & Tobler, 1995; Krauchi & Deboer, 2010), and these data showed a significant positive relationship

between SWA and Tskin (Figure 6), this increase in body temperature may account for such a dramatic percentage increase in SWA. This is supported by the subsequent analysis on SWA data normalised to the ad libitum baseline day, as the majority of reported differences disappear. Furthermore, this dataset suggests that total SWA is significantly lower in the 6 hours following torpor compared to the other conditions, as is peak SWA. This does not necessarily mean that sleep following fasting-induced torpor is not homeostatically regulated, as there may be a combination of both temperature effects and sleep deprivation. Further studies are required to answer this question.

The intensity and maximum SWA were reduced following torpor compared to following the food restricted sleep deprivation. This may be explained by torpor accruing sleep debt at a slower rate than during prolonged wakefulness, as NREM slow waves are still present during torpor bouts, which has previously been suggested when comparing SWA following daily torpor and sleep deprivation in hamsters (Deboer & Tobler, 2003). However, the underlying characteristics of slow waves following daily torpor versus sleep deprivation have been reported to differ (Vyazovskiy et al., 2017), and so may not be fulfilling the restorative processes normally associated with slow waves during deep sleep, therefore explaining why sleep pressure accumulates during torpor even in the presence of slow waves. Interestingly, SWA following food restricted sleep deprivation was no different than following ad libitum sleep deprivation, despite these animals having previously experienced torpor and have then undergone sleep deprivation. If torpor is sleep depriving, it may be expected that this condition would have produced the greatest rebound due to the additive effects of torpor and sleep deprivation as has previously been shown (Palchykova et al., 2002). However, Palchykova and colleagues performed sleep deprivation after spontaneous emergence from photoperiod-induced daily torpor in hamsters, whereas the present study performed sleep deprivation when

the mice would have usually been in torpor. As such, the mice would not have been in torpor for a number of hours before the start of sleep deprivation, resulting in time for euthermic sleep to clear any torpor-associated sleep debt.

When data were normalised to individual baseline days, SWA following torpor and food restricted sleep deprivation were significantly higher compared to ad libitum sleep deprivation. This is most likely explained by the effect of body temperature on EEG power resulting in a greater percentage increase when going from hypothermic conditions on the baseline day, to a significantly higher body temperature post manipulation. This interpretation is supported by comparison of baseline day spectra; notably, more differences between conditions were found for NREM spectra which is the vigilance state in which slow waves occur.

6.4.1 Conclusions

The results presented in this chapter highlight the importance of controlling for body temperature when performing electrophysiological recordings in torpid mice. When accounting for body temperature, analysis of NREM sleep following torpor shows an increase in slow wave intensity compared to baseline recordings, in line with a homeostatic rebound of sleep. Slow wave activity following torpor is less than that following sleep deprivation, therefore supporting the hypothesis that sleep pressure accumulates more slowly during torpor. Overall, these data show initial evidence that fasting-induced torpor is likely a sleep depriving state and provides novel insights into the regulation and modulation of both sleep and fasting-induced torpor.

Chapter 7: Investigating cortical responses to auditory stimulation during sleep, wake, and torpor

7.1 Introduction

Sleep represents an intriguing state of physiologically altered consciousness. Sleep is paradoxical in nature as organisms do not interact with their environment and appear to be oblivious to their surroundings, making them vulnerable to predation and external threats. However, sleep is highly conserved across species and is thought to be essential for optimal brain and bodily function (Cirelli & Tononi, 2008; Siegel, 2005). The development of the EEG has provided an insight into the sleeping brain, showing that the brain remains active during sleep (Adamantidis et al., 2019), and that environmental stimuli can elicit an electrophysiological response which, if sufficient, can translate into a behavioural response (Blume et al., 2017; Kouider et al., 2014). Such observations have led to questions as to whether we can determine the presence of conscious awareness in light of sensory perception, with investigations into how the brain gates and regulates responses to such stimuli. This has become particularly relevant in a clinical setting with research focusing on anaesthesia, coma, and vegetative states (Fu et al., 2021; Padilla & Domina, 2016).

Hibernation and daily torpor represent other physiological states of altered consciousness but have remained largely unexplored in comparison. As shown in Chapters 5 and 6, in addition to elsewhere, brain activity during shallow torpor is markedly similar to that observed during sleep (Huang et al., 2021). However, the threshold for a stimulus to induce arousal appears to be much greater during torpor compared to sleep (Ruf et al., 2022). Further, it is unclear whether sensory processing is preserved, or whether external information reaches higher brain levels, such as the cortex, during torpor. The focus of this chapter was to investigate whether

electrophysiological and/or behavioural responses to sensory stimuli are maintained during torpor, and how this may differ from sleep. It is hoped that this will provide insights into torpor neurophysiology, in addition to further investigating sleep and torpor as related states.

7.1.1 Sensory perception during sleep

From our daily lives, we are most likely aware that we have the ability to remain asleep despite a degree of environmental disturbance. However, if the stimulus is particularly disturbing, e.g., an alarm clock, then this will be sufficient to induce a behavioural response. It has been suggested that this sleep disconnection may be due to "thalamic gating" where peripheral sensory inputs are not relayed effectively to the cortex, but if the stimulus is strong enough then this gating can be bypassed (McCormick & Bal 1994; Steriade 2003). Such sensory gating enables sleep disturbances to be minimised, whilst maintaining the ability to respond to threat.

Presentation of auditory tones during sleep has shown that sensory stimulation modulates the proportion of time spent in each sleep state (Amici et al., 2000). Moreover, tones played during sleep reliably result in cortical evoked response potentials (ERPs), indicating that higher brain regions are perceiving the stimulation to some degree (Atienza et al., 2001; Nir et al., 2015). ERPs across vigilance states have been well characterised in mice (Hall & Borbely, 1970), with the ERP components appearing to be analogous to those observed in humans. Interestingly, ERPs were not found to differ between vigilance state when evoked using auditory stimulation in laboratory rats (Nir et al., 2015). Moreover, perception of a deviant tone amongst a sequence of familiar tones also remained intact across vigilance states.

ERPs are manifestations of simultaneous stimulus phase-locked potential changes in populations of neurons. ERPs are composed of positive and negative deflections which in mice

are termed P20, N40, P80 and P120, where P or N refers to whether the peak is positive or negative, and the numbers correspond to the peak latency relative to stimulus onset in milliseconds (Siegel et al. 2003; Connolly et al. 2004). The exact function of these peaks is unknown, but their reliable occurrence following unexpected, or task relevant events, has led to them becoming the focus of sensory and cognitive studies (Ehlers & Somes 2002; Umbricht et al. 2004).

7.1.2 Sensory perception during torpor

Although brain activity during torpor closely resembles sleep, oscillations are dampened due to a slowing of cortical firing (Berger, 1984; Huang et al., 2021). As such, it can be extremely difficult to make electrophysiological recordings from animals in deep torpor (Chatfield et al., 1951), and may suggest a greater degree of dysconnectivity compared to sleep. In addition, data from hibernating ground squirrels demonstrate a significant reduction in synaptic connections and dendritic spines across multiple brain regions (Roelandse & Matus, 2004; Von Der Ohe et al., 2006). As such, it is unclear whether sufficient cortical connectivity is maintained to enable sensory perception during a torpid state and whether response characteristics are altered.

However, a total lack of environmental perception would make torpid animals highly vulnerable to predation and environmental threats. There does appear to be some degree of sensory awareness during torpor as torpid ground squirrels were able to be provoked to arouse following tactile stimulation through handling (Strijkstra & Daan, 1997). Moreover, work in hibernating pygmy possums found that exposure to smoke as a behaviourally relevant olfactory stimulus, was sufficient to induce arousal from torpor, although responses were less pronounced at lower body temperatures (Nowack et al., 2016). One report found that

hibernating ground squirrels uncurled their bodies in response to auditory tones with some animals arousing completely and rewarming to euthermia (Strumwasser, 1958). In addition, tactile stimulation using a shock in hibernating golden hamsters was found to induce evoked response potentials down to a cortical temperature of 9.1^{o}C, although the latency to response increased as temperature decreased (Chatfield et al., 1951). Conversely, it has been suggested that one of the wider functions of torpor may be to aid in the survival from predation and environmental threats by ensuring animals are hidden in shelters which would suggest an evolutionary advantage for not perceiving environmental stimuli during torpor (Nowack et al., 2017).

There have also been several studies that have recorded auditory brainstem responses (ABR) during torpor. Hamill and colleagues observed that ABRs were present in hibernating ground squirrels but only as they began to rewarm to a body temperature of >10^{o}C. They also report a temperature dependent effect on ABR peaks and latencies which increased and decreased respectively as body temperature increased (Hamill et al., 1989). In addition, a study in torpid deer mice, a species which reduce their body temperature to levels more comparable with laboratory mice but not in response to fasting, found that ABRs were not reproducibly present at body temperatures <19^{o}C. Moreover, wave amplitude increased and latency decreased as temperature increased during arousal from torpor (Katbamna et al., 1996). It should be noted that measurement of ABR demonstrates that the sensory pathways are intact and that auditory tones are being detected and transmitted. However, the ABR does not give an indication of a higher level cortical response and does not determine whether cortical evoked responses are present.

Overall, there is limited existing work on sensory perception in torpid species, especially in higher level cortical regions. Moreover, there does not appear to be any published work investigating sensory perception during fasting-induced torpor in laboratory mice. As such, the focus of this chapter was to investigate whether evoked potentials occur in response to sensory stimuli, and to compare the evoked potential characteristics to those elicited during ad libitum feeding and at euthermic temperatures during food restriction. Moreover, due to the similarities between sleep and torpor, evoked responses were investigated with regard to the vigilance state during which they occurred. This will build on the work presented in Chapters 5 and 6 by further investigating the similarities and differences between sleep and torpor, in addition to providing insight into the neurophysiology during fasting-induced torpor.

Auditory stimulation was chosen as the preferred method of sensory stimulation due to its non-invasive nature, therefore allowing for electrophysiological responses to be recorded with minimal disturbance to the animal. Secondly, the frequency and intensity of each acoustic stimulation can be easily controlled, enabling standardisation between animals. Finally, hearing is one of the primary senses used by rodents to respond to their environment, making auditory responses ethologically relevant. If auditory stimulation were found to induce arousal from torpor in mice, it may also provide an easy, non-invasive method for provoking arousal from torpor prior to an experimental manipulation.

7.1.3 Experimental aims

The experimental aims of this chapter are to:

i) Investigate whether auditory stimulation induces evoked potentials in torpid mice

ii) Compare evoked responses across vigilance states during ad libitum feeding and during food restricted hypothermia and euthermia

iii) Investigate how presentation of auditory stimuli affects torpor characteristics

7.2 Methods

7.2.1 Experimental animals

Adult male C57BL/6J mice (Charles River Laboratories, UK; n=8; aged 9 weeks) were implanted with EEG and EMG electrodes as described in Section 2.6. To reduce the number of animals used across experiments, the data presented in this chapter were gathered from the same cohort of animals presented in Chapters 5 and 6.

Following a one-week recovery period, mice were singly housed in custom made Plexiglass cages, with two cages placed in a single Faraday chamber (Campden Instruments, Loughborough, UK). The C57BL/6J strain is associated with genetic age-related hearing loss due to mutation in the Cdh23 locus (Noben-Trauth et al., 1997). Progressive hearing loss begins from ~2 months old but is most apparent during late adulthood, with loss of sensitivity to high frequency tones being most pronounced (Ison & Allen, 2003). Despite this progressive hearing loss, the sound level pressure used in this study (80 dB), and the frequencies used for stimulation (5-16 kHz), is such that mice are able to hear the tones until reaching 12-18 months in age (Youn et al., 2020).

To investigate responses to external stimuli during torpor, food restriction (as described previously in Section 2.2) was used to induce torpor following completion of an auditory stimulation recording session during ad libitum fed conditions. Mice were continuously monitored using thermal imaging to detect torpor bouts; auditory stimulation was performed following >1 week of food restriction when all mice were reliably entering torpor daily.

7.2.2 Surgical procedures

All animals (n=8) were implanted with EEG (frontal, occipital, and cerebellum) and EMG electrodes as described in detail in Section 2.6 (Figure 39A). Electrophysiological data acquisition, processing, sleep scoring, and analysis were performed as described in Section 2.7. As torpor is not defined using EEG data, standard sleep scoring criteria were applied when scoring food restricted days. This includes the periodic EMG bursts and shivering lasting ≥16s that occurs during torpor bouts being scored as brief awakenings, despite it being unlikely that the mouse was truly awake.

7.2.3 Auditory stimulation paradigm

Open field auditory stimulation was performed to investigate brain responsiveness across vigilance states and during torpor. To this end, pure tones were played through magnetic speakers (MF1 Multi-Field Magnetic Speakers, Tucker-Davis Technologies) mounted on the chamber ceilings. Tones lasted 1 s and were played every 20 s over 24 hrs, resulting in ~4320 tones being played in total (Figure 39B), to ensure that a sufficient number of tones occurred during each vigilance state (wake, NREM sleep, and REM sleep during ad libitum, euthermic, and hypothermic conditions) for comparison. To reduce habituation to the tones, eight different frequencies were used (1000 – 11301 Hz) that ascended in a logarithmic scale across the optimal auditory range for C57BL/6J mice (Ison & Allen, 2003); tones were presented in a pseudorandom order which was generated by the Synapse recording software. Approximately equal numbers of each frequency were presented across the 24 hour recording. Previous work in our lab had found that a sound intensity of 80 dB is optimal for open field auditory stimulation. As such a sound level meter was used to calibrate sound intensity to this level.

7.2.4 Analysis of auditory evoked potentials

Auditory evoked responses were analysed using filtered and scored EEG data (see Section 2.7.2) which was extracted in the window from 0.2 s before stimulation to 0.4 s after stimulation. Manual scoring of EEG epochs was used to determine the vigilance state in which each auditory stimulation occurred. During food restriction, temperature data were also used to determine whether the mouse was hypothermic or euthermic, resulting in the conditions of: (i) ad libitum (AL) wake, NREM sleep, and REM sleep; (ii) food restricted (FR) euthermic wake, NREM sleep, and REM sleep; and (iii) FR hypothermic wake, NREM sleep, and REM sleep. As REM sleep is virtually absent during hypothermia, some mice did not have any stimulations occurring during hypothermic REM, reducing the number of animals for analysis during this condition to n=4. Once the condition for each ERP had been determined, all ERPs occurring during that condition were averaged for each individual mouse (see Figure 40 for representative responses). Analysis of ERP components were conducted by calculating the maximal and minimum peak in the 0.2s following stimulus onset to determine amplitude and latency to peak or trough. The area of each component was determined by integrating the area between the curve and a threshold, determined by calculating the average voltage in the 0.2 s preceding stimulus onset (Figure 39C).

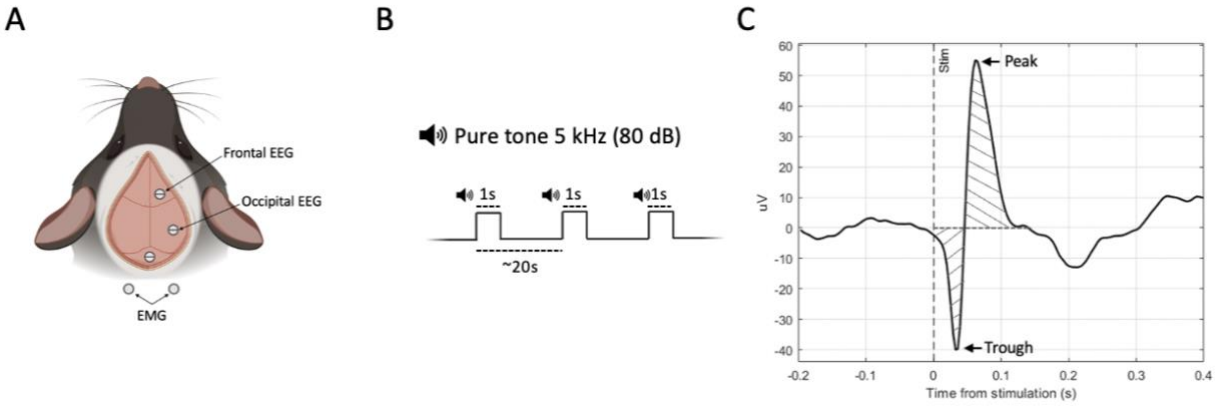

Figure 39: Experimental paradigm. (A) Adult C57BL6/J mice (n=8) were implanted with EEG (frontal and occipital) and EMG electrodes in the configuration shown. EEG electrodes were referenced to an additional electrode implanted in the cerebellum. (B) Implanted mice were exposed to approximately equal numbers of eight pure auditory tones (1000, 1414, 1999, 2827, 3997, 5652, 7992, 11301 Hz; 80 dB, duration 1 s), ~20 s apart. Tones were played across an entire 24 h recording during both ad libitum and food restricted conditions. (C) Evoked response potential (ERPs) characteristics following auditory stimulation were determined using the EEG signal. The maximum and minimum point of the ERP positive and negative deflections were calculated for analysis, as was the area.

7.2.5 Statistical analysis

Data were processed using MATLAB (The Math Works Inc., USA), and analysed using MATLAB and Prism (GraphPad, v9, USA). All data were tested for normality prior to analysis using a Shapiro-Wilk test. Comparisons of sleep-wake architecture between stimulation and non-stimulation days, and comparisons between temperature and torpor characteristics were compared using paired two-tailed t-tests, or a non-parametric equivalent where appropriate (Wilcoxon matched-pairs signed rank test). ERP characteristics were analysed using one-way ANOVAs with repeated measures, or a non-parametric Friedman test, with a post-hoc multiple comparisons test. Sphericity was not assumed whilst performing ANOVAs, therefore, a Geisser-Greenhouse correction was applied. Differences were determined to be statistically significant when $p<0.05$. All data are presented as mean values ± SEM unless otherwise stated. Data analysis was only performed on 7 of the animals, due to the death of an animal during the recording period.

7.3 Results

7.3.1 Sleep-wake architecture

Auditory stimulation resulted in ERPs in all mice across all frequencies and conditions; representative examples of ERPs during each condition are shown in Figure 40. Much fewer stimulations occurred during REM sleep compared to other vigilance states due to REM sleep episodes lasting between 1-2 minutes on average, and much fewer REM episodes occurring during the sleep period (Figure 41 D-F, Figure 42). This was especially prevalent during hypothermia, during which REM sleep is almost completely abolished. As a result, comparisons have been performed between states containing an unequal number of stimulations, which also varies between animals.

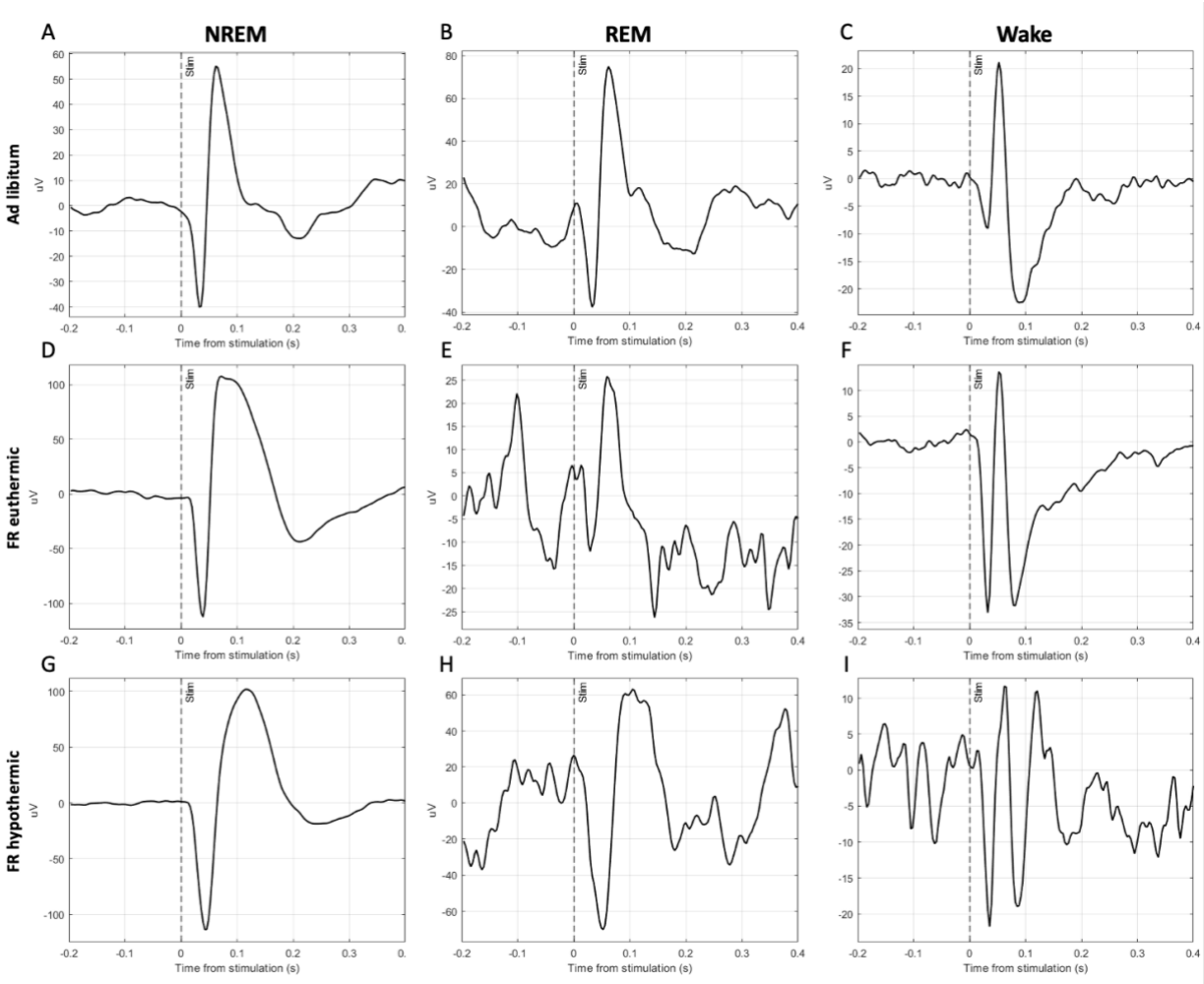

Figure 40: Representative examples of averaged evoked response potentials (ERPs) for each condition during each vigilance state. Examples are from one mouse. NREM: non-rapid eye movement; REM: rapid eye movement; FR: food restriction.

First, the effect of exposure to auditory tones on sleep-wake architecture was investigated. To this end, the total time spent in each vigilance state during auditory stimulation was compared to a non-stimulation day. As previously shown in Chapter 5, food restriction significantly altered sleep-wake architecture, therefore comparisons were only performed between stimulation versus non-stimulation days in the same feeding condition. The time course of vigilance states followed the same temporal patterns on stimulation versus non-stimulation days during each feeding condition (Figure 41 A-C). Quantification of the amount of time spent in each state per hour found no differences in NREM or wake on stimulation versus non-stimulation days (NREM AL stim v AL non-stim: $t(6)=1.16$, $p=0.290$; NREM FR stim v non-

stim: t(6)=1.47, p=0.1913; Wake AL stim v AL non-stim: t(6)=0.946, p=0.381; Wake FR stim v non-stim: t(6)=2.27, p=0.064). However, mice spent significantly less time in REM sleep on the stimulation day compared to the non-stimulation day during ad libitum feeding (t(6)=5.70, p=0.0013); REM sleep was marginally reduced during food restriction stimulation compared to the non-stimulation day during food restriction but not significantly so (FR non-stim: 1.40 ± 0.171 mins/h; FR stim: 1.05 ± 0.133 mins/h; t(6)=1.77, p=0.127). Comparison of number of episodes, episode duration, longest episode, and latency to first episode revealed no differences between stimulation versus non stimulation days, during both feeding conditions, for any of the vigilance states (Figure 42).

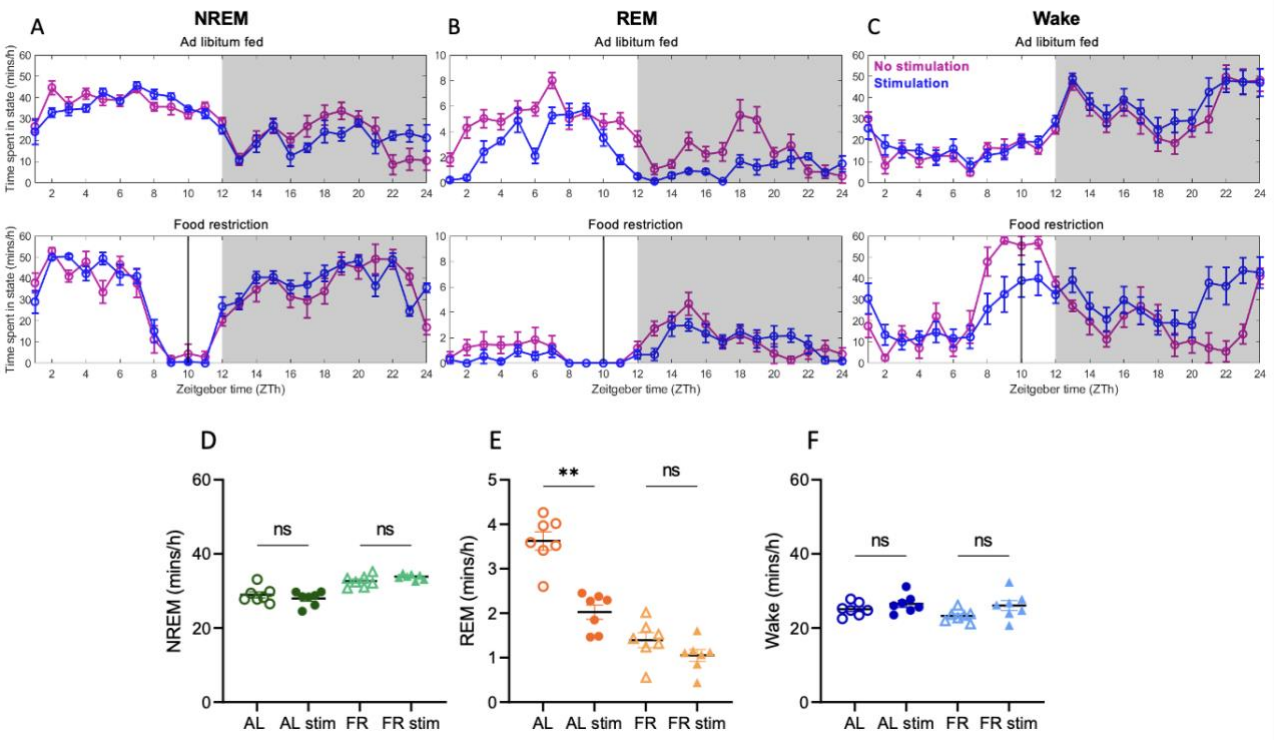

Figure 41: Global sleep-wake architecture during auditory stimulation. (A-C) Time course of time spent in NREM, REM, and wake over a 24h recording session on a stimulation day and a non-stimulation day during ad libitum feeding (top), and food restriction (bottom). Feeding time during food restriction is indicated by the black vertical line. (D-F) Comparison of time spent in each vigilance state on a stimulation vs non-stimulation day during food restricted and ad libitum conditions. **p<0.01, paired t-tests. NREM: non-rapid eye movement sleep; REM: rapid eye movement sleep; AL: ad libitum fed; FR: food restriction.

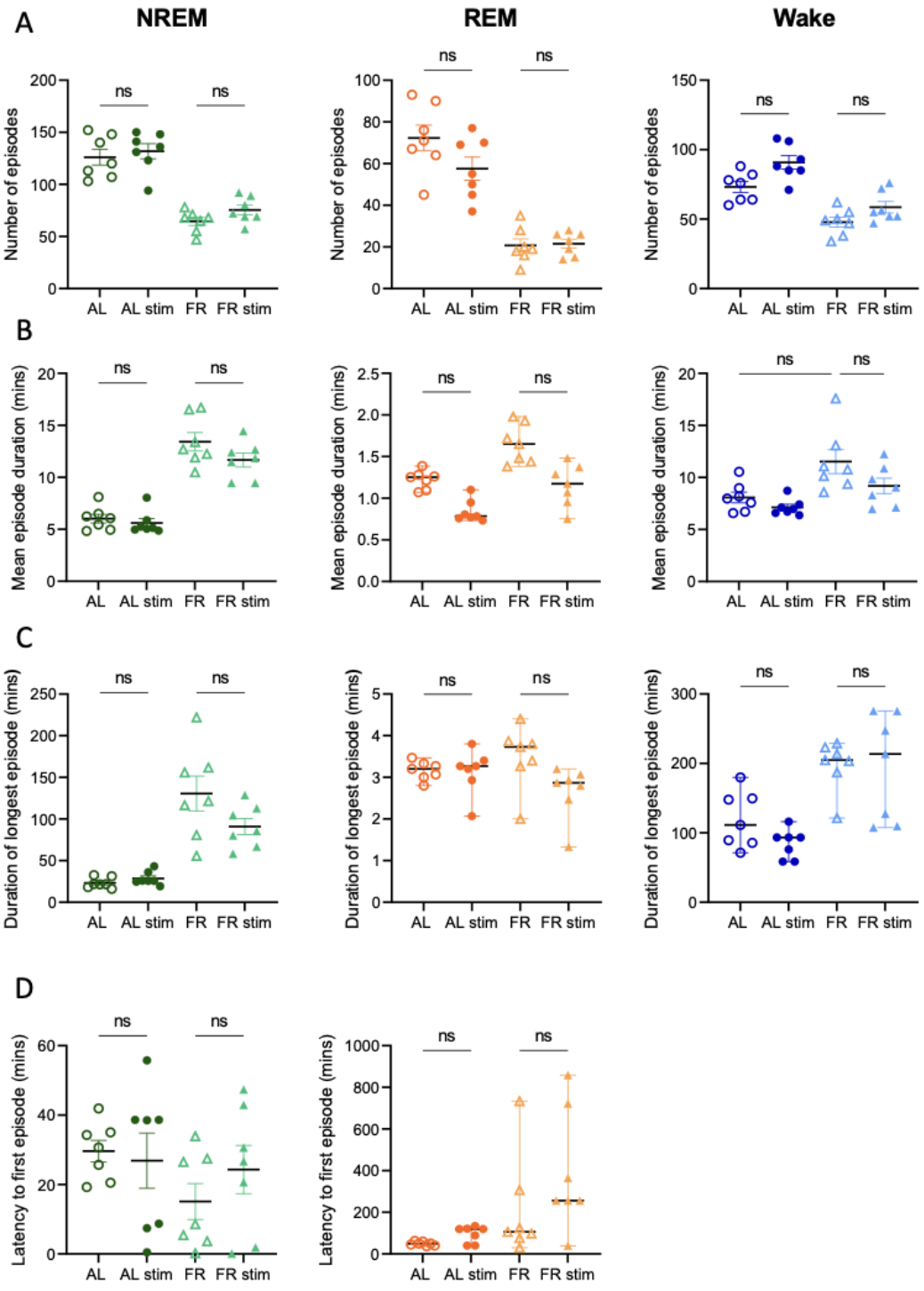

Figure 42: Auditory stimulation does not alter characteristics of sleep-wake architecture. Comparison of the number of episodes (A), episode duration (B), maximum duration (C), and latency to first episode (D) for each vigilance state on a stimulation vs non-stimulation day during ad libitum and food restricted conditions. ns: non-significant ($p>0.05$), paired t-tests. NREM: non-rapid eye movement sleep; REM: rapid eye movement sleep; AL: ad libitum fed; FR: food restriction.

7.3.2 Torpor characteristics

One of the aims of this chapter was to determine whether auditory stimulation would alter torpor characteristics. If, for example, auditory stimulation prevented or blunted torpor then it could be used as a non-invasive method for ensuring mice were not torpid in the hours preceding an experimental manipulation or a behavioural testing session.

Analysis of Tskin during the food restricted stimulation and non-stimulation days revealed a significant main effect of time and a significant interaction between time*condition, although no main effect of condition was found (Figure 43A; main effect of time: $F_{(23,138)}$=12.5, p<0.0001; main effect of condition: $F_{(1,6)}$=0.6085, p=0.4650; time*condition: $F_{(23,138)}$=2.93, p<0.0001; 2-way ANOVA with repeated measures). In comparison, no effect of auditory stimulation on Tskin was found when comparing to a non-stimulation day (Figure 43B; main effect of time: $F_{(23,115)}$=1.506, p=0.0823; main effect of condition: $F_{(1,5)}$=0.010, p=0.9239; time*condition: $F_{(23,115)}$=1.083, p=0.3749; 2-way ANOVA with repeated measures).

Next, Tskin data were used to investigate whether auditory stimulation altered torpor characteristics when compared to a non-stimulation day. No effect of auditory stimulation was found on the average length of torpor bouts (t(6)=0.233, p=0.824), the longest torpor bout (t(6)=1.00, p=0.356), the number of torpor bouts (Z=3.00, p=0.750), the latency to torpor (Z=19.0, p=0.125), or the depth of torpor (t(6)=1.52, p=0.180; calculated as the area below the torpor threshold for that individual) (Figure 43 C-G). This indicates that auditory stimulation is not sufficient to prevent or blunt torpor bouts which may be due to the paradigm being designed to allow for stimulation during sleep, so as not to induce a startle response. In addition, the drive for energy conservation during food restriction may outweigh any drive to remain

euthermic in the presence of tones. Stimulation with a more ethologically relevant stimulus, e.g., predator odour, may result in a different response but would be a more stressful approach.

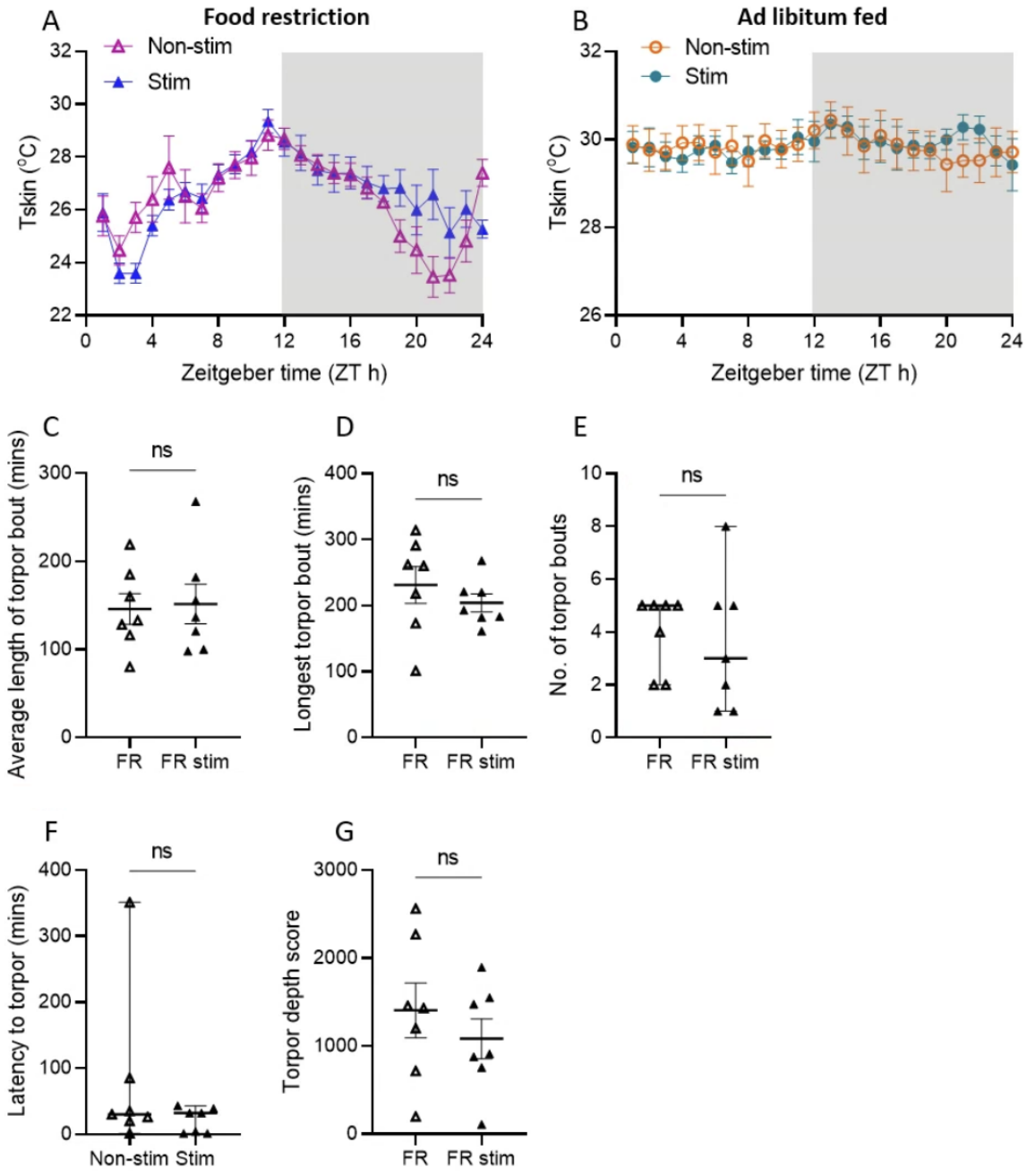

Figure 43: Torpor characteristics in response to auditory stimulation. (A) Time course of hourly peripheral body temperature measurements (Tskin) over 24h on a stimulation vs non-stimulation day during food restriction. Dotted line indicates when food was provided. (B) Comparison of Tskin on stimulation and non-stimulation days during ad libitum and food restricted conditions. Comparison of torpor bout length (C), longest torpor bout (D), total number of torpor bouts (E), latency to first torpor bouts (F), and the depth of torpor bouts (G) over a 24h recording on a day with auditory stimulation vs a day without. ns: non-significant, $p>0.05$, paired t-tests. AL: ad libitum; FR: food restriction.

7.3.3 ERP characteristics in response to auditory tone frequency

Multiple auditory tone frequencies were used during this study to reduce habituation to auditory stimulation, as habituation can alter ERP characteristics (Scourse & Hinde, 1973). Although not a focus of the study, it was important to determine whether the auditory frequency itself influenced ERP characteristics to aid with interpretation of the data. As such, ERP characteristics were compared between each of the auditory frequencies by calculating the maximal and minimal peak of the positive and negative deflections in the 0.2s following stimulation.

During ad libitum auditory stimulation, the amplitude of both the ERP positive and negative peaks did not differ between auditory tone frequencies, although a significant main effect of individual mouse was found for the positive peaks (Figure 44 A&C; negative peak: Friedman statistic=12.1, p=0.0975; Friedman test; positive peak: main effect of tone frequency: $F_{(4.15,24.9)}$=1.06, p=0.396; main effect of individual: $F_{(6,42)}$=63.1, p<0.0001; RM one-way ANOVA). Interestingly, a significant effect of auditory tone stimulation was found on the positive and negative ERP peak amplitudes during food restricted conditions (Figure 44 B&D; negative peak: Friedman statistic=42.2, p<0.0001; Friedman test; positive peak: main effect of tone frequency: $F_{(3,15,18.9)}$=7.76, p=0.0013; main effect of individual mouse: $F_{(6,42)}$=54.2, p<0.0001; RM one-way ANOVA). However, a post-hoc Tukey's multiple comparisons test for the food restricted positive peak reported only one significant difference which was between auditory tone frequencies 1414 Hz and 5652 Hz (p=0.0322).

Next, the area of the ERP positive and negative peaks relative to the mean EEG voltage in the 0.2s preceding the auditory tone were calculated for ad libitum fed and food restricted conditions (Figure 39C). No differences were found in the area of the positive and negative

ERP peaks in response to different auditory tone frequencies during ad libitum feeding conditions (Figure 45 A&C; Positive peak: main effect of treatment: $F_{(2.29,13.7)}$=1.11, p=0.3655; main effect of individual mouse: $F_{(6,42)}$=63.1, p<0.0001, RM one-way ANOVA; Negative peak: Friedman statistic=12.8, p=0.0781, Friedman test). Conversely, a significant difference in the areas of the ERP positive and negative peak were found during food restricted conditions (Figure 45 B&D; Positive peak: main effect of treatment: $F_{(3.06,18.3)}$=6.55, p=0.0033; main effect of individual mouse: $F_{(6,42)}$=54.8, p<0.0001, RM one-way ANOVA; Negative peak: Friedman statistic=34.4, p<0.0001, Friedman test). However, post-hoc analysis for the food restricted condition reported very few significant differences between tone frequencies.

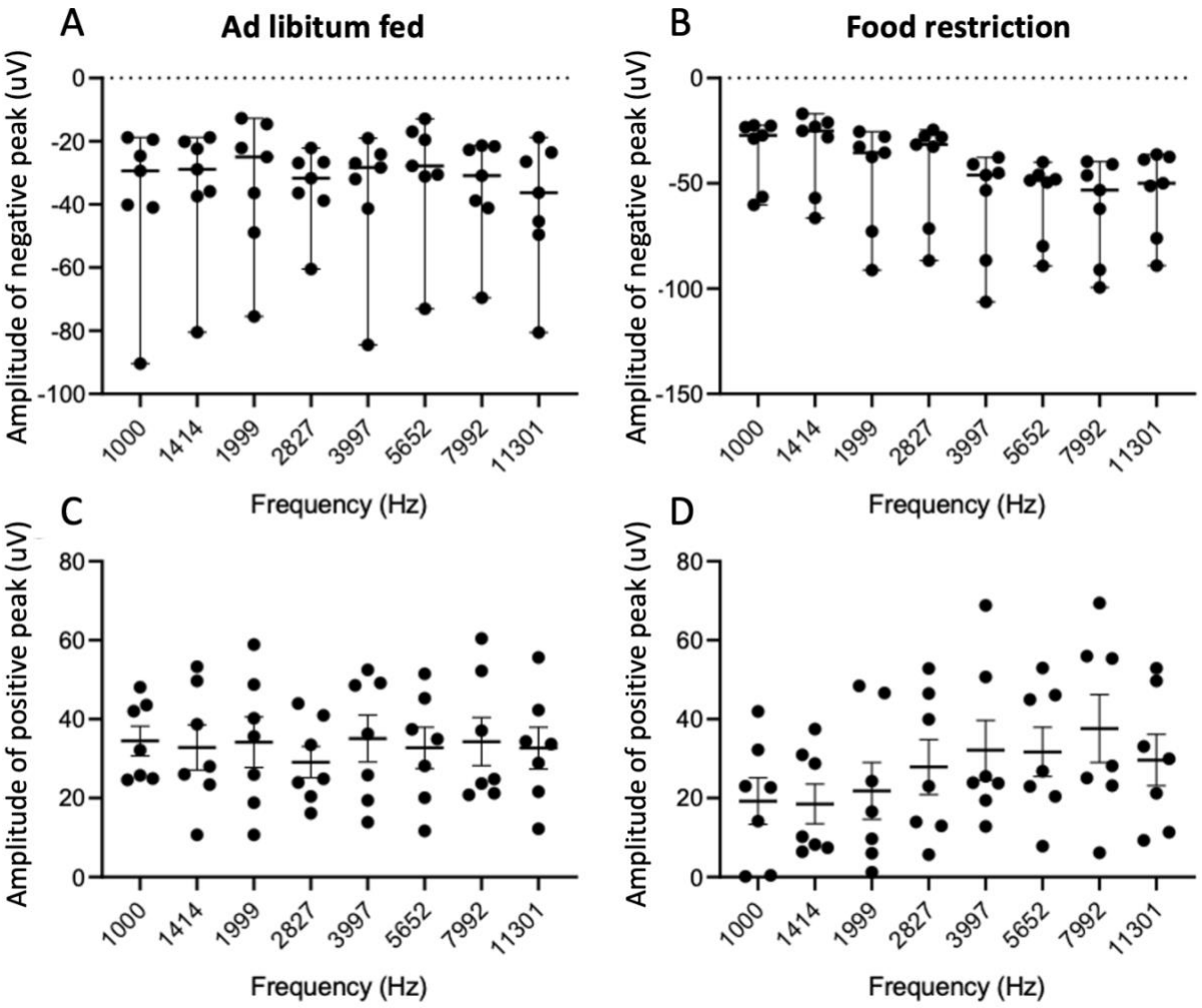

Figure 44: Amplitude of auditory evoked responses across a range of auditory tone frequencies. Comparison of the minimum point of the negative peak of the auditory evoked response in the 0.2 s following stimulation between different frequencies during ad libitum fed conditions (A) and during food restriction (B). Comparison of the maximal point of the positive peak of auditory evoked response between different frequencies during ad libitum feeding (C) and during food restriction (D). One-way ANOVA with repeated measures (C-D) or Friedman test with repeated measures (A-B). Data in A and B are presented as median values with 95% confidence intervals. Data is C and D are presented as mean values ± SEM.

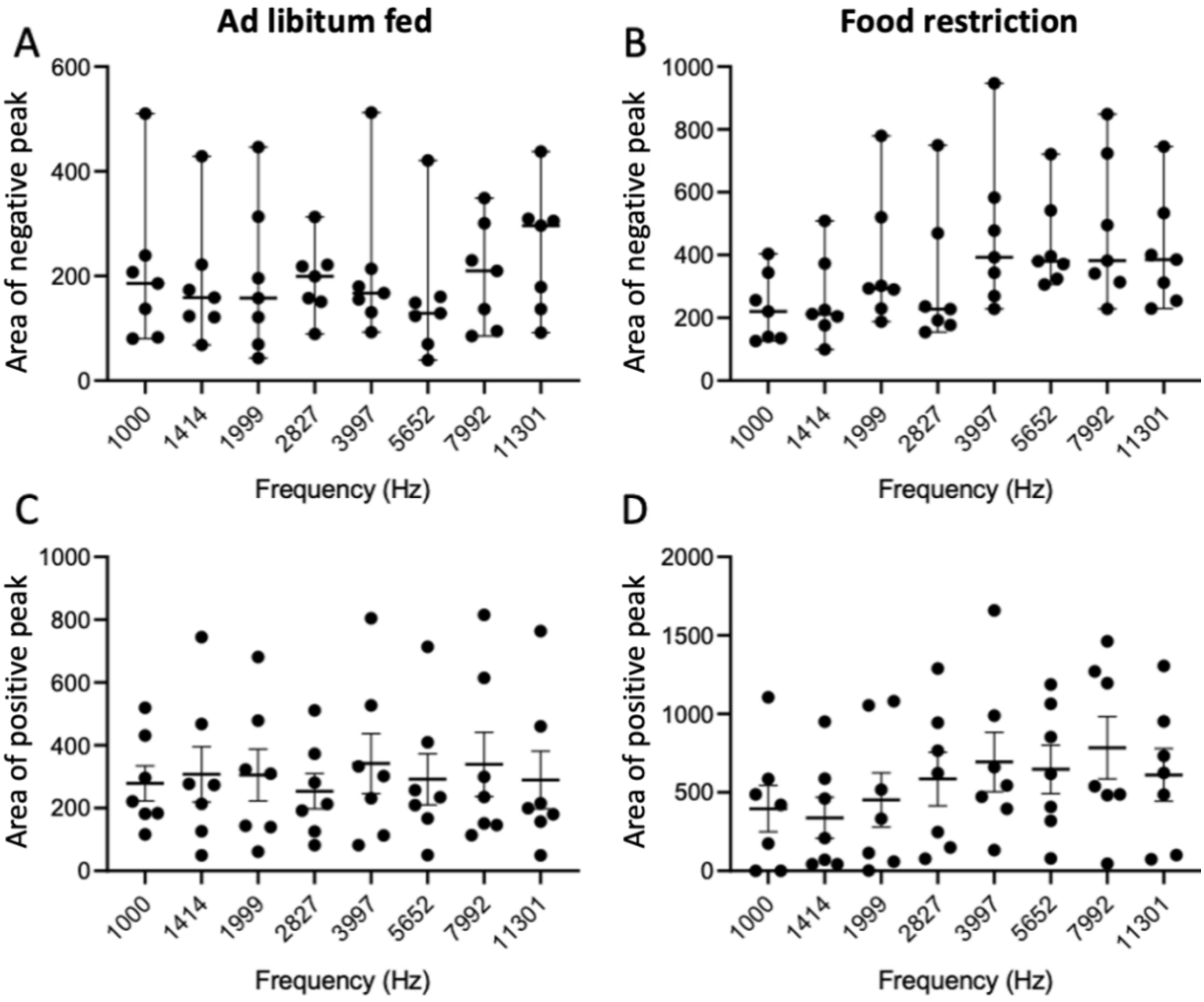

Figure 45: Comparison of the area of auditory evoked potentials in response to different frequencies. Area of negative peak during ad libitum (A) and food restricted conditions (B). Area of positive peak during ad libitum (C) and food restricted conditions (D). One-way ANOVA with repeated measures (C-D) or Friedman test with repeated measures (A-B). Data in A and B are presented as median values with 95% confidence intervals. Data is C and D are presented as mean values ± SEM.

7.3.4 ERP characteristics during each vigilance state

Although some differences in ERP characteristics were found in response to different frequencies, ERPs were not stratified by both frequency and vigilance state. This is because some vigilance states (e.g., hypothermic REM) had so few stimulations occurring during the state that further classification would reduce the already small sample size. Moreover, the primary purpose of using multiple frequencies was to reduce habituation to auditory stimulation, rather than as an additional research question.

No main effect of condition (ad libitum, food restricted euthermic, and food restricted hypothermic) was found for the amplitude of the positive peak during NREM sleep and during REM sleep (NREM sleep: $F_{(1.65,9.87)}$=0.3505, p=0.6728; REM sleep: $F_{(1.13,3.40)}$=4.45, p=0.1146; RM one-way ANOVAs), whereas a significant main effect of condition was observed during wake ($F_{(1.37,8.19)}$=6.685, p=0.0256; RM one-way ANOVA). However, post-hoc analysis for wake found no difference between each condition (Figure 46 A-C). Analysis of the amplitude of the negative peak revealed a significant effect of condition during NREM sleep and during wake, although no difference was observed during REM sleep (Figure 46 D-F; NREM: Friedman statistic=10.3, p=0.0036; Wake: Friedman statistic=7.14, p=0.0272; REM: $F_{(1.01,3.04)}$= 2.37, p=0.2205, RM one-way ANOVA). Post-hoc analysis using Dunn's multiple comparisons tests found a significant difference between ad libitum NREM sleep and hypothermic NREM sleep (p=0.004) and between euthermic wake and hypothermic wake (p=0.0226).

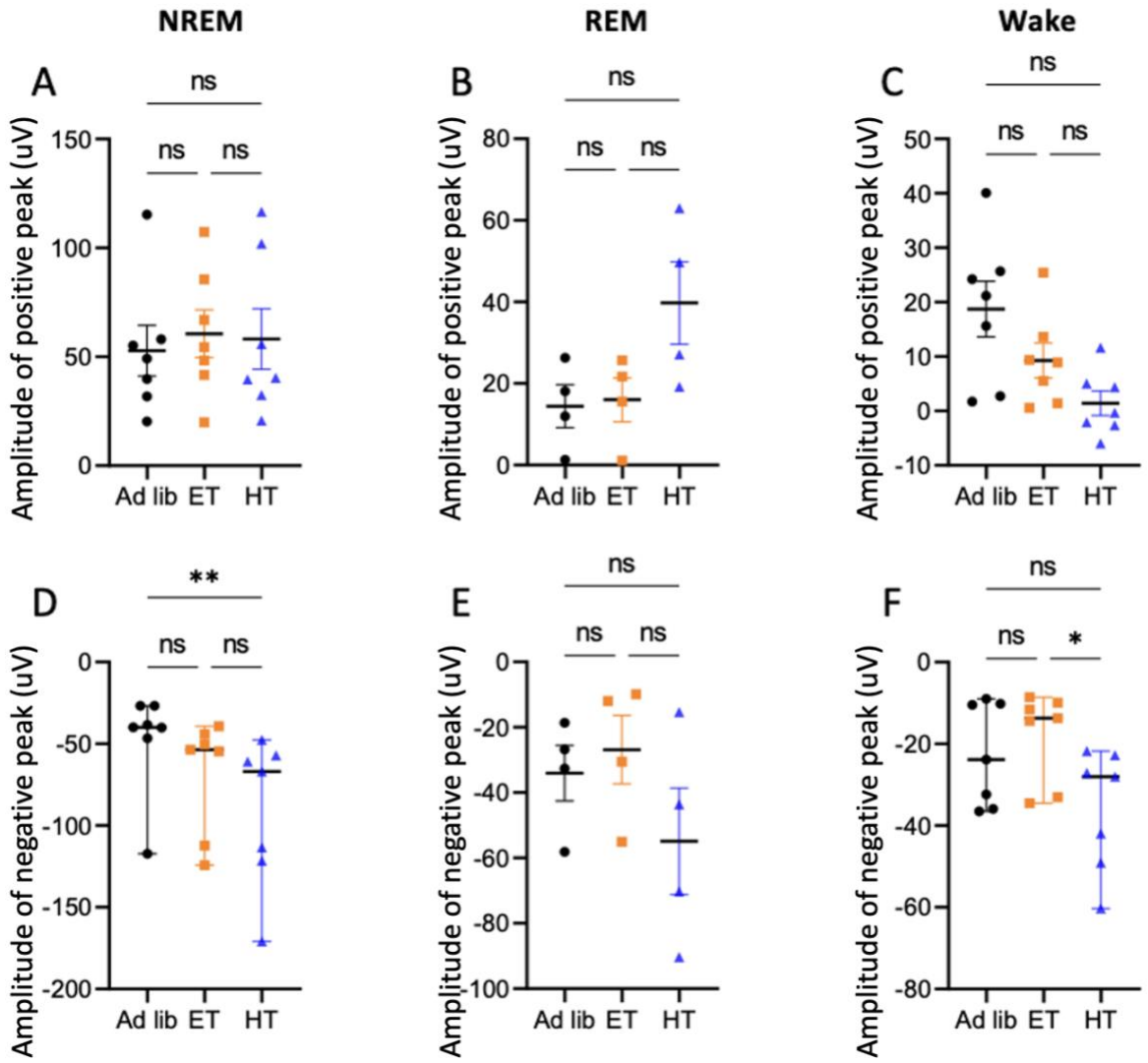

Figure 46: Auditory evoked potentials during different vigilance states. Comparison of the amplitude of the peak (A-C) and trough (D-F) of ERPs evoked by auditory stimulation during NREM sleep, REM sleep, and wake during ad libitum, food restricted euthermic, and food restricted hypothermic conditions. One-way ANOVAs with repeated measures or Friedman test (D, F). ns: non-significant ($p>0.05$), *$p<0.05$, **$p<0.01$. NREM: non-rapid eye movement; REM: rapid eye movement; ET: euthermic; HT: hypothermic.

Following comparison of ERP amplitudes, the latency to the positive and negative ERP peaks were compared between conditions for each vigilance state (Figure 47). This was achieved by calculating the time to reach the maximal or minimal points of the positive and negative peaks, respectively, in the 0.2s following the onset of the auditory tone. A significant effect of condition was observed for the latency to both the positive and negative peaks during NREM sleep (Positive: Friedman statistic=13.6, $p<0.0001$; Negative: Friedman statistic=14.0, $p<0.0001$; Friedman tests). Post-hoc analysis revealed that the latency to both peaks were significantly prolonged during food restricted hypothermic NREM sleep compared to ad libitum NREM sleep ($p=0.0005$; Dunn's multiple comparisons test). A significant main effect of condition was also found for the latency to the negative peak during wake but not for the positive peak (Positive: Friedman statistic=2.15, $p=0.3836$; Negative: Friedman statistic=6.00, $p=0.0453$; Friedman tests). Despite a significant main effect being found, post-hoc analysis did not find any differences in the negative peak latency between conditions during wake. Finally, investigation of latency to ERP peaks during REM sleep found no effect of condition for both positive and negative peaks (Positive: $F_{(1.16,3.49)}=0.8023$, $p=0.4484$, RM one-way ANOVA; Negative: Friedman statistic=0.8148, $p=0.6154$).

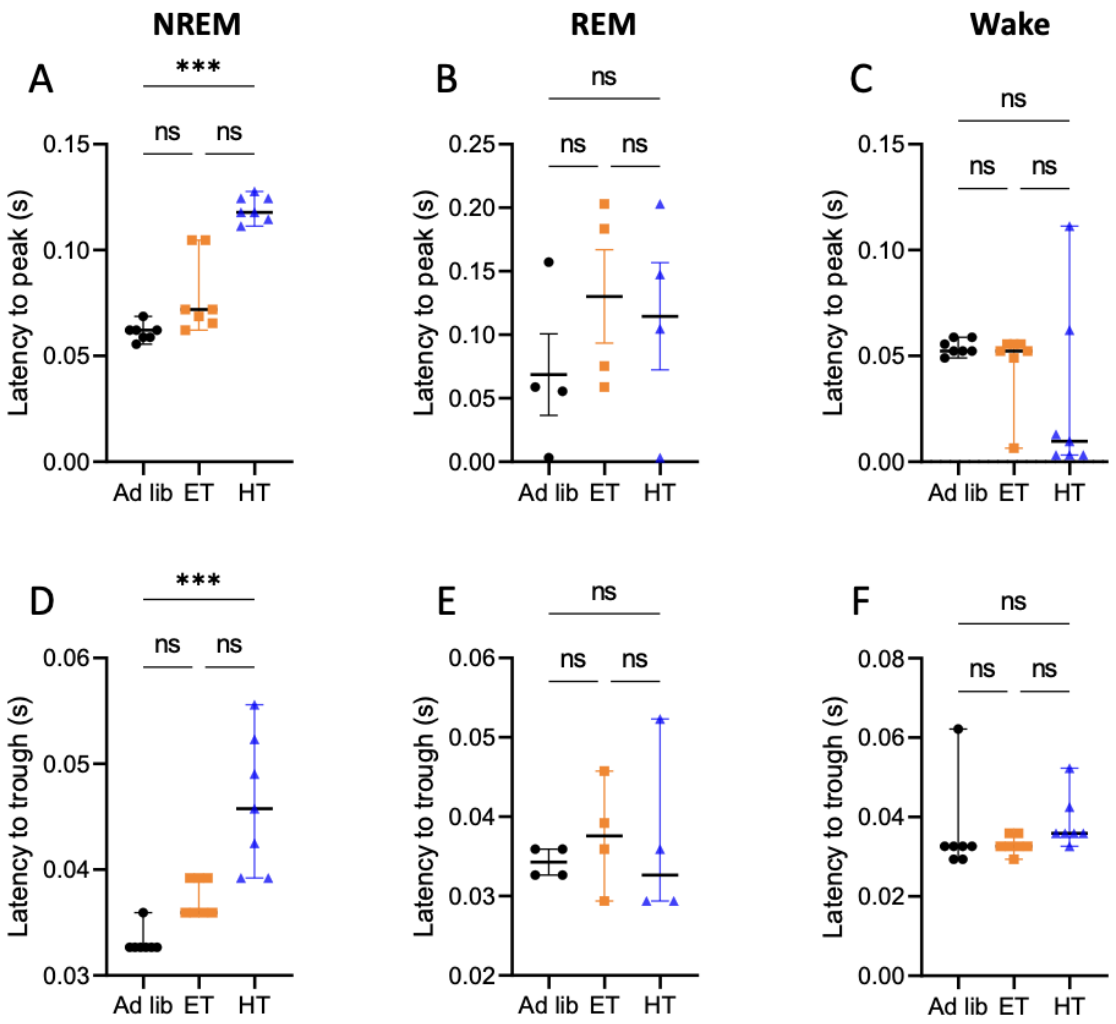

Figure 47: Latency to auditory evoked potential components across vigilance states. Latency to the positive (A-C) and negative (D-F) peak in the 0.2 s following auditory stimulation during NREM sleep, REM sleep, and wake in ad libitum, food restricted euthermic, and food restricted hypothermic conditions. One-way ANOVA with repeated measures (A-B) or Friedman test. ns: non-significant ($p>0.05$), ***$p<0.001$. NREM: non-rapid eye movement; REM: rapid eye movement: ET: euthermic; HT: hypothermic.

Lastly, the area of the positive and negative peaks was compared between conditions for each vigilance state (Figure 48). To this end, the area of each peak was calculated by integrating the area beneath the curve relative to the mean EEG voltage in the 0.2s preceding the onset of the auditory tone (Figure 39C). A significant main effect of condition was found for the area of the negative peak for all vigilance states (NREM: Friedman statistic=12.3, p=0.0003, Friedman test; REM: $F_{(1.28,3.84)}$=8.93, p=0.0402; Wake: $F_{(1.18,7.06)}$=9.270, p=0.0163; RM one-way ANOVAs). Differences were found to be between ad libitum NREM vs food restricted hypothermic NREM (p=0.0015, Dunn's) and between food restricted euthermic wake and food restricted hypothermic wake (p=0.0320, Tukey's). No effect of condition was found on the area of the positive peak for any vigilance state (NREM: Friedman statistic=5.43, p=0.0854; REM: Friedman statistic=0.00, p>0.9999; Wake: Friedman statistic=2.57, p=0.3046; Friedman tests).

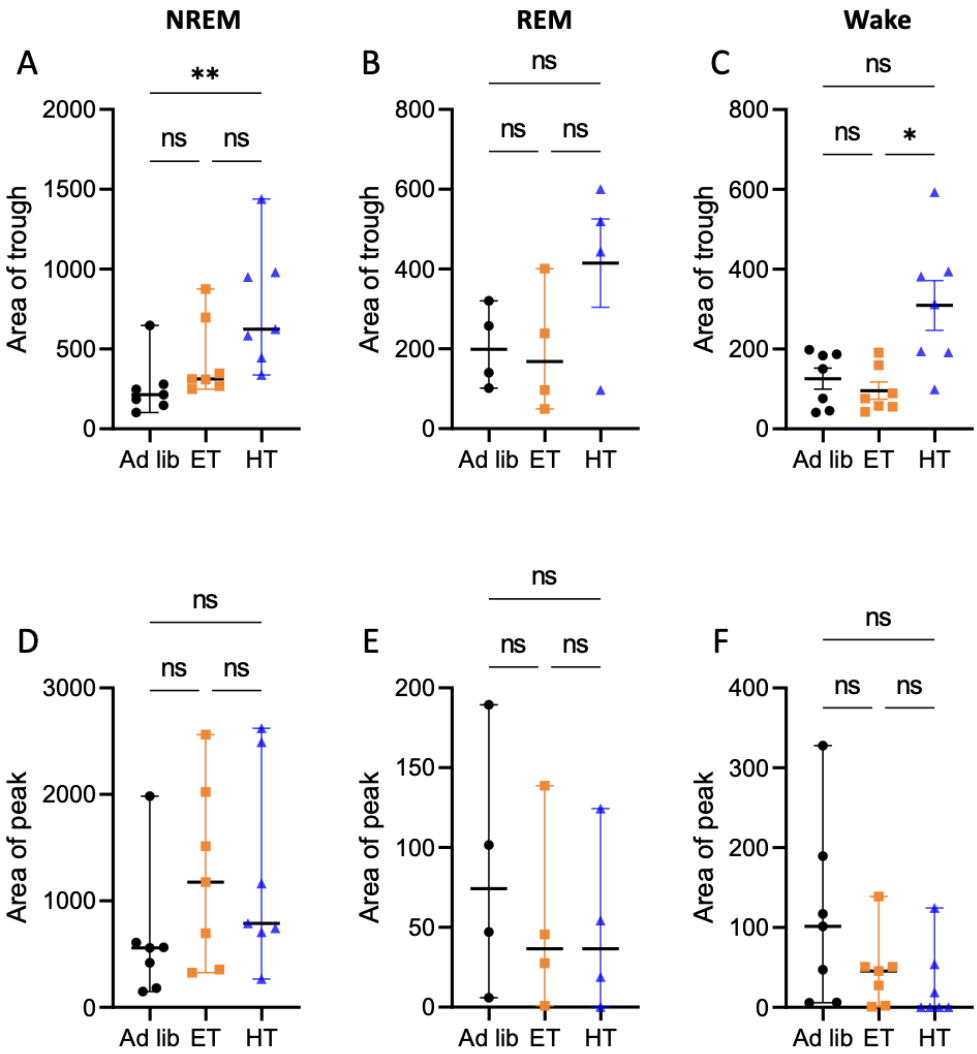

Figure 48: Area of auditory evoked potentials across vigilance states. Area of the positive (A-C) and negative (D-F) peak in the 0.2 s following auditory stimulation during NREM sleep, REM sleep, and wake in ad libitum, food restricted euthermic, and food restricted hypothermic conditions. One-way ANOVA with repeated measures (B-C) or Friedman test. ns: non-significant ($p>0.05$), **$p<0.01$. NREM: non-rapid eye movement; REM: rapid eye movement: ET: euthermic; HT: hypothermic.

7.4 Discussion

This chapter demonstrates that cortical evoked response potentials (ERPs) are elicited following presentation of auditory stimuli during hypothermia. Visualisation of ERPs during torpor suggested an elongation of the response, potentially as a consequence of synaptic dysconnectivity and a temperature-dependent reduction in neuronal firing (Chanaday & Kavalali, 2018; Krilowicz et al., 1989; Van Hook, 2020). Quantification of ERP latency and area support this observation with latencies to both the positive and negative peaks being significantly prolonged, and the negative peak area being increased during hypothermic NREM. As torpor is dominated by brain activity resembling NREM sleep, the responses during this state were considered to be the most representative of torpor electrophysiology, especially as stimulations occurring during hypothermic REM sleep and wake were limited in number. Interestingly, ERP latency and area were only significantly different between hypothermia and ad libitum, but not food restricted euthermic conditions. It is possible that the energetic deficit induced by food restriction may alter neuronal activity. For example, a recent study found that neurons from food restricted mice altered their conductance and input resistance in order to reduce ATP usage and conserve energy (Padamsey et al., 2021).

Amplitude of ERP positive and negative peaks were largely preserved across conditions in each vigilance state, with the exception of ERP negative peaks during hypothermic NREM compared to ad libitum NREM, and hypothermic wake compared to euthermic wake. Padamsey et al reported that neuronal excitability was preserved in food restricted mice which may explain why ERP amplitudes were largely unaltered by conditions (Padamsey et al., 2021). However, the authors of this study did not take body temperature measurements and so it is unclear whether the food restricted mice used to collect data were euthermic or hypothermic.

As such, hypothermia may alter neuronal dynamics due to a temperature-dependent slowing of cellular processes leading to the blunting of the depolarisation observed here.

Previous experiments in cats, rats, and humans have reported that exposure to auditory stimulation increased the proportion of time spent in REM sleep at the expense of NREM sleep (Amici et al., 2000; Drucker-Colin et al., 1983; Mouze-Amady et al., 1986). Here, in contrast, auditory stimulation was found to decrease the proportion of time spent in REM sleep per hour during the ad libitum condition. Interestingly, the number of REM episodes and the duration of REM episodes did not significantly differ between stimulation and non-stimulation days, although an overall reduction was observed for both the total number and duration of episodes. The reduction in REM sleep may be a species-dependent effect as Wang et al found that ultrasonic stimulation in mice also decreased the amount of REM sleep (Wang et al., 2022). No difference was found during food restriction although this may be explained by the overall reduction in REM sleep observed during food restriction as body temperature decreases (Deboer & Tobler, 1995).

Another aim of this chapter was to investigate whether auditory stimulation would modulate torpor characteristics or induce arousal from torpor. No differences were found in mean Tskin, or any torpor characteristics between the stimulation and non-stimulation days. It is possible that a greater sound intensity may have provoked emergence from torpor, as the sound pressure level used (80 dB) was chosen to minimise sleep disruption to allow for measurements to be taken across vigilance states. In addition, all stimuli were pure tones which are less disruptive than complex sounds, such as clicks or white noise, which may also have contributed to the lack of torpor modulation. For example, acoustic stimulation by knocking on the animal's cage

had been used to promote arousal from torpor in mice (Oelkrug et al., 2011), which is an example of a louder, complex auditory stimulus.

7.4.1 Conclusions

The presence of ERPs during hypothermia with largely preserved characteristics provides the first evidence for higher level cortical activation in response to sensory stimulus during torpor. Previous work has shown intact auditory brainstem responses during torpor (Katbamna et al., 1992, 1996), demonstrating functionality of the auditory pathways, but it remained unclear whether the signal was 'perceived' at a higher cortical level. Although this cannot tell us as to whether torpid animals are consciously aware of their environment, it does indicate that sufficient cortical connectivity and activity is preserved in order to elicit an electrophysiological response. Moreover, the similarities in ERP characteristics between ad libitum and food restricted euthermic and hypothermic conditions further supports a close relationship between sleep and torpor, with differences being explained by a slowing of temperature-dependent processes and cortical firing.

Chapter 8: Investigating the effect of water restriction on torpor induction and propensity

8.1 Introduction

So far, this thesis has demonstrated that torpor is readily induced in response to food restriction and has the potential to disrupt performance in behavioural tasks and interfere with sleep regulation. As such, it is necessary to find a suitable alternative to food restriction and to determine whether other restriction protocols are also at risk of torpor induction.

A recent review by Barkus and colleagues demonstrated that there is an increasing popularity in the use of water restriction, or water regulation, for motivating engagement with behavioural tasks (Barkus et al., 2022). This is partly attributed to the rise in head-fixed techniques, such as fibre photometry and intracellular recordings, combined with behavioural responses. For example, a mouse may be required to complete a virtual maze task using a treadmill or 3D tracker ball to navigate. Successful performance will result in a water reward, similar to how food restricted mice will work for a food reward during behavioural tasks (Guo et al., 2014). A liquid reward is preferred to a solid reward in head fixed mice due to it being faster and easier to consume than solid food, allowing for more trials to be completed, and due to the avoidance of chewing artefacts (Barkus et al., 2022). Now, water control is becoming more prevalent in non-head fixed behavioural tasks also.

Due to water restriction being less well established than food restriction, there is a lack of standardisation in the technique, in addition to concerns about the welfare and scientific benefits of water restriction in comparison to food restriction. Both food and water restriction increase levels of stress markers and alter physiology, although differentially (Rowland, 2007;

Toth & Gardiner, 2000). Moreover, a study by Tucci and colleagues suggested that water restriction is better tolerated by mice than food restriction when comparing bodyweight fluctuations, however, both food and water restricted mice were found to show increased levels of aggression compared to non-restricted controls (Tucci et al., 2006). Interestingly, they also found that food restricted mice showed a reduction in exploration of novel environment during an open field task compared to water restricted mice and non-restricted controls. Further, a nose-poke conditional learning task, during which mice learned to poke their nose through a hole to receive a food or water reward in response to a light, revealed that mice were faster to learn the most economically advantageous strategy and maintained a more refined and optimal strategy throughout subsequent trials. Another study reported that water restricted mice reached the learning criteria for a two-choice visual discrimination task significantly quicker than food restricted mice and required fewer training trials to reach the criteria. These data were found to be independent of motivational state or satiation, indicating these differences are due to the effect of the restriction paradigm on other aspects of physiology or cognition (Goltstein et al., 2018). A possible explanation may be found in data reported by Padamsey et al who suggested that food restriction impacts the functioning of the visual cortex due to a decrease in available energy to support synaptic firing (Padamsey et al., 2021). This raises the question of whether behavioural data from food versus water restricted mice are comparable, and what may be underlying these differences.

As such, this led to the question of whether torpor induction may explain discrepancies in behavioural performance between food and water restricted mice. In Chapter 4, it was demonstrated that torpor in response to food restriction can significantly alter behavioural performance, in line with other studies (Nowakowski et al., 2009). However, it has not been established whether or not water restriction induces torpor in mice. Water restriction induces

weight loss of 15-20%, similar to food restriction (Barkus et al., 2022; Guo et al., 2014). Chapter 3 demonstrated that there is a strong relationship between bodyweight and torpor, therefore it is possible that the weight loss observed during water restriction may represent a metabolic challenge that is sufficient to induce torpor. Moreover, it has been reported that water restricted mice show reduced food intake even when food is provided ad libitum (Guo et al., 2014), suggesting that a decrease in food intake is contributing to weight loss and is not solely explained by dehydration.

This chapter aimed to investigate whether water restriction induces torpor in mice to determine whether the same considerations should be taken into account to control for torpor, as with food restriction. Moreover, any torpor bouts induced by water restriction would be compared with those induced by food restriction. If torpor was found to not be induced by water restriction, this may explain why differing behavioural outcomes have been reported but would also suggest that the data gathered from the two techniques should not be compared. Finally, absence of torpor during water restriction may suggest that water restriction provides an alternative to food restriction as it would remove any potential confounds as a result of torpor.

8.1.1 Experimental aims

i) Determine whether water restriction is sufficient to induce torpor in mice

ii) Compare torpor characteristics in food versus water restricted mice

8.2 Methods

8.2.1 Experimental animals and recording conditions

Adult male C57BL/6J mice were used for this study, aged ~9 weeks at the start of the study. The in-house colony of mice from the University of Oxford Biomedical Services was terminated shortly before the start of this study. Instead, mice (n=12) were acquired from Charles River Laboratories, UK, and given 1 week to habituate upon arrival to the facility. Mice were singly housed in either wire top M3 cages (48x15x13 cm) if they were undergoing continuous thermal recordings (n=8), or in individually ventilated cages (GM500, Tecniplast) (n=4) to allow for metabolic recordings to be taken via indirect calorimetry. Cages were housed in custom light tight chambers, with 2-6 cages per chamber depending on the recording device being used. Mice were exposed to a 12:12 light-dark cycle (lights on at 9 am) for the duration of the experiment, with a light intensity of 200 lux at the cage floor. Food was provided ad libitum throughout.

8.2.2 Thermal imaging and metabolic recordings

Torpor is driven by metabolic suppression; therefore, metabolic recordings were conducted in a subset of animals (n=4) to allow for small changes in metabolism in response to water restriction to be detected. In addition, metabolic recordings allow for other parameters to be investigated, such as the primary energy source via the respiratory exchange ratio. Thermal imaging was used for the remainder of the mice (n=8) to allow for comparison with data gathered from food restricted mice in Chapters 3-7 where possible. Additional parameters measured included daily bodyweight and daily food intake.

Where applicable, thermal cameras were mounted ~20 cm above the cage floor, with one camera taking measurements from two cages simultaneously. Thermal imaging data were processed and analysed as described in Chapter 2.3. Metabolic measurements were recorded using the CaloBox system (PhenoSys, GmBh), which uses indirect calorimetry to determine the relative concentrations of CO_2 and O_2 in the home cage compared to the air outside of the cage (Even et al., 2012). The CaloBox software uses the CO_2, O_2, and ambient temperature measurements to determine energy expenditure, metabolic rate, and respiratory exchange ratio. The system was programmed to take measurements every 5 s and to recalibrate every 15 minutes. Calorimetry data from water restricted mice were compared to historic data collected by Dr Laura McKillop in food restricted mice. Torpor was detected using metabolic parameters following the criteria outlined by Oelkrug et al., and Heldmaier et al., in which a mouse was determined to be in torpor when VO_2 dropped below 25 ml per hour for >2 hours (Heldmaier & Ruf, 1992; Oelkrug et al., 2011).

8.2.3 Water restriction paradigm

Water restriction is relatively less well established for behavioural testing, resulting in a lack of standardised protocols, with some users providing a fixed volume of water daily, providing water for a fixed time period, or using weight loss measures (Barkus et al., 2022; Guo et al., 2014). For the purpose of this study, a mixture of the most common paradigms was used. To this end, water was removed at ZT 0 on the first day of water regulation. Water was then provided at ZT 10 at a volume of 1 ml of water per 20 g of bodyweight. Bodyweight measurements were taken daily with mice being maintained at ~85% of their bodyweight prior to starting water regulation. The amount of water provided was titrated based on the individual animal's bodyweight. Baseline measurements of ad libitum Tskin or metabolic parameters were recorded for 1 week prior to the start of water regulation depending on the cohort.

Measurements of bodyweight and daily food intake were also recorded during baseline and throughout the study for all mice (n=12). Daily food intake measurements are missing on days 4 and 5 for all animals due to a technical error. Mice were maintained on water regulation for a total of four weeks. Four weeks was chosen, despite torpor during food restriction typically being induced after ~1 week, due to this being a novel experimental question, therefore it was unclear whether a prolonged metabolic challenge would be required during water restriction to induce torpor.

8.2.4 Food restriction paradigm

Water restricted mice were compared to historic data gathered from food restricted mice undergoing Tskin (n=8), and metabolic recordings (n=2 from beginning of food restriction; n=4 after >1 week of food restriction). Care was taken to select historic data from experiments with a comparable paradigm to the water restricted mice where possible. For example, food restricted mice were fed at the same zeitgeber time as the water restricted mice received water to control for potential circadian influences. Food restriction itself followed the protocol outlined in Chapter 2.2.

8.2.5 Statistical analysis

Thermal and metabolic data were processed using MATLAB (The Math Works Inc, USA, 2022a). Statistical analysis was performed using Prism (GraphPad, USA, v9). All data were tested for normality prior to further analysis using a Shapiro-Wilk test. Repeated measures one-way ANOVA were used to determine the change in parameters over time in water restricted mice. Within group comparisons of mean values were conducted using paired t-tests, whilst between group comparisons of mean values were performed using unpaired t-tests. Where

appropriate, a non-parametric equivalent was used. Comparisons between water and food restricted groups over time were performed using two-way ANOVAs with repeated measures, with an appropriate post-hoc multiple comparisons test. A Geisser-Greenhouse correction was applied in all cases where sphericity was not present. Relationships between bodyweight and Tskin or VO_2 were determined using simple linear regression analysis.

8.3 Results

Bodyweight significantly decreased during water restriction, as expected. Mice were maintained at ~85% of their ad libitum bodyweight, however, bodyweight did start to increase again and got closer to 90% during the third week of water restriction (Figure 49A). Oxygen consumption (VO_2, ml/min), and Tskin both significantly decreased over days of water restriction also (VO_2: $F_{(2.41,7.22)}=6.34$, $p=0.0228$; Tskin: $F_{(2.71,19.0)}=18.0$, $p<0.0001$; RM one-way ANOVAs; Figure 1B-C). Comparison of the respiratory exchange ratio (RER), in the four mice undergoing metabolic recordings, on baseline days versus during water restriction found that RER during water restriction was lower on average, but not significantly so (baseline mean: 0.958 ± 0.0147; WR mean: 0.909 ± 0.0222; $t(3)=2.299$, $p=0.1052$; paired t-test, (n=4)). Food intake also decreased over days of water restriction in all mice (Figure 49D) and was found to be significantly reduced in comparison to baseline with mice eating 3.63 ± 0.151 g of food when water was available ad libitum, compared to 2.90 ± 0.0708 g during restriction (Figure 1E; $t(11)=4.92$, $p=0.0005$; paired t-test, (n=12)). However, food intake during water restriction was still significantly greater than the average food ration provided to food restricted mice to maintain them at ~85% of free feeding bodyweight (Figure 49F; $t(22)=9.69$, $p<0.0001$; unpaired t-test (n=12 for both food-restricted and water-restricted groups)).

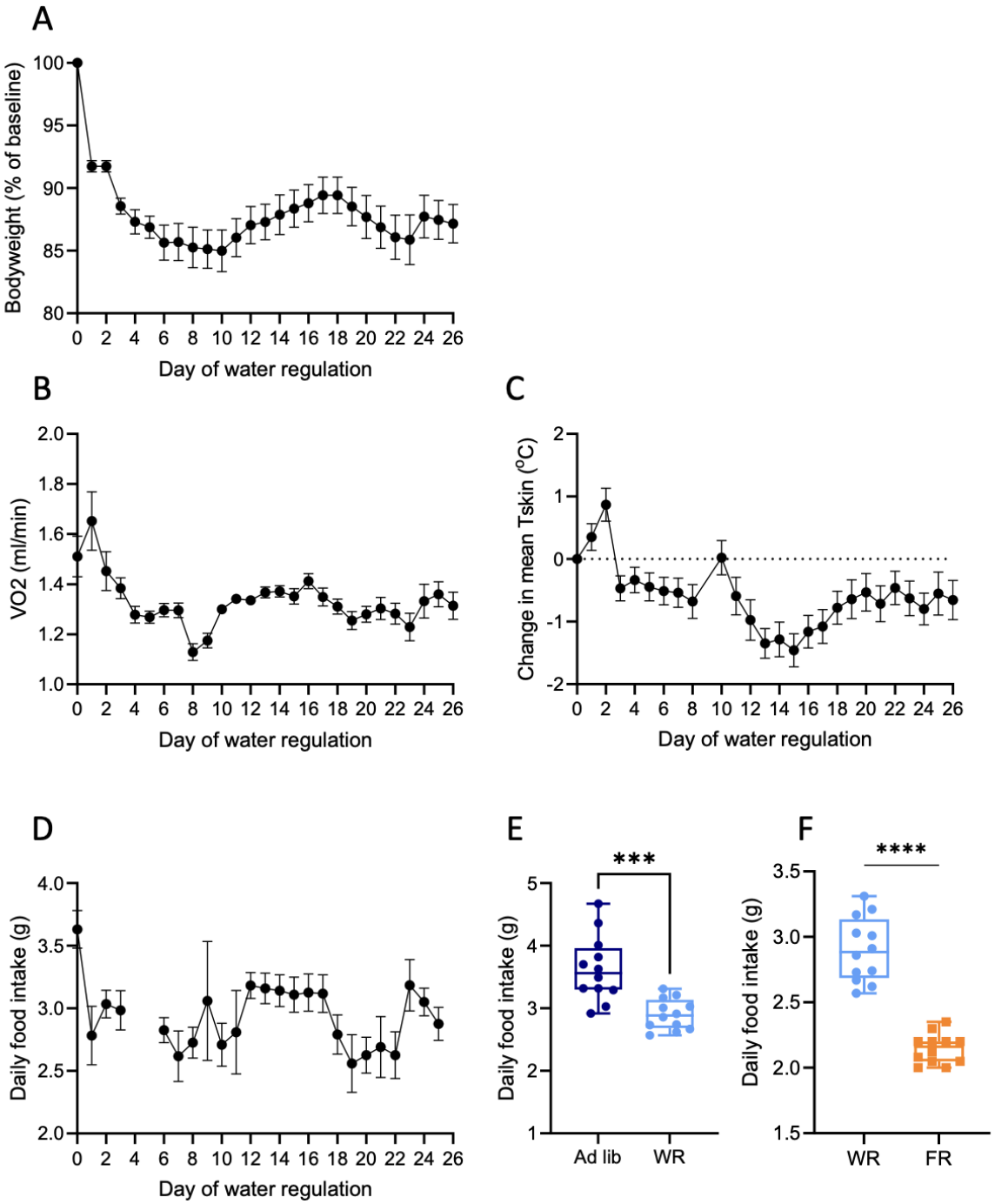

Figure 49: Effect of chronic water restriction on parameters of interest. (A) Change in bodyweight over days of water restriction. Mice were maintained at a target bodyweight of ~85% of ad libitum (n=12). (B) Mean oxygen consumption (VO2) significantly decreased over days of water regulation ($F_{(2.41,7.22)}$=6.34, p=0.0228; RM one-way ANOVA; n=4). (C) Tskin significantly decreased over time ($F_{(2.71,19.0)}$=18.0, p<0.0001; RM one-way ANOVA; n=8). (D) Daily food intake decreased during water restriction despite being available ad libitum (n=12). (E) Comparison of daily food intake when water was available ad libitum and during water restriction (p=0.0005, paired t-test; n=12). (F) Comparison of daily food intake during water restriction and the daily ration of food provided to food restricted mice (p<0.0001, unpaired t-test; n=12). All data are presented as mean values ± SEM. ***p<0.001, ****p<0.0001.

Despite the significant decrease in VO_2 and Tskin during water restriction, no evidence of torpor was found upon visual inspection of raw traces (Figures 50 A & C), which was confirmed by applying the criteria for torpor detection described in Chapter 2 for Tskin measurements, and in the methods section here for VO_2. For comparison, see the Tskin and VO_2 traces from a representative food restricted mouse when torpor is readily occurring (Figures 50 B & D).

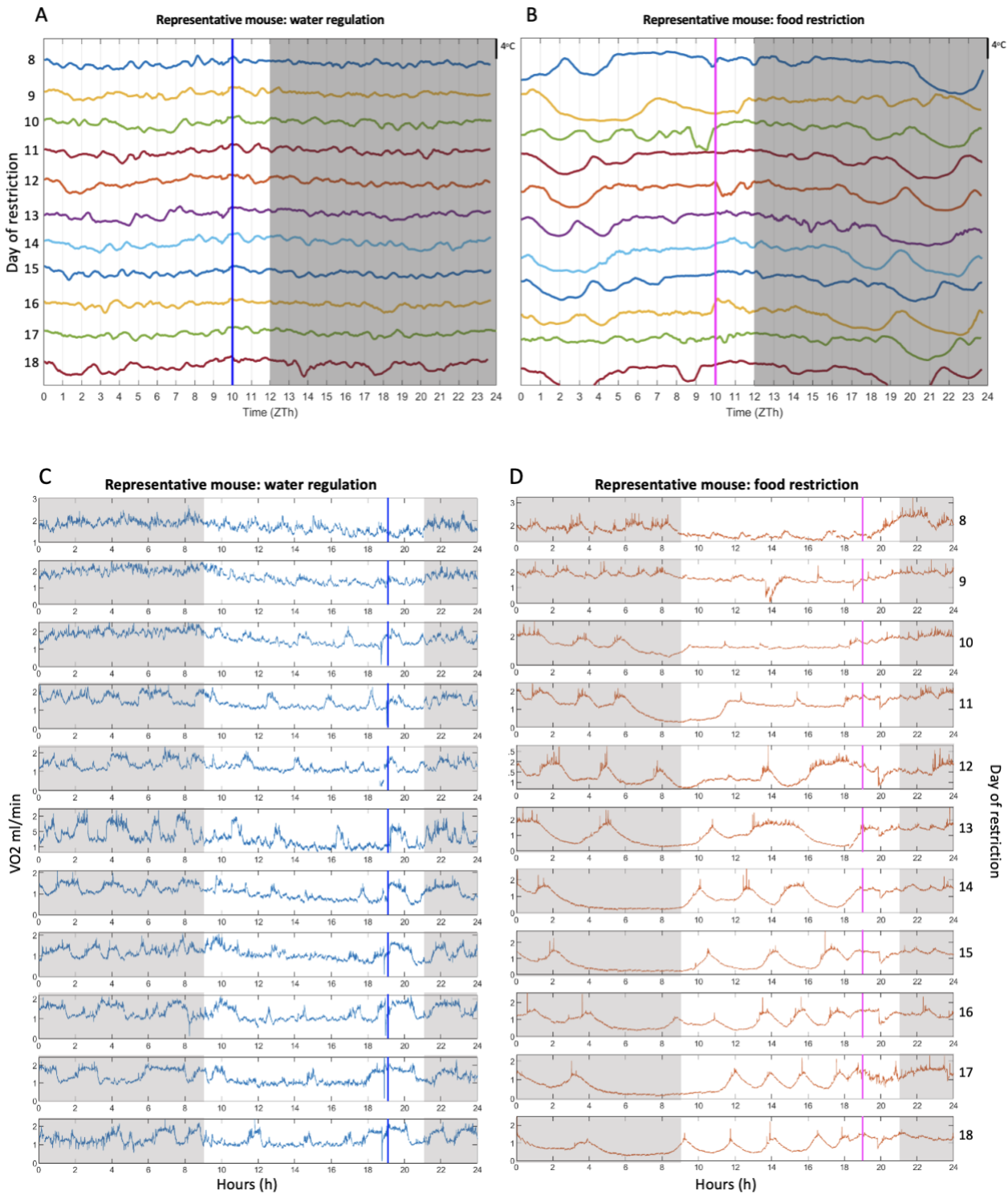

Figure 50: Raw temperature and oxygen consumption traces over days of restriction. (A) Peripheral temperature recordings from a representative mouse over days of water restriction. (B) Consecutive days of temperature recordings from a representative food restricted mouse. Note the sustained large drops in body temperature indicating torpor. (C) Oxygen consumption (VO_2) from a representative water restricted mouse, and (D) a representative food restricted mouse. The vertical blue line indicates when water was provided, and the vertical pink line indicates when food was provided. Grey shading represents the dark period.

Next, data from water restricted mice were compared to historic data collected from food restricted mice. Comparison of bodyweight over days of water/food restriction (n=12 per condition) revealed that water restricted mice lost weight at a faster rate in the initial 5 days of restriction compared to food restricted mice (Figure 51A). Due to some missing values in the food restricted dataset, a mixed-effects model was used to analyse these data. This revealed a significant main effect of day of restriction ($F_{(3.29,66.2)}$=58.5, p<0.0001), no main effect of group ($F_{(1,22)}$=2.55, p=0.1247), and a significant interaction between day of restriction*group ($F_{(17,342)}$=14.95, p<0.0001). Further comparison of bodyweight once weight loss had stabilised found no significant difference between groups (Figure 51B; t(21)=1.79, p=0.0898; unpaired t-test), which can be explained by both groups having the same target maintenance weight.

Both groups showed a significant decrease in Tskin over days of restriction with significant main effects of time and group being found, in addition to a significant time*group interaction (Figure 51C; main effect of day of restriction: $F_{(4.79,67.0)}$=47.2, p<0.0001; main effect of group: $F_{(1,14)}$=29.5, p<0.0001; day of restriction*group: $F_{(17,238)}$=13.8, p<0.0001; 2-way ANOVA with repeated measures (n=8 for each condition)). A between group comparison was also conducted on the final 5 days when Tskin had stabilised, which confirmed that Tskin was significantly higher during water restriction than food restriction, due to torpor only being induced in response to food restriction (Figure 51D; WR: -0.422 ± 0.267; FR: -2.689 ± 0.187; p<0.0001, t(14)=6.96; unpaired t-test). Similarly, VO_2 decreased for both groups over days of restriction, with the VO_2 remaining higher in water restricted animals than food restricted animals. Differences in VO_2 were less apparent than with Tskin measurements, although this may be explained by the smaller sample size (Figure 51E). It should be noted that there was only historic metabolic data from the start of food restricted for two mice. As such, statistical analysis was not performed on the change in VO_2 over time. However, metabolic data were

available from four mice that had been food restricted for >1 week and were readily entering torpor. This dataset was used to compare mean VO_2 with water restricted VO_2, which revealed that VO_2 was significantly greater during water restriction than during food restriction (Figure 51F; $t(6)=3.41$, $p=0.0144$; unpaired t-test ($n=4$ for each condition)).

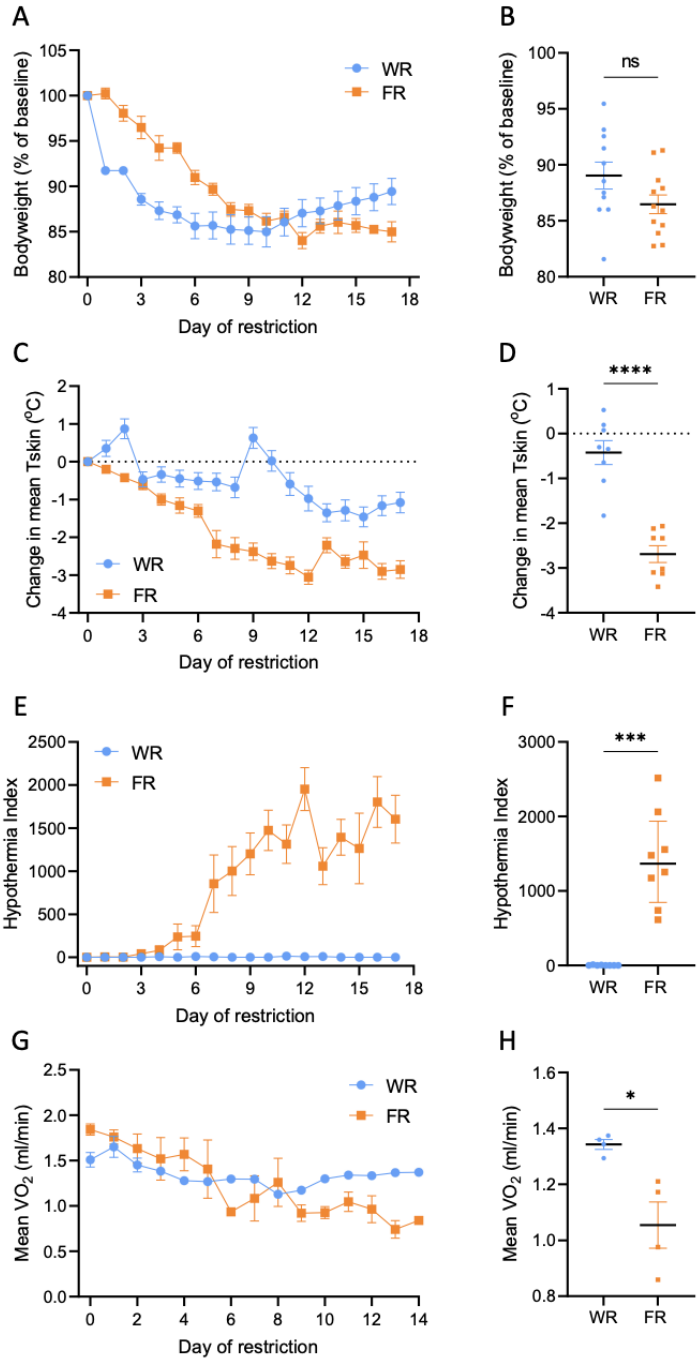

Figure 51: Comparison of the effect of food versus water restriction on parameters of interest. (A) Initial weight loss was more rapid in water restricted mice but both groups stabilised at ~85% (the target weight) after 1 week. (B) There was no difference in stabilised bodyweight between restriction type. (C) Both restriction types resulted in a significant reduction in Tskin over time and a significant main effect of group was found. (D) Tskin was significantly higher in water restricted mice compared to food restricted mice. (E) Average oxygen consumption decreased in both groups over time. (F) Oxygen consumption was significantly reduced during food restriction compared to water restriction. (G) The area beneath the torpor threshold (hypothermia index (HI)) was significantly increased during food restriction but remained close to zero for water restricted mice. (H) The HI was significantly greater during food restriction. Data are presented as mean values ± SEM, except for H which is presented as a median value and IQR. *p<0.05, ***p<0.001, ****p<0.0001.

To further confirm the absence of torpor during water restriction, a hypothermia index (HI) was calculated, in which the area beneath the hypothermia threshold was calculated in line with previous studies (Huang et al., 2021). The HI significantly increased during food restriction as torpor bouts became longer and more frequent, whereas the HI consistently stayed close to 0 during water restriction (Figure 51G). It should be noted that this method did not apply a time threshold, therefore the HI for water restricted mice was above 0 at times. However, upon closer inspection these deviations never exceeded 1 hour in duration so did not meet our criteria for torpor. Comparison of HI towards the end of restriction further confirmed that HI was significantly lower during water restriction than food restriction (Figure 51H; U=0, p=0.0002; Mann-Whitney U test).

Finally, due to the observation of decreased Tskin and VO_2 in conjunction with decreased bodyweight, the relationship between these parameters was investigated. To this end, the mean change in Tskin or VO_2 was calculated for every 1% change in bodyweight for each animal, and simple linear regression analysis performed. A very weak negative relationship was found between change in Tskin and bodyweight, which was not significant for water restricted mice (Figure 4A; r^2=0.01862, p=0.2903). In comparison, a significant positive relationship was found between change in Tskin and bodyweight for food restricted mice (Figure 52B; r^2=0.6198, p<0.0001). No relationship was found between VO_2 and bodyweight in water restricted mice undergoing metabolic recordings (Figure 52C; r^2=0.0022, p=0.7954). Simple linear regression analysis was also performed on VO_2 against bodyweight in food restricted mice; however, there was only metabolic data available from two mice from the beginning of food restriction, and limited bodyweight data available from the other two mice who underwent metabolic recordings after having been food restricted for >1 week. As such, the data are limited and should be interpreted with caution. From the data available, there appears to be a

positive relationship between VO_2 and bodyweight during food restriction which was determined to be significant in this small sample (Figure 52D; r^2=0.6968, p=0.0007), which was in line with expectations. However, this finding should not be over-interpreted and follow up studies using a larger sample size are required.

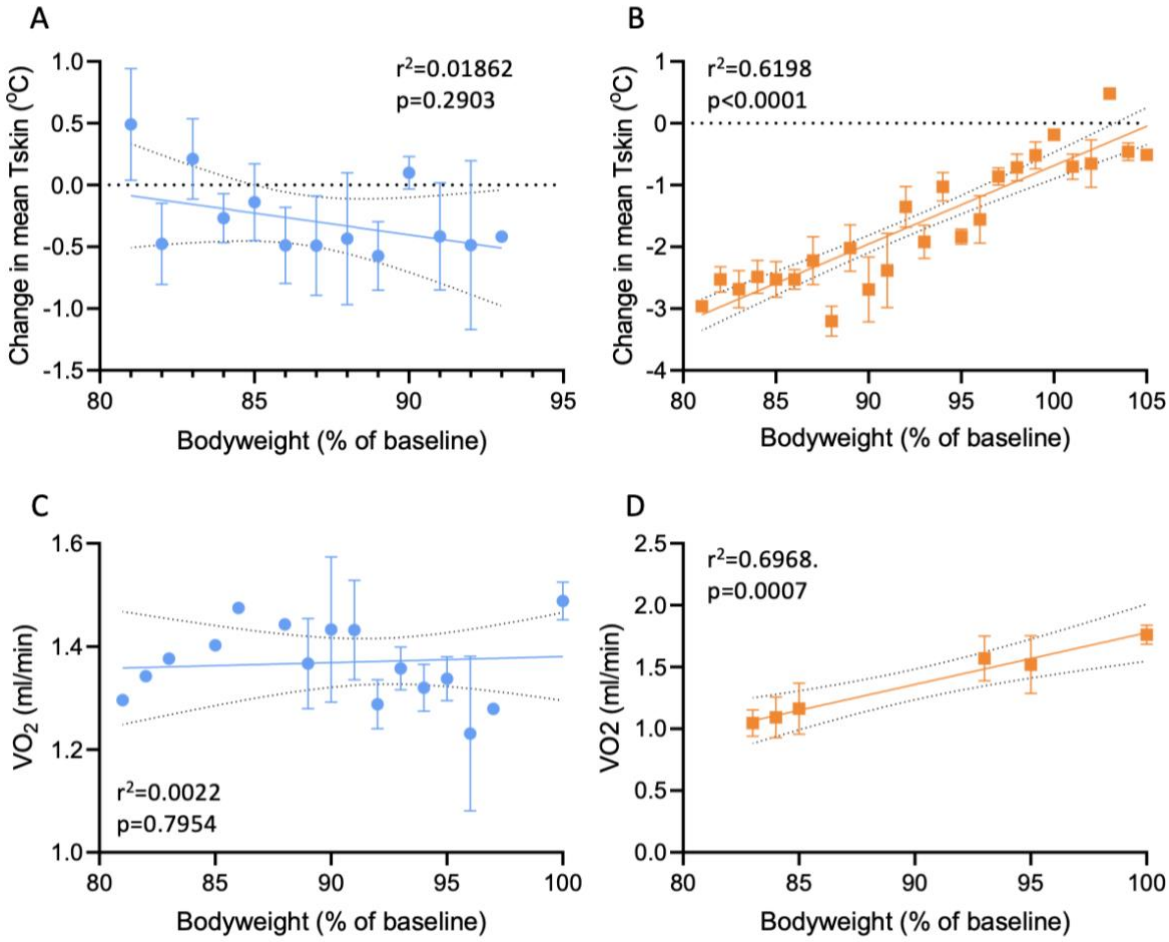

Figure 52: Relationship between bodyweight and Tskin or mean oxygen consumption (VO_2). (A) A non-significant negative relationship was found between change in Tskin and bodyweight during water restriction (n=8). (B) A significant positive relationship was observed between food restricted bodyweight and Tskin (n=8). (C) No relationship was found between VO_2 and bodyweight for water restricted mice (n=4). (D) Bodyweight and VO_2 were found to be significantly positively related during food restriction (n=2). Data are presented as mean values for every 1% change in bodyweight ± SEM.

8.4 Discussion

This chapter presents the novel observation that water restriction alone is not sufficient to induce torpor in mice. It is possible that torpor could be induced if water restriction had continued for longer; however, water restriction was maintained for ~4 weeks here which is much longer than in typical laboratory settings (Barkus et al., 2022). Moreover, this was ample time for bodyweight to drop to and stabilise at 85% of ad libitum bodyweight. Due to torpor and bodyweight having been shown to be closely related, both in this thesis and elsewhere (Kato et al., 2018; Solymár et al., 2015), if torpor were to be induced by water restriction, it would be expected that torpor would have been observed over the course of the experiment. This is further supported by the lack of a relationship found between bodyweight and Tskin or VO_2 in this study in water restricted mice.

It was surprising that mice did not enter torpor during water restriction as, in line with previous studies, bodyweight and food intake significantly decreased over the course of water restriction. However, daily food intake remained higher than the food ration provided to food restricted mice to maintain them at 85% of their free-feeding bodyweight, which may suggest that the weight loss observed is predominantly due to dehydration. This is further supported by the observation that water restricted mice lost weight at a faster rate than food restricted mice, possibly due to depletion of energy stores requiring more time to occur than water loss. Interestingly, a study using hamsters which were entering torpor in response to a short photoperiod, found that water restriction increased torpor duration in comparison to controls but was shorter compared to food-restricted hamsters (Noakes et al., 2022). In addition, there was no difference in torpor between food restricted hamsters and hamsters undergoing simultaneous food and water restriction. Long photoperiod hamsters did not enter torpor in response to either restriction type. The water restricted hamsters were reported to reduce their

food intake by~26%, as such this may have been enough of a metabolic challenge to prolong torpor bouts in hamsters that were already entering torpor. However, as mice only enter torpor in response to actual rather than perceived food shortage unlike hamsters, water restriction alone does not incur enough of metabolic challenge to induce torpor.

It has been assumed that food or water restriction are simply alternative methods for increasing the motivational state of the mouse, however, differing physiological and behavioural states due to torpor induction prompts the question of whether data gathered from each paradigm are comparable. There have already been reports of altered behavioural outcomes when comparing food versus water restricted mice. For example, Tucci and colleagues reported that water restricted mice were quicker than food restricted mice to learn the most optimal strategy in a conditional learning nose poke test, in which mice were trained to poke their nose into a hole in response to light cues to receive a food or water reward. Moreover, food restricted mice were reported to have more incorrect trials (Tucci et al., 2006). Similarly, Goltstein et al. reported that water restricted mice were quicker than food restricted mice to reach the learning criterion in a two choice operant task, despite water restricted mice performing fewer trials overall (Goltstein et al., 2018). The findings presented in this Chapter may help to us begin to understand the reason for such differences.

When evaluating food versus water restriction paradigms, two key factors must be taken into consideration: (i) scientific benefit; and (ii) welfare benefit. It has been established that food restriction readily induced torpor in mice (van der Vinne et al., 2018), and the data presented here suggests that water restriction does not induce torpor in mice. As such, it could be argued that water restriction is preferable for generating more robust scientific outcomes, as there is no potential for torpor history to be confounding results or contributing to variability, by

altering physiology, behaviour, and sleep. However, like food restriction, water restriction is still able to alter physiology and behaviour. In a social dominance task, water restricted mice showed increased aggression compared to non-restricted controls, likely due to resource guarding in times of scarcity (Boukersi et al., 2021; Tucci et al., 2006). Moreover, water restriction has been found to impair growth and evoke a stress hormone response in mice (Vasilev et al., 2021). Stress itself is known to significantly alter physiology, behaviour, and the subsequent quality of animal models, and efforts should be made to refine techniques to mitigate the effects of stress (Gaskill & Garner, 2017). As such, the effect of water restriction should remain an important consideration when drawing conclusions from data gathered from restricted mice, or when attempting to extrapolate behavioural data to not-restricted conditions.

Another important consideration is the effect of restriction type on welfare, as it could be the case that the welfare benefits of one restriction type may outweigh less optimal scientific outcomes, as it is possible to address scientific outcomes with appropriate controls. Although welfare parameters were not a primary measurement for this study, bodyweight was recorded which is often used as an indicator of health. Initial weight loss was more rapid during water restriction than food restriction which may be interpreted as worse welfare outcome. However, this may be explained by initial dehydration rather than depletion of energy stores. Further, mice are able to acclimate to chronic water restriction very effectively, especially if the amount of water is restricted gradually (Bekkevold et al., 2013; Haines et al., 1973). In the present study, bodyweight began to increase and got closer to 90% at ~week 3 of water restriction in line with previous studies (Tucci et al., 2006), which may be due to the increased acclimation. In contrast, this gradual increase in bodyweight over time is not present in food restricted mice. Anecdotally, it has been observed over the course of this thesis that increasingly larger food rations are required to maintain the bodyweight of food restricted mice at appropriate levels

with prolonged food restriction. Other studies report little difference in the welfare of animals on food versus water restriction (Rowland, 2007; Toth & Gardiner, 2000).

8.4.1 Conclusions

Overall, there was no evidence of torpor induction in response to water restriction based on metabolic and temperature recordings. This is in contrast to food restriction which readily induces torpor when weight loss reaches 10-15% of ad libitum bodyweight. As a result, it is likely that mice will be in vastly different behavioural and physiological states depending on the type of restriction paradigm, therefore indicating that data gathered from food versus water restriction may not be directly comparable. As such, the scientific and welfare benefits of each restriction type must both be considered. From a scientific perspective, water restriction may be preferable as there is no risk of torpor confounding data and adding an additional source of variability. In terms of welfare, reports suggest little difference between restriction type, so welfare is unlikely to outweigh the scientific benefits.

Chapter 9: General discussion and future directions

9.1 Summary of key findings

Fasting and food restriction are common techniques used in research involving laboratory mice across disciplines, including but not limited to, behavioural neuroscience (Padamsey et al., 2021), metabolic studies (Ayala et al., 2010), pharmacokinetic and pharmacodynamic studies (Chen et al., 2018; Huang et al., 2015), and circadian neuroscience (Greenwell et al., 2019). Despite the extensive use of fasting and food restriction, the potential for fasting to be a confound for experimental data is rarely considered in spite of the profound changes to physiology that occur in response to fasting (Jensen et al., 2013; Secor & Carey, 2016). This is of particular relevance due to an increasing focus and awareness surrounding the identification of confounds and sources of variability in animal research to support the generation of more robust, reliable, and reproducible outcomes (Begley & Ioannidis, 2015; Ioannidis, 2018).

In this thesis, torpor, a state of metabolic suppression and hypothermia that readily occurs in response to fasting in mice (Hudson & Scott, 1979), was explored from the perspective of gaining insights into the neurophysiology of torpor, and how changes to neurophysiology may interact with sleep and behaviour. In addition, this thesis attempted to identify the potential impact for torpor to be confounding experimental data and contributing to variability, with the purpose of refining techniques to mitigate these unwanted effects of torpor. To this end, a food restriction paradigm that is common across disciplines, but is most frequently used for behavioural studies, was investigated to determine whether torpor may be inadvertently being induced in response. Next, the effect of torpor on performance during behavioural tasks was explored. Following from this, the relationship between sleep and torpor was examined with

the aim of understanding the regulation and modulation of sleep and torpor, and the potential effects of sleep deprivation in response to torpor. Finally, to reflect the growing popularity in head fixed techniques, torpor in response to water restriction was investigated and compared to torpor following food restriction.

9.1.1 Induction of torpor in mice

The first experimental chapter aimed to determine whether a common food restriction protocol would be sufficient to induce torpor in laboratory mice. These data demonstrated that chronic food restriction, during which adult male mice receive food once daily and are maintained at ~85% of their free feeding bodyweight, was sufficient to induce torpor as determined by continuous peripheral body temperature measurements. Interestingly, there was a strong relationship between torpor propensity and bodyweight, with all mice reliably entering torpor daily when at ~85% bodyweight. Despite this, torpor induction and characteristics were highly variable between individual mice and also within mice across days. As torpor is an energy saving response during an energy deficit, it is likely that differences in torpor within and between animals can be explained by differences in activity levels and behavioural state, as these are known to modulate other neurophysiological states such as sleep (Milinski et al., 2021). Moreover, some research has suggested that bodyweight at birth can predict torpor propensity in mice (Kato et al., 2018b), demonstrating that torpor is influenced by multiple factors which can be difficult to control for, even when using an inbred strain of mice which are genetically identical. The data presented in this thesis build on previous reports in the literature in which torpor was induced in mice using acute fasting of between 6-24 hours, involving complete removal of food (Hudson & Scott, 1979; Jensen et al., 2013; Swoap et al., 2006). There has been some work showing that mice enter torpor in response to longer term food restriction protocols (van der Vinne et al., 2018, 2020) and in response to scheduled

feeding (Northeast et al., 2019), however, this is the first example of torpor propensity and characteristics being investigated in response to this particular food restriction paradigm.

Another main finding from this chapter was that some characteristics of torpor were significantly altered depending on when food was provided, with mice fed at the beginning of the night phase entering shorter torpor bouts on average compared to mice fed in the morning. This finding builds on previous reports in hamsters (Paul et al., 2004) and in mice (van der Vinne et al., 2018) that show that torpor is under circadian control. Interestingly, only some torpor characteristics were significantly influenced by feeding schedule, which may be explained by the competing circadian and motivational drives of light timing and food timing, both of which effect the temporal pattern of physiology and behaviours (Mendoza, 2007; Stephan, 2002). It is also possible that the high levels of variation in torpor observed, and the relatively small sample size, may have resulted in some statistical tests being underpowered. Despite these limitations, these findings have important implications for studies that regularly use chronic food restriction techniques as it is likely that torpor will be being induced, which may not be a primary aim of the study. In these instances, torpor has the potential to confound experimental outcomes due to the high level of variability in torpor characteristics, and the profound changes in physiology associated with torpor. Moreover, these data demonstrate the importance of consistency and standardisation of feeding schedules due to the influence of circadian rhythms in torpor control. Doing so should enable more direct comparisons between studies that report the use of long-term food restriction paradigms.

Chapter 8 tested the hypothesis that water restriction would also induce torpor in mice. This hypothesis was developed considering the increasing popularity in head fixed techniques which use water restriction to motivate engagement with behavioural tasks, and to contribute to the

ongoing discussion as to whether food or water restriction is optimal in terms of scientific and welfare considerations (Barkus et al., 2022; Toth & Gardiner, 2000). Moreover, previous studies using water restriction in mice have reported a reduction in both bodyweight and in daily food intake (Guo et al., 2014), which may represent a metabolic challenge sufficient to induce energy saving strategies such as torpor.

Interestingly, this study led to the novel observation that torpor does not appear to be induced in response to water restriction, as no signs of torpor were observed in any of the mice throughout the study's four-week duration, despite both a reduction in bodyweight and food intake being observed. This was in contrast to other data presented in this thesis, which demonstrated that food restriction readily induced torpor in mice and showed a strong relationship with decreased bodyweight. There are no previously published data investigating torpor in response to water restriction in mice. However, water restricting hamsters that were entering torpor in response to short photoperiod was found to increase the length of torpor bouts (Noakes et al., 2022). Hamsters are a species that enter torpor in response to a perceived seasonal food shortage, whereas mice enter torpor in response to an actual food shortage, indicating that water restriction was only modulate torpor in water restricted hamsters due to them already being in a metabolically challenged state. Further, a study in primate species suggested that torpor acts as a method of water conservation in dry environments, but it is unclear whether torpor was induced as a direct consequence of limited water availability as these observations were made in wild individuals (Schmid & Speakman, 2009). In contrast, the data presented in Chapter 8 show no evidence that water restriction is sufficiently metabolically challenging in ad libitum fed mice to result in the induction of torpor. It is possible that water restriction, in combination with food restriction, would affect the length and depth of torpor

bouts and may be an avenue for future research. However, combining both food and water restriction may raise significant welfare concerns.

These findings have important implications for the comparison of data generated using food versus water restricted mice. Due to the induction of torpor in response to food restriction, but not water restriction, it is likely that mice on these paradigms will have profoundly different physiological and behavioural states. As such data generated using these paradigms are unlikely to be directly comparable, which has already been highlighted in a recent paper by Goltstein et al. which reported differential learning outcome in food versus water restricted mice. For example, water restricted mice were found to be less motivated to engage with tasks compared to food restricted mice but were faster to reach the learning criterion during the training period. In addition, food restricted mice ran significantly less distance on a Styrofoam ball compared to water restricted mice (Goltstein et al., 2018). There are a number of reasons why food restricted mice may have different outcomes compared to water restricted mice, including levels of motivation and satiety, but it is also possible that the lasting physiological and behavioural effects of torpor may also be contributing to these differences. For example, the data presented in Chapter 4 which explored the effect of torpor on behaviour and memory found that the time of testing relative to a torpor bout significantly altered locomotor activity whereas this effect would not be present in water restricted mice.

9.1.2 Effects of torpor on behaviour

The next aim of this thesis was to investigate the effects of torpor on behaviour. This was due to torpor representing a profoundly altered physiological state which is likely to translate to altered behaviour. However, the literature regarding torpor's effect on behaviour and memory is mixed (Hensleigh et al., 2022; Nowakowski et al., 2009; Palchykova et al., 2002). Further,

behavioural tasks are recognised as being highly variable and sensitive to confounds (Saré et al., 2021; “Troublesome Variability in Mouse Studies,” 2009), and so the potential for torpor to be a confounding factor was investigated.

Testing shortly following torpor was found to significantly reduce locomotor activity, which returned to levels comparable with non-food restricted mice upon subsequent testing following arousal from torpor. Although this effect was transient, it demonstrates the potential for the physiological changes associated with torpor to influence behaviour. Moreover, many behavioural tests rely on sufficient levels of motor activity for mice to fully engage with and complete the task. For example, many tasks for memory use some form of maze which the mouse must navigate (Wenk, 2004). Moreover, mazes commonly utilise food restriction with a food reward upon completion to motivate mice to engage with the task (Wenk, 2004). Consequently, if a mouse is tested on such a task shortly after torpor, then their locomotor activity may be impaired, therefore confounding the overall outcome of the trial. Interestingly, an increase in exploratory behaviour during subsequent trials was not observed in food restricted mice, despite their inability to explore the arena in the initial trial, leading to the postulation that the mice were still sufficiently aware of their surroundings in order to process their environment and contextual cues, and habituate to their environment despite having just aroused from torpor. In Chapter 7, investigation of sensory perception during torpor suggested that the ability of the cortex to respond to environmental stimuli is preserved, therefore providing some support in favour of the explanation as to why no rebound increase in activity was found on subsequent trials.

Despite Chapter 4 finding that performance in an open field task was significantly affected by time of testing relative to torpor, no robust memory effect was observed in the novel object

task in the torpor group or the control group, with neither group performing better than chance. As such, this makes it difficult to determine from these data whether torpor has a robust memory effect in the novel object recognition task (NORT). The NORT was chosen for this thesis due to it being a non-appetitively driven task, therefore allowing for the investigation of behavioural drive independent of behavioural state. However, it is possible that the NORT was not a sensitive enough task for investigating the effect of torpor on memory, or that torpor effects different modalities of cognition that as not tested in the NORT. For example, a previous study using a Morris water maze found that fasting-induced torpor protected memory retention of the location of a hidden platform in fasted mice compared to non-torpor controls (Nowakowski et al., 2009). There were other differences in the study design compared to that used in this thesis which may also contribute to differences in the findings. For example, Nowakoswki and colleagues used female mice exposed to an overnight fast at a cool ambient temperature (~18ºC), opposed to the data in this thesis which were obtained from male mice under chronic food restriction housed at standard ambient temperatures used in animal housing facilities (~22ºC).

Notably, the behavioural data presented in this thesis were highly variable, especially when assessing memory effects in the NORT. Other chapters in this thesis have shown that torpor itself is high variable between and within individuals. This variability may be due to a mixture of environmental factors, such ambient temperature and activity levels, in addition to epigenetic factors, with some research indicating that torpor propensity is influenced by birth bodyweight (Kato et al., 2018). Further, previous work has demonstrated that behavioural outcomes can be influenced by differences in motivational state and waking experience (Milinski et al., 2021). The waking experience and torpor history were not controlled for during the collection of the behavioural data in this study and so may have contributed to no robust

effect being observed. Subsequent work presented in Chapters 5-7 indicates that torpor and sleep are closely related and interacting states. For example, these chapters present evidence that torpor restructures sleep-wake architecture and may be a sleep depriving state due to an observed increase in sleep EEG slow-wave activity, a hallmark of sleep intensity which increases with a dose-dependent relationship to the length of preceding wake (Borbély et al., 2016). Sleep deprivation is associated with altered behaviours and cognitive deficits (Killgore, 2010), therefore differences in torpor history and the subsequent differential impact on sleep (i.e., increased sleep pressure) may have contributed to the behavioural observations presented here.

Although these data did not demonstrate a robust memory effect associated with torpor, they do emphasise the importance of controlling for confounding variables such as torpor. In addition, the high levels of variability observed, and the subsequent work in later chapters demonstrating a link with sleep, further supports the hypothesis that torpor is a potential confounding factor in behavioural research.

9.1.3 The interaction between torpor and sleep

The final aim of this thesis was to investigate the relationship between sleep and torpor to further the understanding of them as interrelated neurophysiological states. Chapter 5 reported that fasting-induced torpor was entered via NREM sleep which suggests a close relationship between sleep and torpor, with sleep acting as a gateway into torpor. Schmidt has previously suggested that the slight reduction in body temperature observed during euthermic NREM sleep may act as a mild form of energy conservation due to slight metabolic depression, therefore enabling progression into a more profound metabolic depression during torpor (Schmidt, 2014).

The EEG during torpor bouts was also found to resemble the electrophysiological activity associated with euthermic NREM sleep. Notably, the NREM-like brain activity during torpor was lower amplitude than euthermic NREM sleep and was composed of slower frequencies. These data are in line with previous reports in mice (Huang et al., 2021), hamsters (Deboer & Tobler, 1995), and hibernating ground squirrels (Walker et al., 1977). The differences between the NREM-like EEG during torpor and euthermic NREM sleep may be explained by the slowing of temperature-dependent processes as low body temperature can contribute to the inhibition of synaptic vesicle release, signalling cascades, and ionic currents (Sonntag & Arendt, 2019). Alternatively, the differences may be explained by the significant changes in dendritic and neuronal morphology that have been reported to occur in torpor (Von Der Ohe et al., 2006), resulting in a breakdown of network synchronisation, or a reduction in the number of synapses able to contribute to electrical signal.

Further, Chapter 5 presents novel data demonstrating the significant restructuring of sleep-wake architecture that occurs in order to accommodate fasting-induced torpor, in a way that is distinct from other forms of torpor and ad libitum conditions. It is likely that this restructuring of sleep patterns occurs in order to maximise opportunities for energy conservation, sleep, and foraging opportunities which is supported by the findings presented in Chapter 3. In Chapter 3, meal timing was found to significantly alter torpor timing and characteristics to ensure that the mice were active prior to the anticipated feeding time, therefore this temporal shift in activity and torpor patterns would also be impacting sleep-wake architecture.

Building on these findings, the impact of increasing sleep pressure through sleep deprivation on torpor and sleep processes was investigated. This was due to reports from hibernators and daily torpidators suggesting that torpor is a sleep depriving state due to an increase in slow

wave activity, a hallmark of sleep homeostasis, being observed in sleep following a torpor bout (Deboer & Tobler, 2000). It has been suggested that the slowing of EEG frequencies and the reduction in EEG amplitude during torpor compared to euthermic NREM sleep means that sleep-related restorative processes are unable to occur, therefore resulting in an accumulation of sleep pressure which must be alleviated upon subsequent euthermic sleep (Silvani et al., 2018). There are no reports in the published literature as to whether fasting-induced torpor is a sleep depriving state resulting in a significant gap in the current understanding of sleep and torpor.

The presented data in Chapter 6 found that an increase in slow wave activity occurred in sleep following a torpor bout, indicating that fasting-induced torpor may be sleep depriving. These findings are in agreement with previous studies in Djungarian hamsters which reported an increase in slow wave intensity following torpor which was comparable with slow wave intensity following sleep deprivation (Palchykova et al., 2002). In addition, work in hibernators has also demonstrated a rebound-like effect in sleep intensity following bouts of hibernation (Trachsel et al., 1991), with euthermic intervals between bouts of prolonged torpor being dominated by deep sleep (Daan et al., 1991). The rebound in slow wave activity was less following torpor than the slow wave activity rebound observed following sleep deprivation, indicating that torpor is not as sleep depriving as prolonged wakefulness. This may suggest that some sleep-related restorative processes may be able to occur during torpor, possibly due to the EEG being similar to euthermic NREM sleep. However, any sleep-related restorative processes able to occur during torpor would be suboptimal due to the differences in torpor EEG compared to euthermic sleep, and potentially due to a reduction in the number of dendritic spines and synapses (Popov & Bocharova, 1992; Von Der Ohe et al., 2006). This would also explain why deep euthermic sleep is required following torpor bouts, as there may be a need to

rebuild synaptic connections and dendritic spines to facilitate optimal sleep-related restorative processes.

To further investigate sleep homeostasis in relation to fasting-induced torpor, future studies may wish to conduct sleep deprivation either before or after entry into torpor in line with Palchykova and colleagues experimental design (Palchykova et al., 2002). In addition, the relationship between sleep rebound intensity and length of daily torpor bouts may provide further insights into the homeostatic regulation of sleep and torpor. This would build on previous findings in hibernating ground squirrels that found that slow wave intensity increased during subsequent sleep as the length of hibernation increased (Trachsel et al., 1991).

Finally, cortical responses to auditory stimulation during sleep and torpor were explored. For the first time, this thesis showed that a sufficient degree of cortical connectivity is preserved during fasting-induced torpor in terms of cortical evoked response potentials (ERPs). The dynamics of ERPs were altered in comparison to those recorded during euthermic sleep, most notably the latency of positive and negative peaks was increased, as was the area under the curve. In contrast, the amplitude of peaks did not differ between conditions, indicating that the increased area of the peaks is due to slower ERPs. This may be explained by temperature dependent slowing of cellular process, such as ion pumps and vesicle release, contributing to slowed cortical firing (Sonntag & Arendt, 2019). Interestingly, torpor and the accompanying hypothermia do not appear to alter the threshold for evoking a cortical response, possibly due to compensatory mechanisms maintaining overall neural excitability as demonstrated by Padamsey and colleagues (Padamsey et al., 2021). Overall, this dataset provides novel insights into the electrophysiological state of fasting-induced torpor and how environmental stimuli are processed. Moreover, the relatively small differences found between ERPs observed during

euthermic sleep and torpor provides further evidence of torpor and sleep being closely related states. Further work may wish to investigate other forms of stimuli, such as tactile or olfactory, to investigate whether the response is altered depending on the stimulus domain. For example, one study found that the smell of smoke was sufficient to induce arousal from hibernation in pygmy possums (Nowack et al., 2016), whereas auditory stimulation did not alter torpor or induce arousal in this thesis (Chapter 7); these differences may be explained by the differing ethological relevance of these stimuli as it will be more important for survival to arouse from torpor to escape a potential fire.

The data presented in Chapters 5 to 7 provide novel observations into the regulation and modulation of torpor in relation to sleep. Moreover, it provides further evidence that sleep and torpor are closely related states that interact and balance with each other to facilitate both sleep-related and energy saving processes. This is complementary to previous work in daily torpor and hibernators, therefore expanding our knowledge of torpor by investigating the relatively understudied fasting-induced torpor. Further, this work has shown for the first time that fasting-induced torpor is also sleep depriving, in a way that is similar to previous reports in other species. This sleep depriving effect of torpor has implications for cognitive performance on behavioural tasks and may have contributed to the high levels of variability reported in Chapter 4, as it is possible that these mice had experienced varying levels of sleep deprivation depending on their torpor history which would have impacted their behavioural performance to differing degrees.

9.2 Implications of torpor for experimental design

In addition to furthering the scientific knowledge of torpor and sleep, this thesis also aimed to investigate how inadvertent torpor induction in fasted or food restricted mice may be

confounding experimental data. The findings in this thesis were gathered from data in mice undergoing a chronic food restriction paradigm which is used across research areas but is most common within behavioural research. First, it was found that torpor was readily induced in mice under this food restriction paradigm, indicating that the subsequent observations made throughout the experimental chapters will be applicable to experiments using this form of food restriction. However, previous data in the literature suggest that these findings will be applicable to other forms of food restriction also as acute fasting, involving the complete removal of food, has also been shown to result in torpor (Nowakowski et al., 2009), as has scheduled feeding during which food is provided ad libitum for a limited period of time each day (Northeast et al., 2019). As a result, this thesis has the potential to be far reaching into other experimental domains which use food restriction paradigms.

The data collected throughout this thesis suggests that food restricted mice will show profound alterations to their temporal distribution of activity and rest, which are modified to accommodate the induction of torpor. Torpor bouts were strongly influenced by the time of feeding, demonstrating the importance of standardising feeding schedules within and between studies. Lack of standardisation in feeding time can result in mice being in very different physiological states when undergoing testing or when samples are taken between studies, which may be a contributing factor as to why it is often difficult to compare data outcomes across studies. However, this may not be possible in practice due as such standardisation could result in delays in testing and feeding that would disrupt the experimental paradigm. In these cases, more transparent reporting of experimental protocols, including feeding times, may be more appropriate.

An important consideration, as highlighted in Chapter 4, is the time of behavioural testing relative to a torpor bout due to the findings reported in Chapter 4 demonstrated a significant effect on activity levels and exploratory behaviour. Depending on the experimental question and paradigm, studies in food restricted mice may wish to provide food at a time that would cause torpor bouts to be shifted away from the time at which behavioural testing will occur. For example, behavioural tasks could be conducted in the 1-2 hours before feeding time to exploit the high drive for activity at this time, termed food anticipatory behaviour (Northeast et al., 2019). However, this will also be the time at which the mice are the hungriest so it is possible that hunger itself may influence behaviour and level of task engagement. For example, substantially increased hunger levels were found to decrease reaction time, increase impulsivity, and overall decrease the ability of mice to utilise learned strategies in a rule-observance task that required mice to wait for cue in order to receive a reward (Alkahwaji et al., 2023).

However, the effects of torpor on behaviour and physiology may be more chronic than has been previously suggested. The EEG data presented in Chapter 6 indicate that torpor induced by this particular food restriction paradigm is a sleep depriving state; mice are typically maintained on this form of food restriction for many weeks at a time which may be resulting in chronic sleep deprivation. Chronic sleep deprivation is associated with multiple negative health effects including impaired immune responses and increased inflammatory markers (Besedovsky et al., 2012), metabolic alterations such as insulin resistance (Singh et al., 2022), and cognitive and motor impairment (Killgore, 2010). As a result, food restricted mice may be experiencing these effects of sleep deprivation due to regular entry into torpor over a prolonged period of time. Although a robust memory effect was not found here, a significant motor effect was reported which would impair the ability of mice to effectively navigate a task arena. It is

possible that a memory effect would have been observed if mice had been under food restriction for a longer period, or if the food restriction had been more severe. For example, a study by Carlini reported impaired performance during a novel object recognition task in mice chronically food restricted to 50% of their ad libitum food intake for 28 days (Carlini et al., 2008). For reference, mice used across this thesis were provided with 70% of their ad libitum food intake when on food restriction.

A consistent finding throughout this thesis was that a high degree of variability in torpor propensity and characteristics were observed, both between animals and within the same individual across days. As such, animals will be in differing states of physiology compared to each other and when compared to themselves, which may be contributing to the high levels of variability in outcomes being observed in the literature. The increasing awareness of poor reproducibility of scientific research, and the subsequent limitations on knowledge expansion and translation of data between species (Begley & Ioannidis, 2015; Voelkl et al., 2020), is resulting in a recognition for the need to refine experimental techniques. The work presented here highlights a potential avenue for technique refinement by ensuring torpor induction is accounted for when food restriction or fasting is used in mice and implementing appropriate controls depending on the study question. Moreover, increased standardisation of protocols is required to minimise the effects of torpor on experimental outcomes. Examples may include standardisation of food timing during food restriction, standardisation of when behavioural tasks are conducted, or physiological samples are taken and controlling for prior torpor history.

Finally, water restriction was not found to result in torpor induction in mice. This finding has two main implications from a 3Rs perspective. The first being that water restriction may represent a preferable method for motivating engagement with tasks in mice as the effects of

torpor on activity levels, behaviour, and sleep disruption would be avoided. Moreover, previous works has suggested that water and food restriction paradigms are tolerated to a similar degree, with no clear welfare benefit of one over the other (Rowland, 2007; Tucci et al., 2006). As such, the findings from this study suggest that water restriction may be the optimal method for incentivising mice to perform tasks whilst minimising the acute and long-term confounding changes to physiology, behaviour, and sleep explored throughout this thesis and elsewhere.

The second implication of this work is that data gathered using water or food restriction may not be directly comparable to one another, due to the absence of torpor in response to water restriction. Torpor has been shown to profoundly alter physiology and gene expression (Herwig et al., 2006), and this thesis has explored the impact of torpor on circadian rhythms, activity and behaviour, and sleep; therefore, mice on food restriction that are readily entering torpor, will have a significantly different physiological state compared to water restricted mice. A study by Goltstein and colleagues reported that mice undergoing food versus water restriction demonstrated differential learning behaviours in a head-fixed two-choice visual discrimination task, therefore potentially supporting this suggestion. In brief, this study found that water restricted mice were faster to reach the learning criteria when being trained to complete a two-choice operant task despite performing fewer trials overall, compared to food restricted mice (Goltstein et al., 2018). Moreover, food restricted mice travelled significantly less distance than water restricted mice when running on a Styrofoam ball (Goltstein et al., 2018). These findings, in combination with the findings presented in this thesis support the hypothesis that food and water restriction result in different physiological states, possibly caused by the presence of torpor during food restriction, that leads to differences in behaviour and motivation. Overall, these data may suggest that water restriction is preferable over food restriction to maximise motivation without introducing confounding variables due to the induction of torpor. However,

care must be taken not to compare or extrapolate findings observed in water restricted mice to food restricted mice, and vice versa.

9.3 Future directions

This thesis presents novel insights into the effect of fasting-induced torpor in mice on behaviour, activity, and sleep. Due to the exploratory nature of this work, many of the experiments were performed in smaller cohorts of animals which can make the data gathered difficult to interpret and make statistical comparisons challenging; this is especially applicable to the behavioural data in Chapter 4. As such, it will be necessary for future work to replicate these studies in more animals for the effects to be fully understood. Despite this, many of the data presented show a strong effect of torpor, even in the smaller sample size, and highlighted opportunities for further investigation as discussed below.

To build on the finding that food timing altered torpor characteristics presented in Chapter 3, additional studies are needed to further investigate the circadian control of fasting-induced torpor. Previous works have predominantly focused on non-fasted models of torpor such as hibernation (Heller & Ruby, 2004) and daily torpor in response to short day length (Ruby et al., 1989) and have shown that torpor is under circadian control. Moreover, rhythms in torpor induction were lost in SCN-ablated hamsters (Paul et al., 2004). Fasting-induced torpor represents an interesting model for circadian research due to the presence of the two competing Zeitgebers food and light, therefore further work in this model may help to further our understanding of regulation and modulation of the circadian clock. Examples of future work may include investigating torpor characteristics under constant conditions e.g., constant light and constant dark, which could be combined with a random feeding schedule to explore how torpor adapts to a lack of Zeitgeber input. These experiments could be combined with EEG

recordings to further investigate the interaction between sleep, torpor, and circadian rhythms. This would build on work presented here and on a previous study conducted in mice with mutated clock genes which found that feeding schedules outside of the circadian range disrupted torpor, but torpor was still largely driven by the central circadian clock (van der Vinne et al., 2018).

A separate aim of this thesis was to characterise the effect of torpor on subsequent behavioural performance due to conflicting reports in the literature with some showing torpor to improve (Nowakowski et al., 2009), impair (Palchykova, Crestani, et al., 2006), or have no effect (Hensleigh et al., 2022) on behaviour. Although the work presented in this thesis did not show a robust memory effect in the torpor group or the control group, it is important to note the complexity of behavioural phenotypes. As such, future work may refine the experimental procedure conducted here with additional animals or may use alternative tasks to investigate the effect of torpor on other modalities of behaviour and cognitions. For example, this thesis used a novel recognition task which is not appetitive. A non-appetitive task was chosen to allow for behavioural drive to be investigated independently of behavioural state and to maintain a constant level of motivation. However, food restriction is typically used when mice are performing an appetitive task, therefore it is important for a torpor effect to be investigated in a task that does utilise food restriction to motivate behaviour.

A large component of this thesis investigated the neurophysiology of fasting-induced torpor, and the relationship been torpor and sleep. Additional insights may be gained by recording local field potential (LFP) and multiunit activity (MUA) dynamics during sleep and torpor. Previous work has been conducted using LFP and MUA recordings during sleep which has been used to explore neuronal activity during sleep states and at different cortical levels to

explore sleep regulation (Krone et al., 2021), whereas no neuronal data during torpor has been published. Conducting LFP and MUA recordings may also add clarity into the neuronal control of torpor, a topic which is rapidly gaining popularity but more from a circuit perspective (Ambler et al., 2022; Takahashi et al., 2020), in addition to further exploring the similarities and differences between sleep and torpor at the neuronal level. A pilot study was conducted in three mice, each implanted with a laminar probe consisting of an electrode inserted into the brain tissue and spanning all cortical layers. However, due to the fragile nature of the probes and the long-term recording conditions of the experiments, there were not enough good quality recordings across conditions to analyse. Despite this, this provides an exciting avenue for further work to explore the underlying neuronal activity during torpor.

In addition to investigating torpor neurophysiology, this thesis presents novel data indicating that fasting-induced torpor is a sleep depriving state due to the increase in slow-wave activity observed in sleep following torpor which dissipated over time, as is seen following sleep deprivation (Borbély et al., 1984; Trachsel et al., 1989). As such, this provides initial evidence to support the hypothesis that fasting-induced torpor is a sleep depriving state and is aligned with work in hibernators (Trachsel et al., 1991) and species that enter photoperiod induced daily torpor (Deboer & Tobler, 2000, 2003). To further investigate fasting-induced torpor as a sleep depriving state, future studies could investigate whether sleep intensity following torpor changes in relation to the length of torpor. Sleep intensity following sleep deprivation is known to increase with increased time spent awake in a dose-dependent manner, which was used as a key piece of evidence to suggest that sleep is homeostatically regulated (Borbély, 1982). As a result, a similar approach could be taken by provoking mice to arouse after increasing lengths of torpor bouts and measuring the subsequent slow wave activity. This has been previously investigated in hibernating European ground squirrels that were provoked to arouse from

hibernation after increasing lengths of time spent in hibernation (Strijkstra & Daan, 1997). Interestingly, this study reported that EEG slow wave intensity increased with prolonged torpor duration which dissipated during sleep, mirroring the EEG dynamics observed following prolonged wake, therefore providing evidence that sleep following hibernation is homeostatically regulated. However, the findings reported in Chapter 5 demonstrate that there are important differences between fasting-induced torpor and other forms of torpor such as hibernation and photoperiod-induced daily torpor. As a result, these findings would need to be replicated using a model of fasting-induced torpor.

9.4 Overall conclusions

Overall, this thesis integrated a variety of techniques to characterise the effects of fasting-induced torpor in mice on subsequent activity, behaviour, and sleep. Further, novel insights into the regulation and modulation of both sleep and torpor are presented and show initial evidence that fasting-induced torpor is a sleep depriving state. This thesis also highlights the importance of standardising techniques and incorporating appropriate controls for sources of variability in order to produce more robust and reproducible science.

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