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The impact of burn injuries on circadian rhythm and mental health

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Word count = 13, 946

Declaration

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary education institute.

Clare Bandy

Abstract

Background: Burn injuries are well-established to cause severe, systemic repercussions within the human body, leaving few systems unaffected. These consequences extend to include psychiatric health, with studies showing that burns victims are significantly more likely to be diagnosed with an axis one mental disorder. Disruption in circadian rhythm, characterised by sleep changes and hormone secretion abnormalities, among other symptoms, exist as a commonality between the two conditions, being a documented consequence of burn injuries and an enduring theory for the aetiology and maintenance of psychiatric disorders.

Hypothesis: As measured by CBT, there will be a significant difference between the circadian rhythm of the Sham and Excision groups in comparison with the Burn group. After the procedure, burn injury mice will have significantly changed circadian rhythms both acutely (days) and chronically (weeks). Abnormal circadian cycles as a result of thermal injuries could lead to increased rates of mental illness.

Methods: Eight-week-old female mice (n =15) were implanted with a temperature logger into the peritoneal cavity to record core body temperature (CBT) for three months. Two weeks after implantation, surviving mice received a burn injury (n = 6) to 7-8% total body surface area (TBSA), an excision injury (n = 5) to the same surface area or a sham procedure in which no injury was inflicted (n = 5). The animals were euthanised after three months and thermologgers collected. Attained data was manipulated to attain the mesor, amplitude and acrophase parameters per day.

Results: There was a difference in circadian rhythm between Sham and Excision as well as Sham and Burn treatments. Burn injury was the only group to show an acute difference after procedural surgery in all parameters though Excision values also acutely changed after surgery for mesor alone. All three groups differed chronically after surgery, though only the Burn group had significantly altered acrophase in the last time-point of the experiment.

Conclusion: Circadian rhythm disruptions occur after surgery. Given that surgery is so common after burn injuries, in subsequent experiments it would be logical to assess circadian rhythm changes after the procedure using gene analysis as well as by testing other parameters such as melatonin and

cortisol. To link circadian rhythm disruptions to mental health problems, indicators such as mood and sleep in humans both acutely and chronically after burns and surgery should be recorded and compared with before the procedure and with healthy control populations.

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Acknowledgements

I'd like to first thank my supervisors Mark and Sarah, for their continued support and help throughout the year, especially in the past several days, where their availability and calmness, when I certainly was not, was truly exceptional. You have both especially helped with my writing in such a huge way. Thank you to Shane and Grace, who were always willing to help me over the last several months, I most definitely would not have been able to do it without you both and your incredible knowledge of circadian rhythms. Thank you to Fiona and the Fiona Wood Foundation for the opportunity you gave me to help with your invaluable work, there was a never a time I felt unmotivated, I was always proud to say who I worked with and what I worked on. I'd also like to acknowledge my PhD guru Amira, who's kindness and knowledge has been so invaluable. Thank you to my fellow honour's students and lab group, and a special thanks to Kiti, who got the very unfortunate job of helping me learn to cell culture. I thank you so much for your patience despite my clumsy hands and 3 second concentration span! Thank you to Andy who took me under his wing, when he had absolutely no obligation to, and both taught and helped me along the way. Though, arguably your most impressive achievement is getting me to exercise on Fridays.

Finally, thank you to my parents, who encouraged my love of science from the time I could talk, with explosions and flubber galore. Your support through the difficult times alone have got me to the achievements and happy times of today. My successes are yours. And of course, to my brothers who don't call me a dole bludging student nearly as much as they used to. I love you all immensely.

1. Review of literature

1 .1 Long-term systemic impacts of burn injuries

Although treatment of burn wounds has improved drastically, especially in infection control and resuscitation, this form of injury remains devastating in nature. In Western Australia alone, Duke et al. [(1)] found that, between 1983 and 2008, there were 23,450 admissions to hospital with burn injuries. Not only is the injury traumatic on the individual and distressing to loved ones, it is also expensive. Recovery requires a great deal of time and resources, with the average patient costing the health sector over AUD $71,000 [(2)]. Groups of individuals at higher risk of experiencing burns include children younger than 5, adults older than 65, men between the ages of 20 to 24 and Indigenous Australians [(1)]. As survival rates have improved, the focus of large amounts of research has shifted from acute physical care to long-term quality of life and psychosocial functioning [(3)]. The social consequences of such injuries are far-reaching and chronic. Combined with the physiological effect on almost every major organ system in the body, burn injuries are one of the most traumatic experiences a human can endure [(4)]. There exists two major stages post – burn: the "ebb" and "flow" phases, respectively. Taking place within the first 48 hours post-injury, the ebb phase is characterised by decreased bodily functions such as cardiac output and metabolic rate [(5)]. The flow stage is a drastic overcompensation, occurring within the first 5 days of recovery in which the circulatory system responds by pushing the body into a state of increased circulation and hypermetabolism [(5)]. This flow stage was initially thought to end upon wound closure although recent evidence has suggested that it may last several years [(5)].

Having survived the initial injury and the early stages of recovery, risks remain prominent even three years in the future [(5)]. Examples of illness susceptibility include a 50-100-fold full-body increase in bone fracture risk [(4)] as well as elevated chances of developing cardiac dysfunction and immunosuppression due to hypermetabolism [(6)]. Relevant to the central nervous system is the increased levels of endogenous stress hormones post-injury, predominantly adeno-corticotropic

hormone (ACTH) and cortisol. Specifically, cortisol in burn patients has been shown to be roughly 8-10-fold higher than in unburned subjects for a minimum of three years [5]. This hypersecretion is suggested to be a result of disruptions to the sensitive negative feedback loop that occurs following the secretion of cortisol from the hypothalamic- pituitary- adrenal (HPA) axis [7].

Further evidence of the long-term effect of burns can be found in the cardiovascular system [8]. In the acute stages of a traumatic insult to the body, sympathetic activation is vital for survival in order to compensate and heal [9]. However, when this stress response persists long-term, as has been proven in the case of burn injuries [5], the heart experiences unhealthy, damaging levels of stress mediated by catecholamine release [8]. The hallmarks of this state of stress being tachycardia, increased myocardial oxygen consumption and cardiac output, culminating in the cardiovascular system labouring beyond normal levels [8]. As such, it has been found that, after adjusting for demographic factors, those with previous burn injuries are more likely (1.46 - fold) to be admitted to hospital for circulatory system ailments during a span of 32 years, as compared with uninjured controls [9]. Specifically, the at- risk period was the first 10 - 15 years post-burn injury, after which time the cardiac related admissions between the two groups ceased to differ. Specifically, burns cohorts were overrepresented in admission for ischaemic heart disease, heart failure and cerebrovascular disease [9]. Not only are burns patients more likely to be admitted to hospital for the above conditions, their length of stay for circulatory related illnesses were three times longer than that of the uninjured cohort [9].

As alluded to above, risk of mortality remains high decades after the burn injury. In a separate Australian study by Duke et al [10] over a span of 32 years, it was found that, when compared with a control group, those with a burn injury had a 1.4 - fold higher rate of mortality with 29% of these deaths being attributable to the injury itself. Furthermore, the median age of those who died within the burn group (76 years) was lower than those without burn injury (82 years) [10]. A theory for these phenomena lies in the long-term changes that occur after a burn injury, predominantly chronic hypermetabolism [11], oxidative stress [12] and immune alterations [13]. These harmful abnormalities have been shown to increase the risk of sepsis and infection [11], cardiac dysfunction [8] and hepatomegaly [14] and as such, may be contributing factors towards higher risk of early mortality. This

study by Duke et al. [10] shows the long- term effects of burns on the victim leading to higher chances of death at an earlier age.

1.1.1 Mental Health after burn injury

An understudied though highly prevalent long-term consequence of burn injuries, and the focus of this study, is the increased long-term risk of mental illness [15]. As seen below in Table 1, it is evident that study estimates of rates of mental illness in burns cohorts vary between studies, with possible explanations for these differences being discussed below. [15-22]. A Greek study by Madianos et al. [16] found that three weeks after the burn, 46.6% of the 45 patients studied were found to have a clinically diagnosable psychological illness. After 12 months, 30 patients were re-evaluated only to find that the percentage was identical. Post-traumatic stress disorder (PTSD) being the most commonly reported disorder at both three weeks and follow-up meetings. In comparison, Meyer et al. [17] examined the long- term mental health outcomes of 101 young adults who were burned as children (an average of 14 ± 5.4 years postburn). It was concluded that 44.6% of patients had one or more axis one diagnosis at the time of questionnaire - axis one disorders including all major mental health and substance abuse diagnoses, barring personality disorders and mental retardation [23]. The lifetime rate of any mental health diagnosis in this study was 59.6% [17]. The most common diagnoses across the lifetime were of an affective disorder (44.4%) and any anxiety disorder (38%), respectively. The most prominent specific diagnoses within the affective disorder criterion were major depressive disorder (MDD) and within anxiety disorders, PTSD was most common [17].

Table 1. Consolidated rates of mental illness from studies assessing the psychological sequelae of burn injuries.

Burn injury greatly increases the likelihood of developing a psychiatric disorder. Differences between study designs may play a role in the discrepancies seen in psychiatric illness prevalence. For example, the average time between the initial injury and follow-up, number of subjects and the broadness of disorders screened differ between tests. Baseline values refer to the percentage of individuals diagnosable with a mental illness at their first presentation to the service in question after their burn injury.

Study	**Subjects (n=)**	**Diagnosable percentage (%) at baseline**	**Years to follow-up**	**Diagnosable percentage (%) at follow-up**	**Prevalent disorders**
Madianos, Papaghelis, Ioannovich & Dafni	45	45.5	1	40	PTSD & mood disorders
Meyer et al.	101	45.5	14 +/- 5.4	59.4	Any anxiety disorders
El hamaoui et al.	60	-	8 +/- 7.5	39.2	PTSD & MDD *
Dyster- Aas et al.	73	45	1	38	Major/minor depression & PTSD*
Williams & Griffiths	23	-	1	34.7	Anxiety & PTSD
Smitten, Graaf & Loey	90	-	2.2 +/- 0.8	28	MDD & generalized anxiety
Oster & Sveen	67	-	4.6 +/- 1.9	31	Any anxiety disorder

* The study tested for these diagnoses exclusively.

An Australian study of 11,967 adults burned as children replicated Meyer et al.'s finding of poorer mental health in years post-injury [(18)]. The burn cohort saw significantly higher adjusted rates of hospital admission for mental illness in the 32 years of follow-up included in the study. Although all age groups at the time of the burn recorded higher rates of admission than the control cohort (4.6% versus 1.5% respectively), those between 10 and 15 at the time of the burn had the highest rates of

admission for mental health problems as compared to the control cohort, with an adjusted incidence rate ratio of 4.90. The most common reasons for hospitalisation were alcohol-related disorders (52%), MDD (27.8%) and stress and adjustment disorders (24.9%) [18]. Another important finding of this article is that there was no significant difference in mental health admissions associated with varying severity of the burn injury. For example, the rates were similarly elevated for those who suffered severe or minor burns as compared to the uninjured controls [18]. Lastly, a study by El hamoui et al. [19] found that 55% and 23.3% of individuals who had previously reported to the burn's unit of a major Moroccan hospital over a 1- year period met the criteria for MDD and PTSD, respectively when tested 8 years later (± 7.5 years). Patients admitted for self-inflicted burns or with a history of psychiatric symptoms were excluded from this study, thus establishing a possible causal link between burns and mental health diagnoses.

There are several possible reasons for the variation in results between experiments. In some studies, little attention is given to pre-burn psychological functioning. This is especially relevant given that psychiatric ailments have been identified as risk factors in suffering a burn injury [24]. Leaving this patient history unacknowledged could lead to a relationship between burn injury and mental health in which the latter stems from the former when it is more likely mental illness is acting as the predisposing element. Some studies address this challenge by excluding patients with a history of psychiatric illness and those whose burn injury is a result of self- harm. Another explanation for the discrepancy lies in the different ways of measuring psychological distress and clinical features of psychiatric illnesses. Although questionnaires and structured interviews were the most commonly utilised method of data retrieval in the studies analysed, the specifics of the questions and subsequent outcomes can change between the time period [23] and country that the examination is performed in [25].

1.2 Social consequences of burn injuries and effects on mental health

No mental illness exists in clinical isolation. There is a plethora of factors which interact to culminate in either the presence or absence of a psychiatric disorder. Examples of such factors in the context of a burn injury include psychological trauma, social consequences of the injury and abnormalities in

physiology due to the injury [26]. Examining specifically the social domain, paediatric patients experience logistical difficulties associated with time spent away from school [27], coinciding with missed education and social experiences. In adults there is the financial strain [28], self-esteem issues [29] and isolation associated with being functionally unable to work [30]. Inability to re-enter the work force has a large effect on psychosocial wellbeing. In a study by Dyster-Aas et al. [30], it was found that burns patients who were either unemployed or on sick-leave an average of 9 years after the burn injury reported poorer outcomes in affect, body image and interpersonal relationships than those back in the workforce. This is strong evidence for the importance of reintegration after injury and the interplay between psychological health and social functioning.

Given the social stigma surrounding the presence of body abnormalities, such as scarring or amputation, it is unsurprising that issues such as body image distress, social anxiety and depression have been linked back to an individual's sense of physical identity [29]. As such, the effect of visible body disfigurements upon psychological and social wellbeing has been extensively investigated. For example, examining 224 burn survivors seeking reconstructive surgery for body disfigurement post-burn injury, 46% showed at least mild signs of depression [29]. Thombs et al. also found that in the development of depression in a burn population, body dissatisfaction, rather than location, size or severity of scarring, was the most vital predictor [31]. Similarly, it was found that body dissatisfaction was the most reliable predictor of 12-month post-burn psychosocial functioning [31]. Alternatively, a questionnaire-based study by Williams & Griffiths looking at mental health one year post-burn found that not only were over a third of patients suffering from at least one mental health problem, the highest prevalence being anxiety and MDD respectively, the most significant predictor of these psychological issues was visibility of scarring [15]. Findings from these studies show that the effect of body image dissatisfaction cannot be understated, highlighting the need for ongoing support related to body distress in burns victims.

The effects of trauma and social ramifications on the development of mental illness post-burn is eliminated using mouse models. By anaesthetising the animals before the burn surgery and using pharmaceuticals to limit pain, the effect of trauma is made negligible. Similarly, the social issues that

humans face after burns such as isolation, embarrassment due to disfigurements, therapeutic garments and new bodily limitations are not present in a mouse model [32]. It was found by a pilot study by another group within our laboratory that even within this controlled environment, depression- like behaviours were still increased in mice months after a non-severe thermal injury (Allahham, unpublished). This suggests that despite severe social ramifications from a burn experience, there may also be a physiological mechanism underpinning the relationship between burn injuries and increased rates of mental health disorders.

1.3 Sleep changes after burn injury and circadian rhythm disruptions

Sleep is a measurable example of the body's circadian rhythm in action. As such, sleep efficacy can be used as a benchmark to ascertain disruptions in circadian rhythm. Sleep is controlled by the hormone melatonin which is produced and released by the pineal gland situated in the circadian rhythm control centre of the brain, the suprachiasmatic nuclei (SCN) [33]. The hormone is predominantly responsible for the initiation and maintenance of tiredness [34]. Studies have also shown that burns can cause severe, chronic sleep abnormalities [35]. A study of long-term sleep efficacy after burn injury found that quality drops significantly not only acutely but remains poor an average of 9 - years after the injury [36]. Specifically, the researchers reported decreased restorative REM sleep and greater superficial stage one and two sleep. This lack of REM sleep is deleterious for metabolic [37] and immune functioning [38] and quality of life [39]. Another piece of literature, which used retrospective surveys as a mechanism of self- reporting sleep quality, found that 61% of burn patients indicated "poor" sleep while only 31% of controls registered the same deficits [35]. This could potentially be a consequence of decreased night-time melatonin release as was found to take place after a burn [34]. A serious state of pathophysiology, sleep inadequacies have been implicated in hypertension [40], heart disease [41] and stroke [41]. Sleep deficits have also been found to chronically activate the hypothalamic- pituitary- adrenal (HPA) axis [42]. As will be discussed in detail, a great deal of literature focuses on the relationship between hypercortisolemia as a result of a hyperactive

HPA system and mood disturbances. As such, these sleep deficits due to burn injury may have a role to play in increasing the risk of mental health disorders post-injury [43].

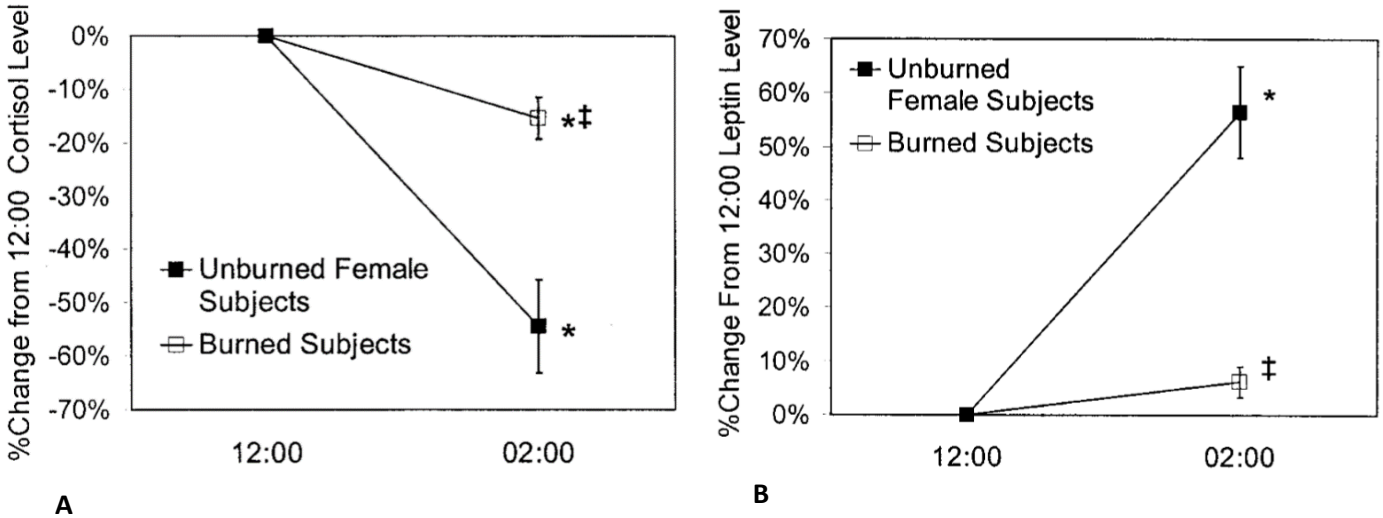


Figure 1. The concentration changes of a.) cortisol and b.) leptin after burn injuries.

There is an evident loss of normal diurnal pattern from the approximate peak (12:00) to trough (02:00), also termed the nadir, in cortisol level. The small change between the two points in burned females shows the blunting of the nadir. Image B shows no evident difference in leptin concentration between the peak and nadir. These two hormones show prominent circadian rhythm abnormalities post- burn. Images adapted from a patient study by Hobson et al. [44] (n = 20).

In conjunction with these sleep changes, the circadian rhythm of endocrine secretions is also lost after burn injuries [44, 45]. Studies examining the diurnal pattern of cortisol secretion found that not only is cortisol significantly oversecreted in patients of both sexes and all age groups as compared with controls, the nadir, or naturally occurring low in secretion, is substantially blunted, as shown in Figure 1 [44] which depicts only female patient's data. This finding is echoed in a small study of 10 burns victims which found that diurnal patterns of cortisol, aldosterone and renin were completely lost. In fact, all three hormones remained high at any given time over a 24 - hour period [45]. Another piece of evidence to suggest that circadian rhythm is altered by burns comes in the form of leptin, a hormone

produced by adipose tissue and associated with appetite and energy intake. Hobson et al. (Figure 1) found that the diurnal pattern of leptin was absent in patients to the point where there was no difference between levels at the normal time of peak and nadir [44].

The before-mentioned sleep deficits and diurnal hormone secretion abnormalities, specifically cortisol, aldosterone, renin and leptin, are indicative of circadian rhythm disruptions as a result of burn injuries. As will be discussed below, circadian rhythm changes are a notable hypothesis in the aetiology of mental health problems and have gathered an extensive amount of literature over multiple decades. As such, the presence of circadian rhythm disruptions after burns may act as a catalyst or exacerbating factor towards the development of mental illness, leading to an over-representation of psychiatric diagnosis within burns populations.

1.4 Mental health disorders

1.4.1 Inflammation theory

It is well established that inflammation, especially long-term, can have a deleterious effect on the human body. Implicated in ailments from cardiovascular disease [46] to cancer [47] and diabetes [48], the negative effects of inflammation have been extended to include the central nervous system (CNS) as well as psychiatric illnesses [49]. Significantly increased expression of pro-inflammatory cytokines, acute phase proteins as well as chemokines and adhesion molecules in MDD have all been widely noted [49-52]. Specifically, the most frequently linked inflammatory factors have been interleukin- 6 (IL-6) and C reactive protein within patient plasma [49]. This upregulation of the immune system is so consistent that measurement of cytokines and soluble receptors in the blood have been proposed as biomarkers for MDD [51].

Studies have shown that inflammation dysregulates concentrations of monoamines implicated in mental illnesses, predominantly serotonin, dopamine and noradrenaline [53]. Once either the cytokine is physically present in the brain or a signalling molecule, such as nuclear factor kappa B (NF-kB), has

communicated the presence of peripheral cytokines to the CNS, neurotransmitter metabolism can be altered in the synthesis, release and/or reuptake phases [53]. In the case of serotonin, inflammatory pathways activate a hormone called indoleamine 2,3 dioxygenase (IDO) which breaks down the major precursor of serotonin, tryptophan, decreasing its overall availability [53]. Supportive of this hypothesis, O'Connor et al. [54] showed that using an anti-inflammatory drug to block IDO activation after injection of lipopolysaccharides into mice allowed for the avoidance of depressive- like symptoms. Similarly, cytokines have been shown to affect the reuptake of monoamines in the brain [54]. With less serotonin available in the synapse of the brain, a pathological state of pronounced deficiency exists that is widely implicated and treated in MDD [55].

Inappropriate inflammation can also be inferred from similar studies performed on patients with a range of anxiety-spectrum illnesses. Reichenberg et al. [56] injected healthy individuals with a harmless endotoxin from the *Salmonella abortus equi* bacteria or with a saline control substance. Those subjected to the endotoxin triggered immune response reported significant anxiety and depressive symptoms, proportional to the level of circulating cytokines, up to 5 hours after the initial injection. Similarly, after adjusting for lifestyle and disease factors, Vogelzangs et al [57]. found that, in a cohort of 1237 men with existing diagnoses along the anxiety disorder spectrum, patients had increased levels of C - reactive protein. However, studies have not all been so supportive of the immune hypothesis with Schuld et al. [58] and Steptoe et al [59] finding no association between inflammation and the presence of depressive symptoms or diagnoses.

The above theory centres around the effect of inflammation on monoamines, however there is a pillar of thought which states that the behavioural changes due to inflammation are induced through the effect of cytokines on the neuroendocrine system [49]. As will be discussed further, hyperactivity of the HPA system due to stress is commonly associated with the development and presence of MDD. The body reacts to peripheral cytokines in the same way it would a physical threat; by stimulating the expression of corticotrophin-releasing hormone (CRH) and ACTH which leads to the release of cortisol [60]. For example, in a study of patients undergoing IFN- α (Interferon alpha) treatment for malignant melanomas, those who subsequently developed depressive symptoms after the

administration of IFN- α had a significantly higher initial cortisol and CRH response [61]. These results appear to display the concept of sensitization in the CRH pathway, which states that after a challenge posed to the body which renders the CRH pathway (and HPA axis) active, both systems are primed for hours or days in the future to be better able to cope with the future stresses [62].

Burn injuries prompt a massive, long-lasting immune response resulting in a surge of inflammatory factors such as cytokines as well as acute phase and complement proteins [63]. Compared with control patients, Kim et al. [64] found that pro-inflammatory cytokines Il-6, IL-8 and TNF-α, and anti-inflammatory cytokines, IL-10 and granulocyte-colony stimulating factor (G-CSF) were significantly higher in burn patients over a period of three weeks post injury with the highest difference being IL-6 on day three, almost 300 times higher in burn patients than in controls. A similar study found that even three years later, IL-6, IL-8, IL-10 and G-CSF, among other serum cytokines, remained significantly elevated, up to 2000-fold, in 977 burns victims compared with healthy controls [5].

The brain has layers of protection against threats in the form of toxins, drugs and cytokines [65]. Predominantly, this defence occurs in the form of a layer of semi-permeable endothelial cells comprising the blood brain barrier (BBB). However, the brain is not invulnerable to the massive systemic inflammation that occurs as a result of a burn injury. In a mouse study of severe burns (n = 28) by Reyes et al. [66], mRNA expression of TNF- α, interleukin- 1β and intracellular adhesion molecule – 1 (ICAM-1) in the brain were elevated compared with the control cohort at the three and 7 hour mark. This evidence of brain infiltration among other research is indicative of disruption to the integrity of the BBB [66, 67]. Given the evidence described above for the relationship between mental health and inflammation, the ability for burn injuries to damage the BBB and increase access for immune factors into the brain may be an explanation for burn patient vulnerability to psychiatric illnesses.

1.4.2. Neuroendocrine theory

Hyperactivity of the HPA system, a neuroendocrine secretion abnormality, within patients with psychiatric ailments is a highly consistent finding in the realm of neurobiology [68]. In response to

stress, and to help the body react to the potentially dangerous situations, the hippocampus secretes corticotrophin- releasing factor (CRF) and vasopressin (AVP). As shown in Figure 2, these hormones activate the secretion of ACTH from the pituitary gland. Finally, cortisol (a glucocorticoid) is released from the adrenal cortex which acts upon both the brain and peripheral target tissues [(69)].

A primed HPA acts peripherally, by regulating metabolic and immune functions, and in the brain by controlling neurogenesis (development of new neurons) [(68)]. Centrally, activation of the HPA axis has a role in higher order functioning within the hippocampus such as emotional evaluation of experiences and the formulation of new memories [(68)]. A vital negative feedback loop exists whereby the presence of cortisol inhibits the secretion of CRF, AVP and ACTH in order to avoid harmfully high levels of cortisol accumulating in the body. Most research has been performed to quantify HPA activity in mood disorders such as bipolar and MDD. It was found that the saliva, plasma and urine of patients with mood disorders contained excessive levels of cortisol [(68)]. It is believed that this hyperactivity is due to a breakdown of the negative feedback loop [(70)]. Support for this theory of inhibited negative feedback comes from a multitude of studies which have shown that patients are unable to suppress cortisol upon administration of synthetic glucocorticoids in contrast with neurotypical subjects [(71-74)]. Putting this evidence together, it is unlikely that an overactive stress response is simply a consequence of psychiatric ailments, rather a contributing predisposition.

Burn injuries, as well as their repercussions, such as multiple surgeries and sleep deprivation, are prime examples of prolonged and extreme stress. Cortisol is an important marker of stress after injury, with levels being directly proportional to the severity of the insult and clinical outcome [(75)]. The consequences of such physiological turmoil are evident, with cortisol in burns patients remaining elevated (initially 8 - to 10 - fold) three years post burn injury [(5)]. Palmieri et al. [(76)] found that cortisol levels did not correlate with levels of ACTH (the precursor to cortisol), the conclusion of which being likely disruption of the adrenal feedback loop in burned individuals. As such, lack of adrenal synchronicity is a probable outcome of the extreme stress following a burn injury and a potential elucidating agent in the development and/or maintenance of mental illness, as will be elaborated upon

below. As such, HPA axis disturbances may be an explanation or confounding factor for the high rates of mental illness seen after burn injuries.

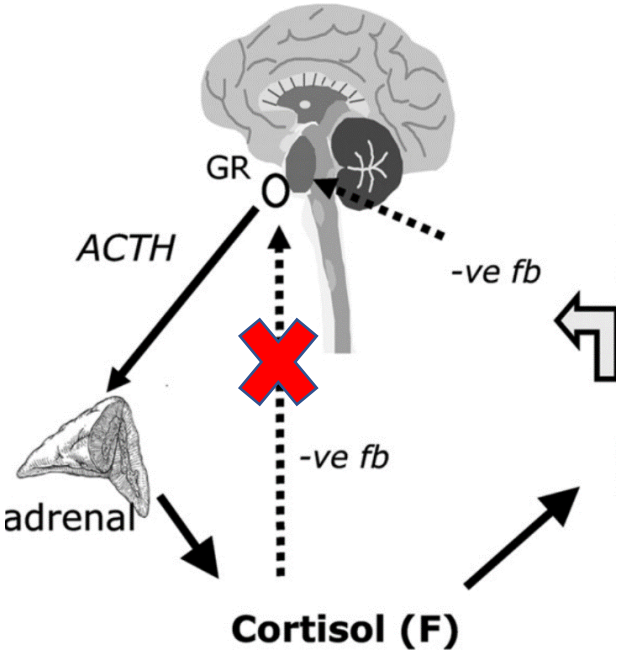


Figure 2. Negative feedback of cortisol is disrupted after burn injury.

A schematic diagram of the HPA axis in which cortisol is released from the adrenal cortex to glucocorticoid receptors in the brain. Negative feedback of cortisol regulates its own feedback through decreasing ACTH. The red cross indicates the disruption of the negative feedback loop as hypothesised in MDD. Adapted from Yehuda & Seckl [(69)].

1.4.3. HPA system in MDD

Evidence for the role of HPA hyperactivity and negative feedback in the example of MDD comes in the form of anti-depressant therapy. For example, Pariante et al. [(71)] found that citalopram, a commonly administered selective serotonin reuptake inhibitor, increases HPA axis negative feedback as a result of improved rates of glucocorticoid binding. This leads to a rate of suppression, upon administration of a steroidal drug, that is over 20% higher. Taking a slightly different approach, Dinan

et al.[72] found that the administration of a glucocorticoid agonist, in this case dexamethasone, which activates the glucocorticoid receptor, improves mood difficulties in those with treatment- resistant depression for a period of 16 days post-discontinuation. Although only a small sample of 10, this finding, which is echoed by others with similar methods [73,74], is interesting in that those with the highest baseline cortisol levels pre-administration were found to respond best to the dexamethasone therapy. The authors believe that these effects can be attributed to dexamethasone's ability to enhance negative feedback within the HPA system to decrease cortisol levels, resulting in mood improvements [72].

Studies into a group of patients with Cushing's syndrome, a disorder in which hypercortisolemia is so clinically extreme it leads to cognitive dysfunction, muscle weakness and decreased immune competence, shows that out of 29 patients, 25 also displayed significant depression [77]. Likewise, Sonino et al. [78] found that 62% of Cushing syndrome patients showed major depressive symptoms. Although a distinct diagnosis, Cushing's syndrome shares certain physiological traits with MDD. Cerebrospinal fluid CRH levels in MDD have even been found to overlap the clinical range diagnosable for Cushing's syndrome [79]. This link between extreme cortisol levels and mood disruptions shows the relevance of corticosteroids in the development of mood disorders and could share clinical similarities to patients with hyperactivity of the HPA axis, as is often found in unipolar depression.

1.5 Circadian rhythm and mental health

Circadian rhythm is a cyclical phenomenon maintaining the body's endogenous period of roughly 24 hours [33]. The orchestrater, or master clock, is within the hypothalamus, specifically the SCN, which sends output signals to other parts of the brain as well as to peripheral targets which regulate cell physiology [33]. In these target cells, transcription factors CLOCK and BMAL1 drive the rhythmical expression of clock genes, *Period* and *Cytochrome,* that act as negative feedback to coordinate their own expression [81]. The SCN is entrained by light/dark cycles received by retinal photoreceptors that provide synchronisation of molecular oscillators present in most body tissues. The sleep/ wake cycle

is the most recognised circadian rhythm, however, multiple systems, such as core temperature, endocrine release [81] and bodily healing [82] also reflect a 24 - hour rhythm.

The association between circadian rhythm disruptions and neuropsychiatric illnesses is by no means a modern realisation. Even from the late 1880s sleep abnormalities begun to be associated with mental health ailments [83]. Sleep complaints are recorded in 80% of schizophrenic and MDD patients and in conjunction, drugs which target circadian rhythm, specifically *CLOCK* genes, have been found to be beneficial in the treatment of mental illnesses [83]. A well-documented example being the bipolar medication, lithium, which works to lengthen the circadian period by increasing *Per2* transcription [84]. Genetic studies in humans have supported a link between circadian genes and mental illness though attaining conclusions have proved difficult, given the shared symptoms and genes across diagnoses and polygenic nature of mental illnesses [85]. However, genome-wide association studies (GWAS) have ascertained a linkage between circadian rhythm genes and bipolar and schizophrenia. For example, *DISC1* gene variants have been found to be prevalent in schizophrenia and bipolar disorder though also associated with homeostatic regulation of sleep [86].

1.5.1 Measuring circadian rhythm

Early experiments focusing on circadian rhythm collected data through the examination of physical activity among mammals in relation to light/dark hours [33]. As technology improved, techniques for monitoring circadian cycles shifted to more quantitative measures. The first example being the measurement of melatonin secretion over 24 hours which, as discussed previously, acts as a robust measure of the individual's sleep cycle and reaction to external stimuli [87]. Similarly, cortisol secretion from the adrenal cortex displays a 24 - hour pattern, being measurable in saliva and serum [87]. To analyse and draw statistically significant conclusions from these values of secretion, a least-squares cosinor fitting analysis is applied to attain measurable parameters [88]. These parameters include the mesor (mean level of, in this scenario, cortisol, based on the distribution of values), amplitude (the height around which the value deviates from the mesor) and the acrophase (time of maximum value) [88], all of which depicted in Figure 3. These results can be compared among

subjects, pre- and post-intervention or the like. The above-mentioned statistical parameters will be applied to our analysis of core body temperature (CBT). CBT displays a reliable, roughly 24 - hour rhythm, reaching its minimum at approximately 5 AM and maximum at around 5 PM and, as such, can be used to measure circadian rhythm [87].

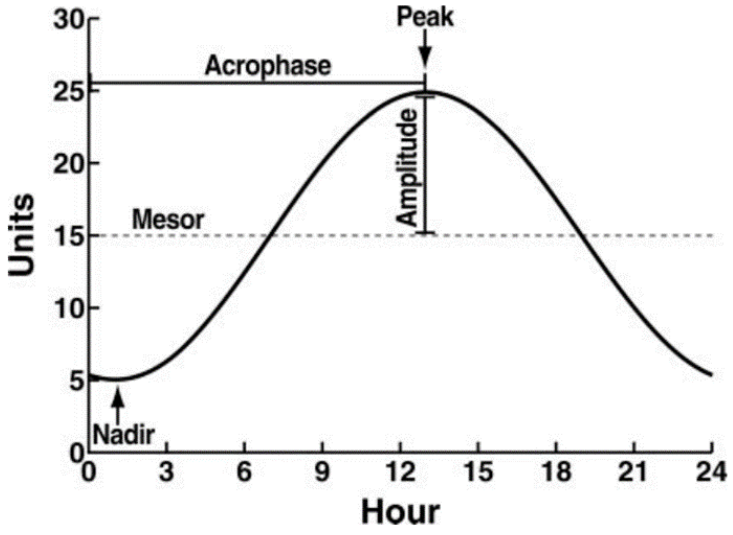


Figure 3. Example circadian rhythm trace with depiction of the statistical parameters utilised in the current experiment.

Specifically, mesor, the midpoint value, amplitude, the degree difference between acrophase and mesor, and acrophase, time of peak temperature in hours relative to midnight. Adapted from O'Dell et al. [89]

1.5.2 Depression and circadian rhythm abnormalities

Several features of major depression have associations with human circadian rhythm. Physiological premises such as natural daily mood variations [90], sleep and excretory patterns of hormones [91] have all been found to be abnormal in MDD. Cortisol is seen to be chronically hyper- secreted in MDD, though the specific chronobiological abnormalities are inconsistent. In a study by Linkowski et al. [91] of 18 depressive patients and 7 age-matched controls, it was found that cortisol was released in greater magnitude per secretory episode as opposed to an increase in the number of secretory episodes.

Furthermore, the nadir for both cortisol and its precursor, ACTH, arrived earlier than in the control patients, suggesting that circadian timing abnormalities, as well as errors in the function of the HPA axis, may characterise MDD. Alternatively, a study by Deuschle et al. [92], found that pulse magnitude remained unchanged, but secretory frequency rose significantly in depressed males. Furthermore, the study did not show differences in nadir timing rather a shortened quiescent period duration (phase of inactivity) by almost 50% in depressed patients [92].

With 90% of patients experiencing sleep changes during an acute depressive episode, it is unsurprising that sleep has been a major point of interest for researchers in the last 50 years [83]. In neurotypical individuals, an event called the dim light melatonin onset (DLMO), in which melatonin levels reach a predicted peak, occurs roughly one to two hours before the onset of sleep [93]. In women with MDD, the time period, or phase difference, between DLMO and sleep onset is significantly longer [94]. Furthermore, the length of this interval was directly correlated with symptom severity, for example, the longer the phase difference, the more severe the depressive symptoms [94]. This desynchrony was also found when comparing midsleep (middle point between sleep onset and sleep offset) to core body temperature minimum, which saw time periods significantly shorter in depressed patients as compared with healthy controls [95]. Release of melatonin is the predominant factor of analysis in these tests and is directly controlled by the circadian "master-clock", the SCN, as such, these phase differences are strongly indicative of significant circadian misalignment.

In regards to the physiological abnormalities related to sleep in depressed patients, rapid eye movement (REM) sleep is commonly found to be irregular. REM latency, the time between the onset of sleep and the first REM period, is often shortened as well as the duration of this first REM period being significantly increased [96]. Slow-wave sleep, which is the least superficial, most restorative non-REM sleep period, is decreased in patients diagnosed with MDD, evidence of marked sleep fragmentation. These abnormalities in sleep architecture are also associated with increased risk of a relapse in symptoms [96].

1.5.3 Anxiety disorders and circadian rhythm abnormalities

In comparison with mood disorders, less is known about the relationship between circadian rhythm abnormalities and anxiety spectrum disorders. An important finding in this area was the confirmation of a widely held clinical observation; there is a tendency for patient anxiety symptoms to be worsened late in the afternoon/ early evening as compared with other times in the day. Cameron et al.'s finding from 86 patients with agoraphobia or panic disorder was the first demonstration of circadian driven mood variations in a mental illness other than MDD [(97)].

Circadian rhythm genes analysed through single nucleotide polymorphisms (SNPs) have been shown to play a role in genetic predisposition to several clinically recognised anxiety disorders. Six SNPs from two circadian-clock-related genes were found to be relevant to social phobia, generalized anxiety and anxiety disorders collectively [(98)]. This study of 321 Finnish patients displays evidence for the role of circadian rhythm gene polymorphisms in predisposing individuals to anxiety disorders of any form, consequently providing further linkage between circadian rhythm and specific mental illnesses [(98)].

1.6 Burns, circadian rhythm and mental health: Putting it all together

In the research reviewed, there appears to be a complicated relationship between the presence of mental health issues after burn injury and circadian rhythm disruptions. Numerous studies have analysed one or two of these phenomena collectively though none, to our knowledge, have attempted to ascertain any causal link between the three. Mouse models of injuries have already shown that rates of depression are significantly higher in burned mice versus control and excised mice (Allaham, unpublished). In human observational studies it is evident that mental health issues are more prevalent in burns populations than in uninjured counterparts with the risk ranging from 25% [(99)] to 55% [(19)] in the literature reviewed. Despite being a prevalent, distressing complication for burns patients, the mechanisms behind these increased rates are unknown. Circadian rhythm disruptions act as a common thread between both burns injuries and mental health issues, specifically as a consequence of the former and a theorised causative factor or exacerbating agent of the latter.

As previously reported, mental health disorders including MDD, anxiety and schizophrenia, have substantial circadian rhythm abnormalities with symptoms that largely overlap with the long-term consequences of a burn injury, predominantly hyperactivity of the HPA axis, hormone secretion abnormalities and sleep inefficiencies.

1.7 Present study

In this project, a mouse model of study was used to investigate the body's in-built endogenous rhythms, namely circadian rhythms, which have been shown to be disrupted in burn injury patients. 18 mice were allocated to one of three groups, Burn, Excision or Sham. The inclusion of an Excision group allowing for the differentiation of circadian rhythm disruptions between generic trauma and burn injuries. All 18 female mice were anaesthetised and shaved, and the burn and excision surgeries performed to an area of 7-8% TBSA (total body surface area) - a non-severe burn. Female mice alone were used due to evidence that women are significantly more likely to report psychopathologies following burn injury [17, 100].

Without knowledge about the ways in which burns lead to increased negative mental health outcomes, medical health professionals are forever one step behind, treating psychopathologies as they arise even years in the future. Understanding how circadian rhythms change after burns and contribution of this phenomena to mental health issues would be the first step towards developing preventative measures post-injury to minimise harm. An example of such being through the prophylactic administration of circadian rhythm correcting drugs, such as melatonergic antidepressants, to burns survivors deemed in a "high-risk" category to avoid the development of psychopathologies [101].

1.8 Aims

Aim 1: To ascertain circadian rhythm disruptions in core body temperature (CBT) present after burn, excision and sham surgeries through computational analysis. Comparing diurnal CBT patterns

between the three surgical groups allows for abnormalities present to be attributable to either the procedure, generic trauma or the burn injury itself.

Aim 2: To quantify differences between pre- and post- surgery periods, both acutely and chronically, for Burn, Excision and Sham group's mesor, acrophase and amplitude.

Hypothesis: Burn injury will result in circadian rhythm disruptions, as displayed by abnormal CBT patterns, specifically, in comparison to the Sham surgery. There will be differences in the Burn group between pre- and post-surgery and although disturbances will be present in the Excision group after surgery, the burned mice will have more pronounced and severe changes. The Sham group will see no significant changes between time points.

2. Methods and materials

2.1 Animals

18 mice of the strain C57BL/6J were procured from the Animal Resource Centre and housed at the Pre-Clinical Facility (PCF) on the University of Western Australia, Crawley campus, for the duration of the experiment. The 8-week-old female mice (n = 18) were kept in spacious caging in groups of three to allow for social interaction. Standard hard food was provided for the mice, excluding two days after the surgical procedure where soft food was administered to ease digestion. Enviro-dri nesting material, paper towels and tissues as well as gnawing blocks, tunnels and tubing were included in the cages for hygiene and mental stimulation, respectively. In the PCF, a standard light/ dark cycle of 12:12 hours was strictly adhered to in which lights were turned on at 0700 and turned off at 1900 hours.

All individuals handling or working with the mice completed the Programme in Animal Welfare, Ethics and Science course and attained a Permission to Use Animals license. An ethics approval number was also acquired from the Animal Ethic Committee at the University of Western Australia (RA/3/100/1624).

2.2 Experimental Overview

Mice were randomly assigned to one of three groups, Burn injury (n = 6), Excision (n = 6) or Sham (n = 6). The mice were allowed to acclimatise to the PCF facility for 5 days in order to eliminate the effect of an inevitable stress response as a reaction to a change of environment. As shown in Figure 4, on the fifth day, temperature loggers were surgically implanted into the peritoneal cavity of all 18 mice. After the procedure (see section 2.3 below), and for 5 days to come, the mice were monitored daily to search for signs of distress, post-surgery infection or anxiety. Specific symptoms of distress searched for included lack of standard socialisation habits, poor bodily conditioning, orbital tightening and flattened ear positioning among others (See Burkholder et al. for comprehensive description and list) [102]. Temperature was continually recorded every 5 minutes for a further 14 days to accumulate baseline data to be used as a control and point of comparison between the post-burn/excision/sham results. On Day 15, the 16 mice – due to the death of two mice as a result of the thermologger implantation surgery- underwent the intervention procedures, being either exposed to a burn (n = 6), an excision wound of the same surface area (n = 5) or to a sham method (n = 5), all of which under general anaesthetic. For 5 days after the operations, the mice were monitored daily for signs of pain and distress which, if present, would be further examined and treated by trained veterinary staff. The mice were then examined twice weekly until the three-month mark where they were euthanised and temperature loggers retrieved for computational data analysis.

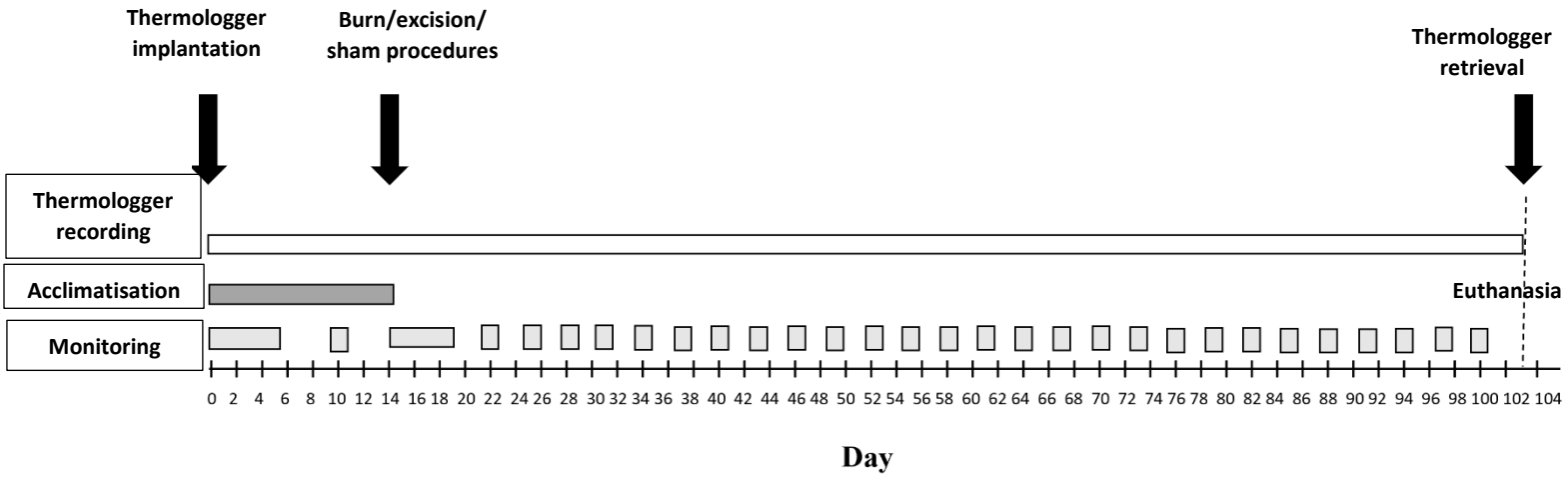


Figure 4. Timeline of experimentation.

2.3 Temperature Logger Implantation

The temperature loggers were of the model DST nano – T, 6 mm in diameter and 17mm in length. Each logger weighed one gram and was guaranteed for accuracy within ± 0.2 °C of the true temperature [103]. The loggers recorded CBT once every 5 minutes for a total of three months. Although unable to be irradiated or autoclaved, the thermologgers were sterilized overnight with a chlorhexidine/alcohol (75%) solution and rinsed with sterile water before the implantation procedure.

The animals were anaesthetised through inhalation of isoflurane in its liquid vapour form (100% v/v). During the initial induction, which took place with the mice inside a chamber, isoflurane was administered at 4% oxygen rate. For the remaining procedure, isoflurane was maintained at 2% oxygen rate via a mask. The abdomen of each mouse was shaved with fine-toothed clippers and scrubbed with 4% chlorhexidine thrice followed by a final surgical scrub with 0.5% chlorhexidine in a 70% ethanol solution. Following the application of a sterile drape, the incision site was injected with a solution of 0.25 mL of 2% lignocaine and adrenaline subcutaneously to reduce pain in the area. To insert the thermologger, a 4 mm laparotomy was performed along the midline (linea alba) of the stomach and into the peritoneal cavity of each mouse.

The muscular layer of the incision wound was closed using dissolvable sutures. To reduce the risk of gnawing wounds and self-inflicted injuries, intradermal sutures were used for skin layers. To aid in recovery, the mice were left on heating pads until deemed completely dry. As a method of post-operative analgesia, the mice were injected with 0.05-0.1 mg/ kg buprenorphine subcutaneously prior to regaining consciousness.

2.4 Burn/sham/excision procedure

14 days later, the surviving mice (n = 16) underwent the experimental procedure. The experimental surgery was carried out by chief investigator, Dr Mark Fear, to limit error and ensure consistency of results. The same anaesthetic method was utilised as with the temperature logger implantation,

through 100% (v/v) vapour liquid isoflurane administered at 4% oxygen rate within the chamber and then maintained at 2% oxygen rate. The right dorsal flank of all animals was shaved, without generating injury. To avoid pain and restriction to movement, the area shaved and operated upon was distal to the limb joint. The sham group had no further procedures performed and were left to recover, with initiatives to aid in comfortable recuperation described below.

Whilst anaesthetised and having been shaved, 4% chlorhexidine scrub was administered to the injury site to minimise the risk of infection. In the Burn cohort, a 19 mm (diameter) brass rod was heated to a temperature of >90 ^{o}C. The rod was applied to the shaved dorsal flank for 10 seconds, creating a full thickness burn wound of 7-8% TBSA. Full thickness injuries damage both skin layers and disable nerve endings, limiting the pain associated with recovery.

4% chlorhexidine scrub was similarly applied to the shaved area of the Excision mice. To provide a guide for the excision procedure, a 12 mm diameter punch biopsy was used to score the skin. A full thickness excision was created covering the same TBSA (7-8%) as the burn injury. After completion of the excision wound only, 0.1 mL Xylocaine was administered to provide numbing to the area whilst recovery takes place. Similarly, analgesia was provided in the form of 0.1 mg/kg dosage of Buprenorphine.

After the procedure, the mice were housed in separate cages to recover before being moved back to their communal cages containing two to three mice each. 1 mg/mL of Paracetamol was integrated into each cage's drinking water to reduce recovery pain. As an approximation, each mouse consumed roughly 20 mg of Paracetamol per day.

2.5 Euthanasia and logger retrieval

At the three- month timepoint, the mice were euthanised for collection of the temperature loggers and analysis of results. One mouse at a time was injected with Pentobarbitone at a dose of 160 mg/kg into the peritoneal cavity and monitored until unconscious. Once sufficiently anaesthetised, the mouse was sacrificed through decapitation. The logger was removed by making a sagittal excision along the linea

alba and manually retrieving the device. Until use, the loggers were housed in phosphate buffered saline (PBS) solution at room temperature.

2.6 Data Analysis

2.6.1 Calibration and drift correction

Upon calibrating the thermologger data from known bath temperatures (33, 36, 39 and 42 °C), it was found that there was a small but significant drift in temperatures over the three month time frame. Before adjusting the data, the thermologgers varied from 0.008 °C to 1.7 °C (χ= 0.554 °C ± 0.624 °C) during the second calibration period. A sliding correction was performed on all data points using the discrepancies found from the first calibration. The linear slope of the standard errors per calibration time point against the mean temperature per calibration point was taken for each thermologger along with the intercept, giving a complete correction formula. Each logger's correction values are shown in Table 2.

The second calibration was used to validate the changes. It was deemed to be successful given that the error of thermologgers subsequently varied between 0.0013 °C to 0.048 °C (χ= 0.0026 °C ± 0.0206 °C), evidence of drift correction. After removing the calibration periods, analysis was performed purely on the drift amended data.

Table 2. Accumulated correction formula slope and intercepts for each thermologger.

Values used to develop the corrected data points based on the first calibration period, with the equation for each calibrated data point being:

Corrected data point = (Original temperature * Slope + Intercept) * (Row # -252)/ 32262

	CORRECTION FORMULA	
THERMOLOGGER	SLOPE	INTERCEPT
N3899	-0.000338951	-0.358590968
N3900	-0.00005.022	0.032466229
N3901	-0.002365116	0.091714822
N3902	-0.001830845	0.060878426
N3906	0.008731465	-0.319124331
N3945	0.031067016	-2.415627034
N3846	0.005432412	-0.266571861
N3948	0.026308	-1.907352953
N3949	-0.001944337	0.054996516
N3950	-0.00177327	0.071183216
N3951	0.00426375	-1.753440486
N3954	-0.005079103	0.20679759
N3955	0.015698422	-1.73701326
N3956	0.052804919	-3.594326879
N3957	0.00428538	-0.125764445

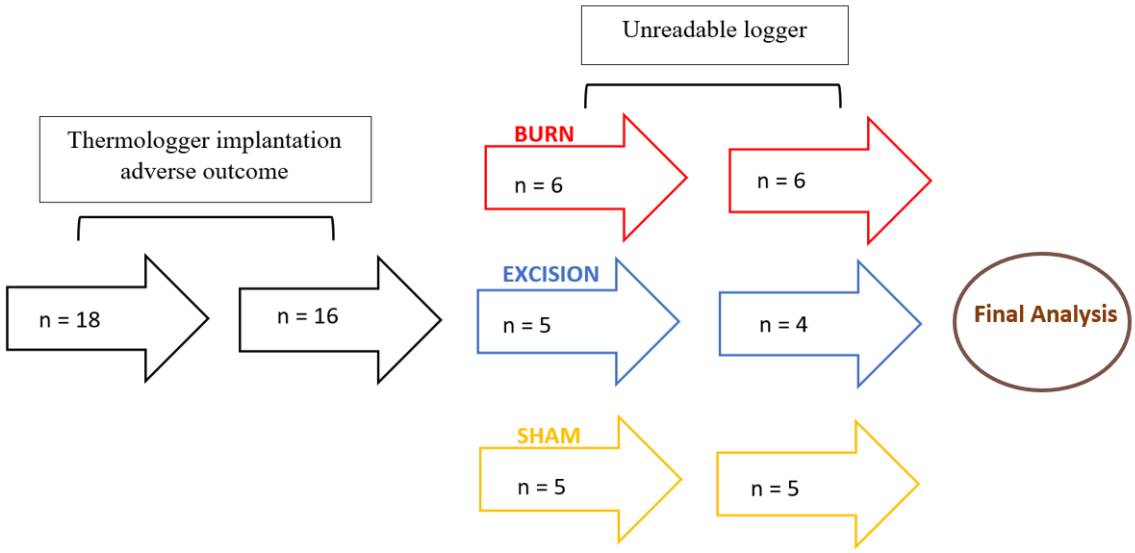


Figure 5. Changes in the amount of viable thermologger data per surgical group.

Before insertion of the thermologgers, each mouse was randomly allocated to one of three groups, Burn, Excision or Sham (all n = 6). Two mice died unexpectedly as a result of logger implantation, one from the Sham group (n = 5) and one from the Excision group (n = 5). Upon animal euthanasia and logger retrieval, one thermologger from the Excision group was unreadable (n = 4; total n = 15).

2.6.2 Cage cleaning effect

Mice cages were cleaned professionally within the PCF fortnightly starting from the 12th of April (starting date of experiment being 1st of April). Upon visual examination of the data, body temperature is significantly altered on the day of, and several days post, cage cleaning in all three statistical parameters. This is likely due to the anxiety associated with human handling, noise and movement. As shown below in Figure 6 of daily amplitude (though also true for acrophase and mesor), the cleaning days and days post- cleaning have sustained, irregular averages. However, the mice varied on how many days taken to return to a steady state. At a maximum, it was decided that this return took 7 days. It was therefore decided to discount all data points 7 days post-cleaning for the amplitude, mesor and acrophase of the data set. The days after the thermologger surgery (one to four) and that of the surgical procedures (Days 14 and onwards), also had large deviations, due to stress of

surgery, drugs, or anaesthetic and were removed from further analyses as part of the 7 days post cleaning. Each set of 7 days (Period) included in the analysis was ordered from one to 7, as displayed in Figure 6.

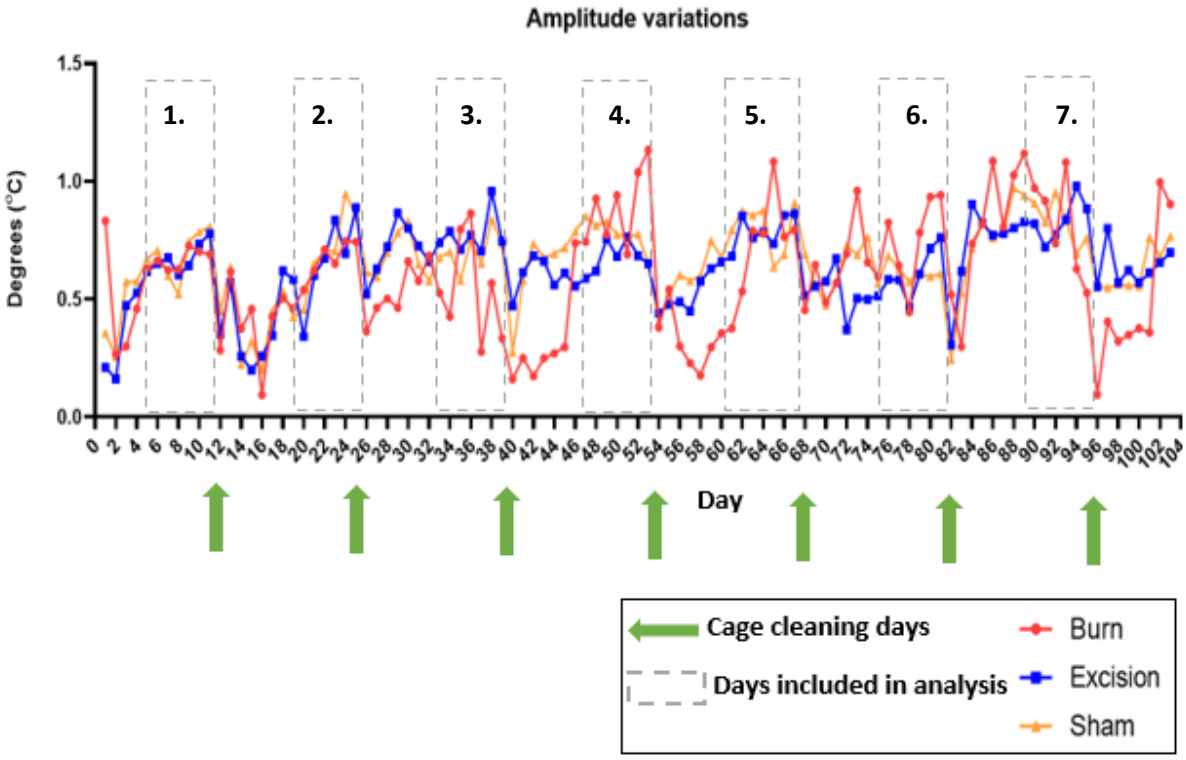


Figure 6. Fortnightly cage cleaning has an observable effect on circadian rhythm of all procedural groups for several days.

The mean amplitude of all mice per surgical procedure is shown for each day as evidence of the effect of the cage cleaning procedure on CBT. There is a visible change in amplitude on the day of cleaning, characterised by a sharp decrease. Also highlighted in the data, this change is also sustained over several days, in which results slowly rise back to pre-cleaning levels over a period of roughly 7 days. The boxed areas indicate the days included in analysis and the number of its corresponding Period. The same patterns are visible in all statistical parameters.

2.6.3 Statistical analysis

Using Microsoft Excel, the remaining data points were fitted with a least-squares cosinor regression curve to gain statistical parameters. These parameters per day include the mesor, amplitude and the acrophase. All subsequent statistical tests were performed using R studio and graphical constructions developed using Prism 8. A test for outliers found no significantly irregular points in the mesor, acrophase and amplitude data. Grouped data was checked for Gaussian distribution by using a Shapiro- Wilk test in conjunction with analysing corresponding frequency histograms for symmetry.

Significant issues were found with the assumption of normality and, as such, non-parametric tests were used throughout. Procedural group variations were tested using a Kruskal -Wallis test given independence between assumptions and one independent variable with three levels. Similarly, differences between the 7 time Periods were compared using a Kruskal- Wallis test. Differences between acute pre- and post-surgery days were attained using a non-parametric test, Wilcoxon signed-rank test (Wilcoxon test), given a continuous dependent variable. Specifically, paired Wilcoxon tests were used given that the same mice were being tested before and after the intervention. Given that there were differing amounts of days per period in the chronic testing (pre: 7 days; post: 42 days), a pairwise Wilcoxon test was used though with a false discovery rate correction, due to the effect on error attributed to multiple tests. Similarly, to decipher whether circadian rhythm changes were still present before euthanasia (Period 7), a paired Wilcoxon test was utilised.

3. Results

To calculate statistics and be able to compare and analyse circadian rhythms, data was transformed into amplitude, mesor and acrophase values, as demonstrated in Figure 7. CBT circadian rhythm results will henceforth be displayed and discussed in terms of these statistical parameters.

In Section 3.1, the three parameters were compared between the Burn, Excision and Sham using each group’s compiled averages per day to ascertain significant differences attributable

to surgical procedure. In Section 3.2, acute differences between pre- and post- surgery acrophase, mesor and amplitude in all procedural groups were analysed by comparing the averages from two days before surgery (Days 13 and 14) and two days after surgery (Days 16 and 17). In 3.3, which looks at the chronic effect of burns, Periods (which were blocks of 7 compiled days grouped together and included in analysis - as depicted in the dotted boxes of Figure 6) one through 7 were compared between each other for each group and parameter, again by compiling each group's averages per day. In conjunction, Period one (the before surgery timepoint) was compared with Period 7 (the last Period before euthanasia) for each group and parameter.

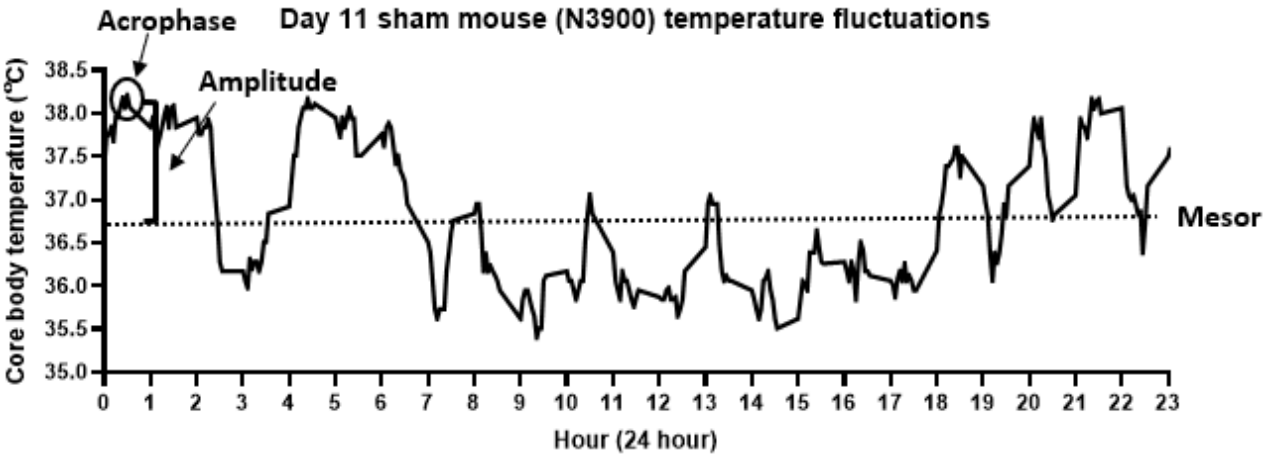


Figure 7. Sham mouse (N3900) Day 11 CBT trace.

A trace of a single Sham mouse's (N3900) mean CBT per hour on Day 11, illustrating the key CBT parameters considered in this thesis. Acrophase is the time of peak temperature in hours relative to midnight. Mesor is the mean value based around the distribution of data in degrees Celsius and amplitude is the temperature difference between acrophase and mesor, also in degrees Celsius. In this example, the acrophase is 40 minutes past midnight, mesor is 36.8 °C and amplitude is 1.4 °C.

3.1 Impact of experimental condition on daily CBT fluctuations

There was no significant difference between Burn, Excision and Sham groups in mesor or amplitude (Figure 8A and C). There was a significant difference, however, between acrophase in the Excision group and in the Sham group with those having undergone the Excision procedure reaching their peak CBT roughly 28 minutes earlier than those of the Sham procedure (Kruskall Wallis, $P < 0.001$). The acrophase of the Burn group was roughly 16 minutes later than that of the Excision group, as displayed in Figure 8B (Kruskall Wallis, $P < 0.05$).

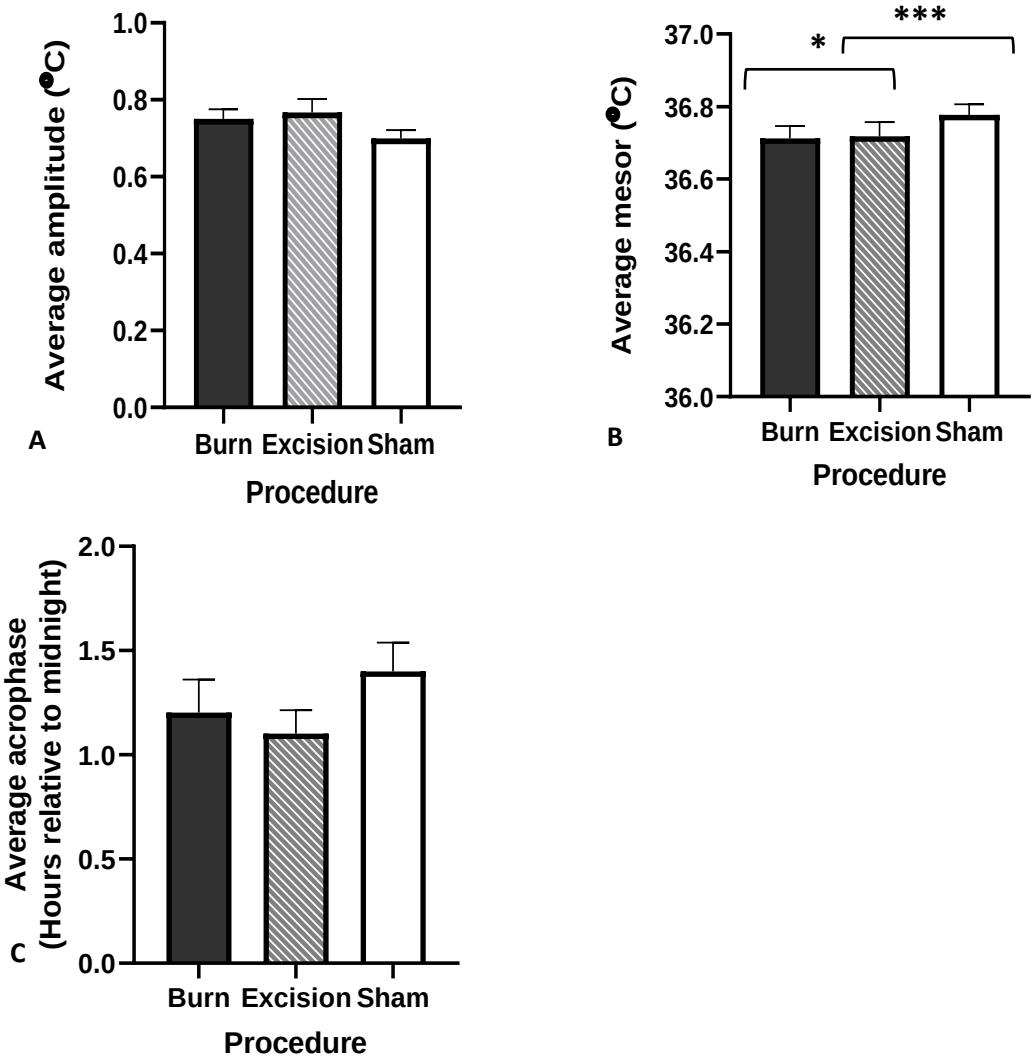


Figure 8. CBT acrophase, though not mesor or amplitude is differentially impacted by Burn injury compared with Excision or Sham surgery.

CBT acrophase (A) was significantly later in Burn (black bars, n = 6), compared with Excision (hashed bars, n = 4) animals (*, Kruskall Wallis, $P < 0.05$). By contrast, acrophase in Sham animals (white bars, n = 5) was later than for Excision animals (***, Kruskal-Wallis test, $P < 0.001$). Neither mesor (B) nor the amplitude (C) of variations were different between the three conditions. The error bars in this figure and all subsequent figures represents standard error mean (SEM), in this case for each procedural group.

3.2 Acute CBT circadian rhythm changes after surgery

Next examined was the acute effect of surgery on circadian rhythm, depending upon the injury received. Comparing two days before and after the surgical procedure for all groups allows conclusions to be drawn regarding the acute (daily) effect rather than purely the long term, chronic, (weekly) consequences of surgery. Similarly, it allowed for comparisons to be made between the current study and other pieces of literature, most of which rarely examining beyond several days either side of the surgical procedures [104-108]. As such, two days pre- surgery (Day 13 and 14) were used as base levels with Days 16 and 17 acting as acute post-surgery values. Given that the same mice were being sampled on either side of the surgical procedure the assumption of independence for an unpaired test was not met, therefore a paired Wilcoxon signed rank test was used.

The burned mice were the only procedural group which demonstrated significant differences between pre- and post- surgery time points in all three parameters, being significant for mesor and acrophase values. Burn group CBT amplitude (Wilcoxon, $P < 0.05$) and mesor (Wilcoxon, $P < 0.001$) decreased pre- to post-surgery both at a magnitude of 0.24 °C. Burn group acrophase decreased (Wilcoxon, $P < 0.001$), moving earlier in the day, by four hours and 48 minutes (from approximately 3 am to 10:20 pm). The Excision group's mesor also decreased significantly (0.13 °C) (Wilcoxon, $P < 0.05$). Sham values were not significantly affected in any parameter. This suggests that trauma, though not generic surgery is capable of changing circadian rhythm in the first two days after the procedure.

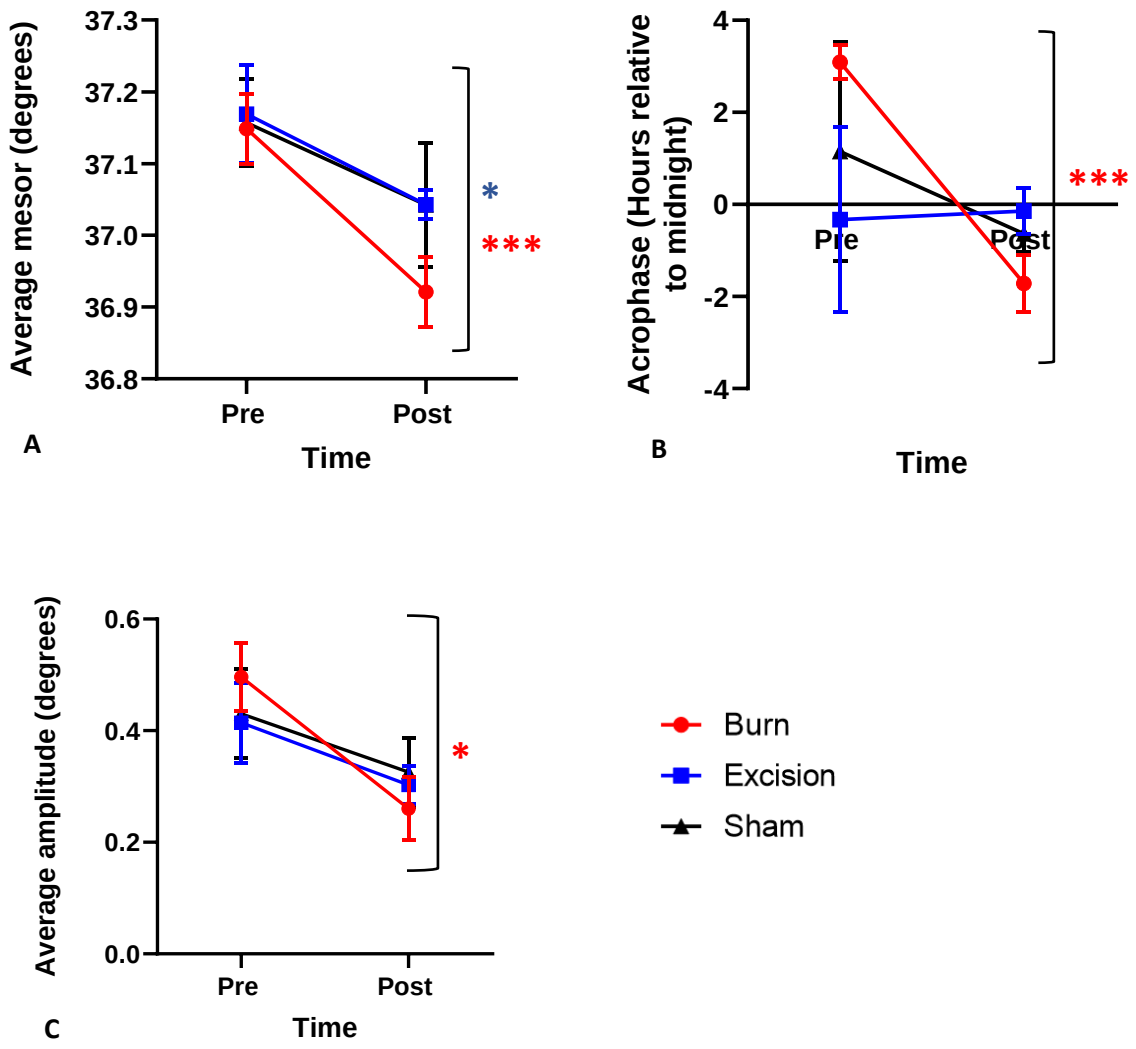


Figure 9. Mesor, acrophase and amplitude were disrupted acutely after surgery in the Burn group, but not in the Sham group, whilst Excision animals decreased in mesor alone.

CBT mesor (A) before surgery was higher than after in the Burn group, (n = 6, red line) (***, Wilcoxon, $P < 0.001$). Similarly, Burn acrophase (B) (***, Wilcoxon, $P < 0.001$) was shortened and amplitude (C) (*, $P < 0.05$) increased from pre-intervention to post-intervention. The Excision group (n = 4, blue line) had also decreased in mesor acutely after surgery.

3.3 Chronic CBT changes after surgery

Long-term circadian rhythm changes after surgery were present for all surgical groups. Using a pairwise Wilcoxon, each Period (two to 7) was compared with the baseline (Period one) to give individual P- values for the difference between the two time points per group and parameter. For burned mice, mesor values were significantly different from baseline during Periods four (Wilcoxon with FDR correction, $P < 0.001$) and persisted to Periods 6 ($P < 0.001$) and 7 ($P < 0.001$), decreasing by 0.241 °C, 0.293 °C and 0.250 °C, respectively, being depicted in Figure 10, in which dotted lines indicate where the baseline Period ends and post-surgery time points begin, and Table 3A. In Table B, Burn group acrophase only showed changes early in the chronic time periods, Periods two (Wilcoxon with FDR correction, $P < 0.001$), three ($P < 0.01$) and four ($P < 0.05$), which were absent or recovered in Periods 5, 6 and 7. These three early Periods all moved closer to midnight by 47 minutes, 46 minutes and 18 minutes for Periods two, three and four, respectively. Burn amplitude changed significantly from baseline to Period four (Wilcoxon with FDR correction, $P < 0.001$) and to Period 7 ($P < 0.001$), increasing by 0.207 °C and 0.155 °C.

For the Excision group, a chronic change was evident in CBT mesor and amplitude from baseline levels. Interestingly, both of these parameters were significantly different exclusively at Periods three (Wilcoxon with FDR correction, $P < 0.01$, both), 5 (mesor: $P < 0.001$, amplitude: $P < 0.01$) and 7 ($P < 0.001$, both). Like the Burn mice cohort, mesor values decreased for the Excision group (Period three: 0.217 °C; Period 5: 0.292 °C; Period 7: 0.336 °C). Amplitude increased by 0.103 °C, 0.118 °C and 0.163 °C for Periods three, 5 and 7, respectively. Acrophase values showed no significant differences between baseline and subsequent Periods.

Mesor, acrophase and amplitude all experienced significant changes chronically after surgery in the Sham group, as compared with baseline. Sham group mesor decreased chronically in Periods 5 (Wilcoxon with FDR correction, $P < 0.05$), 6 ($P < 0.05$) and 7 ($P < 0.01$) (0.148 °C, 0.173 °C and 0.341 °C, respectively) though no changes in Periods proceeding this. On the contrary, Sham acrophase changed chronically only in early Periods, two (Wilcoxon with FDR correction, $P < 0.05$)

and four ($P < 0.05$) (shortening, as such moving closer to midnight). Period two shortened by 47 minutes and Period four shortened by 37 minutes. In Sham mice, amplitude values, again, significantly increased, specifically in Periods four (Wilcoxon with FDR correction, $P < 0.05$), 5 ($P < 0.05$) and 7 ($P < 0.01$) (Period four: 0.088 °C; Period 5: 0.113 °C; Period 7: 0.163 °C). In conclusion, chronic circadian rhythm changes were present for all groups, suggesting that the effect of surgery or anaesthesia is the main effector in long-term circadian rhythm changes.

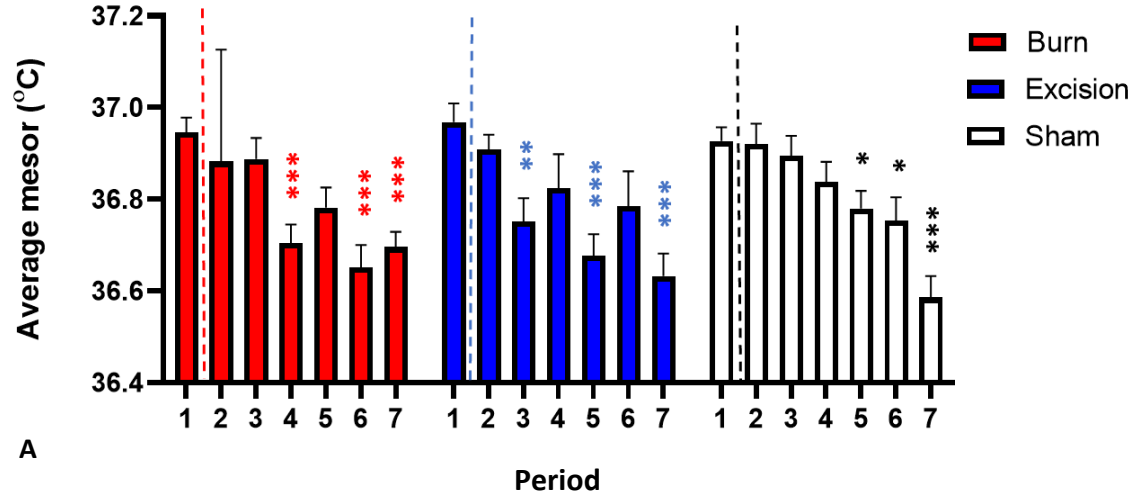
Table 3. Differences in mesor, amplitude and acrophase when comparing mean Periods two through 7 individually to baseline (Period one) for Burn, Excision and Sham groups.

For Periods which returned a significant P-value in the pairwise Wilcoxon test in any of the three parameters, the Period value was taken away from the baseline (Period 1) value to obtain the difference in degrees (mesor and amplitude) or hours (acrophase) between pre-surgery and the Period in question. As such, negative values depict changes in which the specific post – surgery Period mean exceeds the baseline level. Dotted lines indicate non-significant changes.

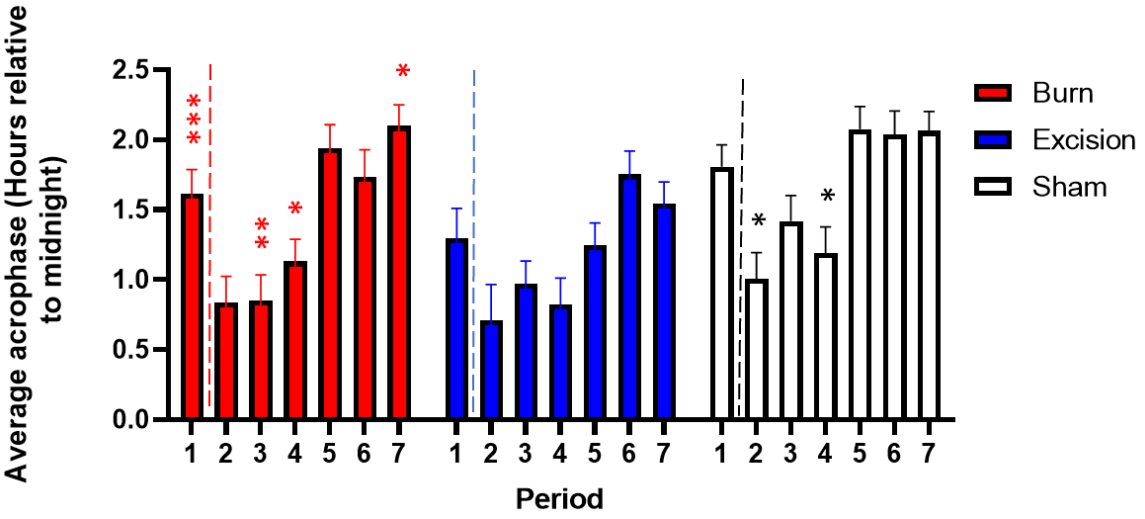
		Burn					
		2	3	4	5	6	7
Mesor *(°C)*	1			0.241***		0.293***	0.25***
Acrophase *(Hours relative to midnight)*		0.780***	0.767**	0.306*			
Amplitude *(°C)*				-0.207***			-0.155***

		Excision					
		2	3	4	5	6	7
Mesor *(°C)*	1		0.217**		0.292***		0.336***
Acrophase *(Hours relative to midnight)*							
Amplitude *(°C)*			-0.103**		-0.118**		-0.163***

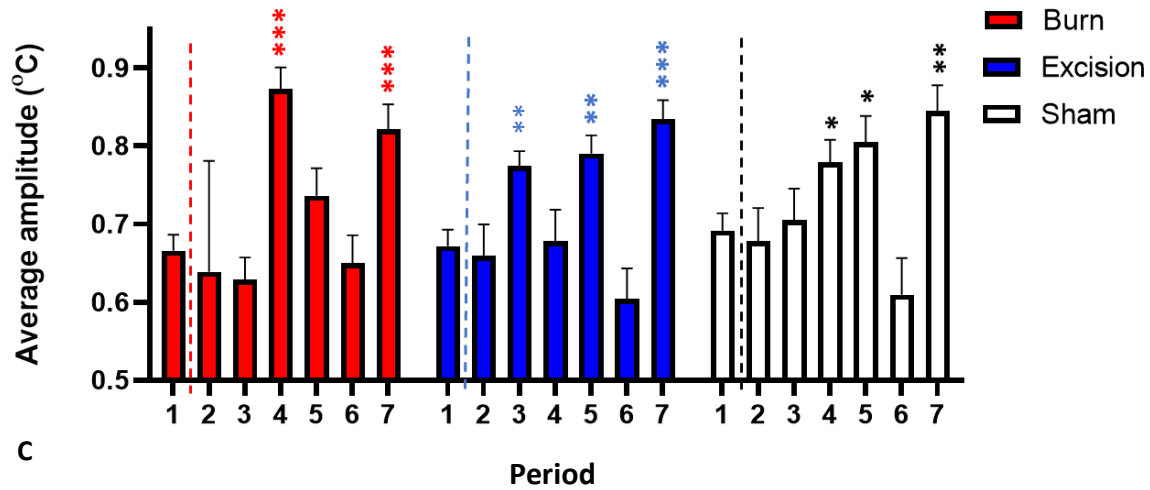
		Sham					
		2	3	4	5	6	7
Mesor *(°C)*	1				0.148*	0.173*	0.341***
Acrophase *(Hours relative to midnight)*		0.794*		0.613*			
Amplitude *(°C)*				-0.088*	-0.113*		-0.153**


Average mesor (°C)
37.2
37.0
36.8
36.6
36.4
1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7
Period
Burn
Excision
Sham

A


Average acrophase (Hours relative to midnight)
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7
Period
Burn
Excision
Sham

B


Average amplitude (°C)
0.9
0.8
0.7
0.6
0.5
1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7
Period
Burn
Excision
Sham

C

Figure 10. Burn, Excision and Sham mice CBT mesor and amplitude were disrupted chronically after surgery, Burn and Sham acrophase were also significantly altered long-term.

Dotted lines indicate where baseline values end, and chronic post-surgery Periods begin. Specifically, mean mesor for the Burn group (red bars, n = 6) was significantly decreased in Periods four, 6 and 7. Mean CBT amplitude in burned mice was decreased significantly in Periods two, three and four, whereas Burn group acrophase shortened in Period 4 and 7. Excision acrophase values compared with baseline levels were unaffected though Excision (blue bars, n = 4) mesor were decreased and amplitude values were increased for Periods three, 5 and 7. Sham (black bars, n = 5) CBT mesor was significantly decreased in Periods 5, 6 and 7, whilst Sham amplitude increased in Periods four, 5 and 7. Sham acrophase shortened in Period two and Period four compared with baseline timing. See above text for relevant P – values.

3.3.1 Longevity of chronic circadian rhythm changes after surgery

When examining the chronic effect of the surgical procedure/ injury, it was of interest to ascertain whether changes observed reverted to pre-surgery levels (Period one) by the end point of the experiment (Period 7). Given that the same mice were being analysed in both Period one and Period 7, a paired Wilcoxon test was utilised. It was found that for all group's mesor and amplitude parameters, there was indeed a significant difference. The Burn, Excision and Sham mesor (Table 4) experienced a significant drop between Periods one and 7 (Wilcoxon, $P < 0.001$, all) by 0.25 °C, 0.34 °C and 0.17 °C, respectively. Amplitude values in all three groups increased from Periods one to 7 (Wilcoxon, $P < 0.001$, all). The Excision and Burn group amplitudes both increased by 0.16 °C. The Sham group amplitude value rose significantly by 0.053 °C. The Burn group alone showed a change in acrophase at Period 7 (Wilcoxon, $P < 0.05$), specifically lengthening by 29 minutes. Although all groups saw Period 7 differences, which highlights the prolonged effect of surgery, only burn injury created

significant acrophase changes (lengthening) at the end of this study, suggesting that burns have a more severe impact on circadian rhythm than excision or surgery alone.

Table 4. Mesor values for all procedural groups and Excision and Burn group amplitudes had not reverted to baseline by the final Period of the experiment (Period 7)

There was a significant drop between Periods one and 7 in Burn, Excision and Sham mesor (***, Wilcoxon, P < 0.001). All three procedural group amplitudes increased from Periods one to 7 (***, Wilcoxon, P < 0.001). The Burn group acrophase alone was different between the two timepoints, significantly lengthening (*, Wilcoxon, P < 0.05).

		Mesor *(°C)*			**Acrophase** *(Hours relative to midnight)*		
	Period	**Mean**	**SEM**	**P-value** *(Wilcoxon test)*	**Mean**	**SEM**	**P-value** *(Wilcoxon test)*
Burn	**One**	36.946	0.0323		1.616	0.171	
	Seven	36.696	0.0339	1.272e-06 ***	2.0989	0.150	0.0258 *
Excision	**One**	36.968	0.0406		1.299	0.211	
	Seven	36.633	0.0493	1.02e-06 ***	1.544	0.155	0.236
Sham	**One**	36.946	0.0195		1.595	0.104	
	Seven	36.775	0.0117	1.609e-07 ***	1.430	0.0457	0.100

		Amplitude *(°C)*		
	Period	**Mean**	**SEM**	**P-value** *(Wilcoxon test)*
Burn	**One**	0.666	0.0206	
	Seven	0.821	0.0321	0.000289 ***
Excision	**One**	0.672	0.0207	
	Seven	0.835	0.0238	7.965e-05 ***
Sham	**One**	0.676	0.0123	
	Seven	0.729	0.00859	0.000444 ***

4. Discussion

Burn injuries are detrimental to the human body, with prolonged physiological changes. On top of these deleterious effects are well documented mental health issues shown to be overrepresented in burned individuals. With the aim of elucidating the causative agent behind these increased rates of psychiatric illness after burn injury, this study examined disruptions of circadian rhythm, as measured by CBT. The logic behind this focus was the evidence that circadian rhythm abnormalities are present after thermal injury as well as theorised to cause or exacerbate mental health problems, existing as a commonality between the two conditions. Specifically, we hoped to determine if/how burn injury circadian rhythm disruptions differed between the generic trauma and surgery alone as well as before and after the surgical procedures both long and short term. Although the differences between Burn and Excision group acrophases and between Excision and Sham group acrophases do not support the hypothesis of burn injuries being more severe, burn procedure mice were the only cohort which

displayed disrupted acrophase during the last recorded Period, suggesting that burns have an additional effect on circadian rhythm not seen after surgery and generic trauma. It was also found that circadian rhythm was distinguishably altered after surgery, both acutely and chronically, besides the Sham group which, acutely, saw no difference between pre- and post- surgery.

Our hypothesis postulating that the sham surgery will have no effect on circadian rhythm was supported by the lack of difference between the acute measurements after surgery for the Sham group, suggesting that generic surgery does not have an instantaneous effect on circadian rhythm. However, in contrast to our hypothesis, the Sham procedure was as disruptive to chronic circadian rhythm post procedure as the burn and excision injuries, suggesting that the trauma of these injuries is not alone responsible for the long-term circadian rhythm disruptions, potentially incriminating the effect of anaesthesia or surgical stress. Although burn surgery was the only procedure which affected every parameter in acute measurements, there was also a difference between the acute pre- and post- surgery time points in the Excision group, suggesting the effect of trauma is significant regardless of its nature. However, as previously mentioned, burn injury alone culminates in disruptions to acrophase parameters at the conclusion of the study over 11 weeks later.

4.1 Changes pre vs. post: Burn surgery

In amplitude and mesor, circadian rhythm was changed chronically after the Day 15 burn procedure, increasing and decreasing, respectively. Simply put, long-term mean CBT of burned mice decreased after surgery and the distance, in degrees, between the peak temperature point (acrophase) and mesor value became wider. Similar to our chronic findings, acute mesor for Burn mice decreased, though acutely, amplitude decreased. Several studies have been performed comparing burn patient CBT chronobiology to that of healthy controls, however, findings presented are reversed from those found in the current study [34, 107-110]. For example, in Pina et al. [34], which examined melatonin and temperature profiles in burns patients, amplitude was significantly smaller and mesor significantly higher than in control participants. Increased mesor values were also seen in Vaughan et al. [107], where it was found that rectal temperature over the first 24 hours post burn was elevated compared with

control participants. Circadian rhythm analysis per say was not completed, though references to "mean" temperatures are most reminiscent of mesor calculations, suggesting that mesor was raised [107]. These studies analyse data that is very short-term and related more to our acute measurements of burn effect in which we saw both amplitude and mesor decrease. Although our amplitude findings are consistent with those of Pina et al. [34], mesor directional changes in burns subjects (our current study seeing mesor decreases whereas previous literature found increases) was evidence of a discrepancy between our findings and those in previous literature.

4.2 Explanations for burn result discrepancies between past studies and current findings

4.2.1 Uncontrollable variables in human studies

Several explanations exist for why these beforementioned discrepancies in mesor directional change may have occurred between our study and those previous performed. Both Vaughan et al. [107] and Pina et al. [34] used human subjects, in which variables are difficult to control for [111]. Medications, such as sedatives and long- term use of opioids for analgesia, were taken by all participants at varying strengths and doses, as well as multiple surgeries taking place before or throughout the course of the experiments [34, 107]. These variables cannot be controlled, being unethical to withhold from patients. In Pina et al., day/night periods are undefined, with all participants residing in the same Intensive Care Unit, where noise, light and intermitted wakings are common [34]. In our study, these variables were eliminated, for example, set lights on/off time, no administered drugs or surgical procedures other than those scheduled. These uncontrolled factors in the studies by Pina et al. [34] and Vaughan et al. [107] may have profound effects on circadian rhythm.

4.2.2 Burn severity

Another notable difference between the current study and those often cited in literature is burn size. The burn injuries studied in Pina et al. [34] (TBSA average = 53% ± 16%) as well as in Vaughan et al. [107] (TBSA average 41% ± 6%) would be deemed severe. With our study inflicting a small, non-severe burn (7-8% TBSA) it could be possible that the increased mesor/ temperature profiles, which

were not found in our experiment, are present only after burns of a greater severity [107]. Non-severe burn sizes were chosen in our study due to the lack of literature focusing on small burns, despite small burns being the most common severity in Australia, with 96% of burn TBSA's being under 10% [112]. It is difficult to determine the effect of wound size on circadian rhythm in smaller burns as, to our knowledge, there are no studies which examine the effect of non-severe burns on CBT.

It is well documented that the capability of the body to regulate core temperature is lost in severely burned patients [34,107-110], leading to distinct circadian rhythm changes. This is due equally to damage to the outer skin layer allowing added evaporative heat loss, increasing proportionately to the percentage of the body burned [108], as well as increased glycolytic- gluconeogenic cycling after severe burns in human subjects (TBSA: 74% ± 2.75%) [109]. These factors cause core and peripheral temperatures to rise a maximum of 2 °C above normal controls in a study following patients an average of 20 ± 4.5 days after the burn injury [109]. This significant hypermetabolic response has been shown to exist even three years post-burn [5]. As such, it would have been reasonable to assume that core temperatures would be elevated in the current experiment, even purely in the early days or weeks following surgery. Given this is not the case, small burn sizes are likely not to produce the extreme evaporative temperature loss, hypermetabolism and circadian rhythm abnormalities characterised by severe burns. Given the active decrease in mesor seen chronically in our procedure, burn injury alone is not likely to be a major contributing factor in the presence of circadian rhythm changes.

4.3 Changes pre vs. post: Excision surgery

7-8% TBSA excision wounds caused significant pre- and post- surgery effects on mesor both chronically and acutely (decreasing in both) as well as on amplitude effects chronically (increasing). The inclusion of the generic trauma group allows for distinguishability between the effects of the burn injury itself and the acquirement and healing of an injury. Acutely after surgery, the Excision group only recorded significant changes in mesor, whereas the Burn group was changed in all three parameters. With no difference being found between the circadian rhythm of Excision and Burn groups overall, it is likely that burns create the same changes as excision injury, suggesting a role for

generic trauma. There are studies which analyse circadian rhythm after trauma, the majority focusing on ICU patients with highly severe injuries, often septic and mechanically ventilated [113 - 115]. With the excision injury in our experiment being so mild in comparison, it is difficult to determine whether the noted circadian rhythm effects, especially those experienced acutely, come from the specific injury or the mere presence of a wound or surgical strain on the body.

4.3.1 Excision findings and the effect of surgical stress response

Burn, Excision and Sham mesor and amplitude were all still disrupted in Period 7 (80 days post-surgery). Given the presence of Sham long-term changes, the effect of surgery is likely to be the aetiological agent of these circadian rhythm abnormalities. If not specifically attributable to the trauma of burn or excision injuries, it is possible that a physiological stress response to surgery culminates in circadian rhythm changes that possibly last weeks.

Corticosteroid secretion has long been noted to rise significantly after surgery due to physiological stress [106, 116, 117]. Relating back to circadian rhythm, cortisol (a corticosteroid) has shown to have an inverse relationship with melatonin, a circadian rhythm modulator [106]. Specifically, it is theorised that corticosteroids decrease the activity of the N-acetyltransferase enzyme in the pineal gland, negatively impacting the synthesis of melatonin [118]. There is a strong link between melatonin and CBT [112]. At night, raised melatonin levels sharply decrease body temperature through vasodilation, leading to greater propensity towards sleep, with the opposite occurring in the morning [119]. With cortisol at elevated levels after surgery, melatonin could be decreased, and well as its secretory pattern disrupted, and as such, circadian rhythm would be changed as a result.

It is possible that the stress from surgery, anaesthesia or excision/body manipulation, present in all experimental groups is the major source of long-term circadian rhythm variations between pre- and post-surgery time points seen in the current study. Post-surgery studies are limited by short time periods though some found that the hypercortisolism was still unresolved at the end of data recording [109,111]. As such it is unknown how long this stress response persists.

4.4 Changes pre vs. post: Sham Surgery

Perhaps the most surprising finding of the current experiment is that the sham procedure saw significant changes in circadian parameters after surgery, chronically but not acutely. This suggests that the mechanism behind the long- term circadian rhythm alterations may indeed be the surgical process itself, whereas trauma is the most likely effector in the short-term. This hypothesis is not without backing, with several studies looking at the effect of surgery alone on chronobiology [104-106, 116, 117].

In a study by Gögenur et al. [117], which analysed the circadian rhythm of patients undergoing abdominal surgery under general anaesthetic, it was observed that median temperature was significantly elevated on both the first and second days post-operatively. Similarly, acrophase was delayed by up to four hours. Rhythmicity of cortisol secretion and melatonin onset were also disturbed [117]. A second study by the same group found that circadian rhythm was completely abolished on the second postoperative day, which is where the sampling ended [108]. These two studies suggest that major surgery disrupts acute CBT circadian rhythm. This was not found in our study, with no significant changes occurring acutely in the Sham group for any parameter. Potential explanations for the source of this discrepancy are outlined below.

4.4.1 Explanation for Sham finding discrepancies: General anaesthesia

A suggested culprit for the lack of acute sham surgery effect could be the specific anaesthetic agent, which in our procedure was isoflurane (100% v/v). Sessler, Lee & McGuire [104] tested the effect of isoflurane on circadian rhythm by measuring CBT every 5 minutes for two days for baseline measurements. On the third day, 1% isoflurane was administered for three hours continuously. After ending anaesthesia, temperature was recorded for a further two days and it was found that isoflurane did not produce any clear differences in acrophase, mesor and amplitude on the post-surgery days [104]. Given that Sham changes were only seen chronically in our results, isoflurane may be incapable of

creating circadian rhythm change as seen in Gögenur's studies which used bupivacaine and morphine [108, 117].

4.5 Limitations

4.5.1 Sample size and demographic

Due to cost, logistics and unforeseen loss of data, animal numbers per group, Sham, Excision and Burn, were small (n = 5, 4, and 6, respectively). With greater time available, the data from this experiment could have been paired with that of an identical study (n = 17) performed by another member of the laboratory group. It was found that, given significant differences between the two experiment results, more complex statistical techniques would be needed to make evaluations from the combined set. This raise in sample set would drastically improve the power and reliability of conclusions drawn. Similarly, a conscious decision was made to only include female mice, given evidence that women are disproportionately diagnosed with psychiatric issue after burn injury in comparison with men [17, 100]. Including males in conclusions developed from this study should be done tentatively given that sex differences exist in circadian rhythm under controlled conditions and would likely persist in response to injury and surgery [120-122]

4.5.2 Short pre-surgery period

This experiment went for a total of 103 days, with recordings being taken every 5 minutes, which allowed for a large amount of data points to be collected and analysed. However, the surgical procedure was performed only 14 days after the initial thermologger implantation and, after having to remove 7 days due to cage cleaning, the pre-surgery Period (7 included days) was small in comparison to the postoperative period (42 included days). Had the experimental procedure been performed further along the experimental timeline, there would be a greater amount of data to compare between pre- and post-surgery, thus enhancing the strength of conclusions for the effect of surgery.

4.5.3 Effect of cage cleaning

Fortnightly cage cleaning had a significant effect on CBT within the mice. In acrophase and mesor parameters, there was a distinct, visible increase on cleaning days, whereas amplitude decreased, as a response to acute stress, which did not return to baseline for several days. Studies suggest that it takes mice 60 minutes to decrease their CBT back to baseline levels after simply being moved whilst still within their cage [123]. The investigators of this study also saw a rise in body temperature (0.5 °C), as was displayed by our mesor data for all three groups due to cage cleaning, displaying a well-known phenomenon termed "stress induced hyperthermia" [123]. If this hypothesis is true, it would be logical that the single day results were spiked and abnormal though would be back to baseline by the next day. This was not found in our mice however, leading us to exclude 7 days after the procedure. The specific choice of 7 days was chosen through visualisation and not statistical measurements per say. As such, it is unlikely but cannot be ruled out that the cage cleaning processes had an effect longer than 7 days after the procedure. If this is the case, it would be indecipherable whether circadian rhythm changes, especially in mesor values, are due to the stress from handling or the experimental procedures themselves. In future studies, further research and statistical tests should be performed to quantify the changes occurring due to cage cleaning. These conclusions can be used to decipher how many days to exclude after the procedure in a more analytic fashion.

4.5.4 Circadian rhythm measurement

There are numerous regularly utilised measures of circadian rhythm. CBT was chosen due to the relative simplicity of implantation and the large amount of data that can be acquired. However, conclusions would be stronger had more than one measurement of circadian rhythm been collected. An effect of the burn on the central circadian pacemaker would show abnormalities in all measures [124]. For example, if abnormal pre- and post- surgery circadian rhythm results were found with both CBT and melatonin, it eliminates the risk of conclusions being incorrect if body temperature was changed by variables not relevant to the study, such as increased evaporative heat loss from

hypermetabolism after burn injuries. Similarly, it gauges a more "whole-body" response to the injury with further evidence of a multiorgan response.

4.6 Implications

From the current study it has been found that burn and excision trauma disturb circadian rhythm acutely, in the days following the injury. Chronically (up to three months later) circadian rhythm is still disrupted after sham procedure and burn and excision injury which likely highlights the substantial and long-term effect of surgery on circadian rhythm. In the clinical realm, especially that which looks at the mental health of burns victims, this is an important finding, given that circadian rhythm disruptions have been attributed to large amounts of psychiatric pathologies [83, 85, 124]. If the changes occurring acutely after burns and surgery indeed contribute to long-term abnormalities, being able to minimise or eliminate these changes as soon as the burn injury takes place may limit chronic disruptions and long-term repercussions. For example, sleep quality in burns patients and after surgery should be prioritised given that disordered sleep from circadian rhythm abnormalities has been shown to lead to mental health problems in predisposed individuals [124]. Furthermore, knowledge of the deleterious effects of surgery on circadian rhythm can give vital further information to clinicians considering the pros and cons of proceeding with a procedure when compared with the desired outcome.

Pharmacological treatments for circadian rhythm misalignment also exist for patients experiencing chronic and unwavering disruptions [125, 126]. Synthetic melatonin, if administered at the appropriate phase, has been shown to both induce sleepiness and synchronise the circadian system [125,126]. This treatment could be especially well utilised in burns patients with a previous or current mental health problems and/or a strong family history of mental illness.

4.7 Future directions

Molecular studies are a logical next step given these results. Brain tissue from the hippocampus could be tested using RT – PCR (real-time polymerase chain reaction) for gene expression, specifically key circadian rhythm genes such as *BMAL* and *PER-2* [127]. An increase or decrease in circadian RNA

between sham, burn and excision injury would provide further evidence for circadian rhythm disruptions and assist in deciphering the effect of surgery versus trauma.

In order to link these findings to mental health after burns, it would be necessary to perform behavioural analysis in conjunction with circadian rhythm tests on a murine model. To do so, it is possible to create circadian rhythm disruptions by using *BMAL1* knockout mice [(129)]. Performing behavioural tests on these mice compared with controls in a large sample would allow for comparison between symptoms of depression and anxiety. This would be able to further test the theory that circadian rhythm is a cause, rather than an effect or irrelevance, in the development of mental health problems.

In humans, it would of use to analyse the chronobiological markers of circadian rhythm after burn, such as melatonin, cortisol and sleep quality, in conjunction with mental health and quality of life surveys such as, the Paediatric Quality of Life Inventory [(126)] and Patients Health Questionnaire [(127)]. For the purpose of deciphering changes between time periods these tests would be performed, and markers taken, immediately (hours), acutely (days) and chronically (months) after the burn injury occurs as well as following up long term (years after). On enough patients, this data would be invaluable, giving estimates of the quality of life and rates of mental illness within burns patients at varying time points whilst attempting to decipher risk factors in the development of psychiatric illness. With this knowledge, patients who displayed such symptoms could be supplied with early psychological interventions.

5. Conclusions

Our results indicate that trauma, in the form of burn or excision injuries, lead to acute circadian rhythm changes, with no observable effect of surgery. Chronically, it is more likely that surgery is the causational agent for long-term changes as oppose to the nature of the injury. Although burn injury was not significantly different to Excision and Sham group circadian rhythm in the way that was expected, it was the only parameter which was still affecting acrophase after 11 weeks, suggesting a greater impact than the Excision or Sham group. Conclusions drawn from this research broaden our understanding of possible links between circadian rhythm and burn injuries and surgery. Before clinical implementations can be developed, such as the administration of circadian rhythm correcting drugs to burn patients and those undergoing surgical procedures, further investigation is required to genetically link circadian rhythm disturbances to burn injury and surgery and/or anaesthetic. Similarly, to strengthen the hypothesised link between circadian rhythm abnormalities and mental health problems, behavioural testing in a murine model is needed to measure for depressive and anxious symptoms.

6. References

1. Duke J, Wood F, Semmens J, Spilsbury K, Edgar DW, Hendrie D, et al. A 26-Year Population-Based Study of Burn Injury Hospital Admissions in Western Australia. Journal of Burn Care & Research. 2011;32(3):379-86. doi: https://doi.org/10.1097/BCR.0b013e318219d16c
2. Ahn CS, Maitz PKM. The true cost of burn. Burns. 2012;38(7):967-74. doi: https://doi.org/10.1016/j.burns.2012.05.016
3. van Baar ME, Essink-Bot ML, Oen IMMH, Dokter J, Boxma H, van Beeck EF. Functional outcome after burns: A review. Burns. 2006;32(1):1-9. doi: 10.1016/j.burns.2005.08.007
4. Herndon DN, Tompkins RG. Support of the metabolic response to burn injury. The Lancet. 2004;363(9424):1895-902. doi: https://doi.org/10.1016/S0140-6736(04)16360-5
5. Jeschke MG, Gauglitz GG, Kulp GA, Finnerty CC, Williams FN, Kraft R, et al. Long-Term Persistance of the Pathophysiologic Response to Severe Burn Injury. PLoS ONE. 2011;6(7):1-12. doi: 10.1371/journal.pone.0021245
6. Williams FN, Jeschke MG, Chinkes DL, Suman OE, Branski LK, Herndon DN. Modulation of the Hypermetabolic Response to Trauma: Temperature, Nutrition, and Drugs. Journal of the American College of Surgeons. 2009;208(4):489-502. doi: https://doi.org/10.1016/j.jamcollsurg.2009.01.022
7. Palmieri TL, Levine S, Schonfeld-Warden N, O'Mara MS, Greenhalgh DG. Hypothalamic–Pituitary–Adrenal Axis Response to Sustained Stress after Major Burn Injury in Children. Journal of Burn Care & Research. 2006;27(5):742-8. doi: 10.1097/01.BCR.0000238098.43888.07
8. Williams FN, Herndon DN, Suman OE, Lee JO, Norbury WB, Branski LK, et al. Changes in cardiac physiology after severe burn injury. J Burn Care Res. 2011;32(2):269-74. Doi: 10.1097/BCR.0b013e31820aafcf
9. Duke JM, Randall SM, Fear MW, Boyd JH, Rea S, Wood FM. Understanding the long-term impacts of burn on the cardiovascular system. Burns. 2016;42(2):366-74. doi: https://doi.org/10.1016/j.burns.2015.08.020
10. Duke JM, Boyd JH, Rea S, Randall SM, Wood FM. Long-term mortality among older adults with burn injury: a population-based study in Australia. World Health Organization Bulletin of the World Health Organization. 2015;93(6):400-6. doi:10.2471/BLT.14.149146
11. Williams FN, Herndon DN, Jeschke MG. The hypermetabolic response to burn injury and interventions to modify this response. Clin Plast Surg. 2009;36(4):583-96. doi: 10.1016/j.cps.2009.05.001

12. Liu D-m, Sun B-w, Sun Z-w, Jin Q, Sun Y, Chen X. Suppression of inflammatory cytokine production and oxidative stress by CO-releasing molecules—liberated CO in the small intestine of thermally-injured mice. Acta Pharmacologica Sinica. 2008;29(7):838-46. doi: 10.1111/j.1745-7254.2008.00816.x
13. Jeschke MG, Barrow RE, Herndon DN. Extended Hypermetabolic Response of the Liver in Severely Burned Pediatric Patients. Archives of Surgery. 2004;139(6):641-7. doi: 10.1001/archsurg.139.6.641
14. Jeschke MG, Micak RP, Finnerty CC, Herndon DN. Changes in liver function and size after a severe thermal injury. Shock. 2007;28(2):172-7. doi: 10.1097/shk.0b013e318047b9e2
15. Williams EE, Griffiths TA. Psychological consequences of burn injury. Burns. 1991;17(6):478-80. doi: https://doi.org/10.1016/0305-4179(91)90075-R
16. Madianos MG, Papaghelis M, Ioannovich J, Dafni R. Psychiatric Disorders in Burn Patients: A Follow-Up Study. Psychotherapy and Psychosomatics. 2001;70(1):30-7. doi: https://doi.org/10.1159/000056222
17. Meyer WJ, Blakeney P, Thomas CR, Russell W, Robert RS, Holzer CE. Prevalence of Major Psychiatric Illness in Young Adults Who Were Burned as Children. Psychosomatic Medicine. 2007;699(4):377-382. doi: 10.1097/PSY.0b013e3180600a2e
18. Duke JM, Randall SM, Vetrichevvel TP, McGarry S, Boyd JH, Rea S, et al. Long-term mental health outcomes after unintentional burns sustained during childhood: a retrospective cohort study. Burns & Trauma. 2018;6. doi: https://doi.org/10.1186/s41038-018-0134-z
19. El hamaoui Y, Yaalaoui S, Chihabeddine K, Boukind E, Moussaoui D. Post-traumatic stress disorder in burned patients. Burns. 2002;28(7):647-50. doi: https://doi.org/10.1016/S0305-4179(02)00100-6
20. Dyster-Aas J, Willebrand M, Wikehult B, Gerdin B, Ekselius L. Major Depression and Posttraumatic Stress Disorder Symptoms Following Severe Burn Injury in Relation to Lifetime Psychiatric Morbidity. Journal of Trauma and Acute Care Surgery. 2008;64(5):1349-56. Doi: 10.1016/j.burns.2006.10.240
21. ter Smitten MH, de Graaf R, Van Loey NE. Prevalence and co-morbidity of psychiatric disorders 1–4 years after burn. Burns. 2011;37(5):753-61. doi: 10.1016/j.burns.2010.12.018
22. Öster C, Sveen J. The psychiatric sequelae of burn injury. General Hospital Psychiatry. 2014;36(5):516-22. doi: 10.1016/j.genhosppsych.2014.05.003
23. Regier DA, Kuhl EA, Kupfer DJ. The DSM-5: Classification and criteria changes. World Psychiatry. 2013;12(2):92-8. doi: 10.1002/wps.20050
24. Patterson DR, Everett JJ, Bombardier CH, Questad KA, Lee VK, Marvin JA. Psychological effects of severe burn injuries. Psychological Bulletin. 1993;113(2):362-78. doi: 10.1037/0033-2909.113.2.362

25. Alegría M, Kessler RC, Bijl R, Lin E, Heeringa SG, Takeuchi DT, et al. Comparing data on mental health service use between countries. In: Andrews G, Henderson S, editors. Unmet Need in Psychiatry: Problems, Resources, Responses. Cambridge: Cambridge University Press; 2000. p. 97-118.
26. Corry N, Pruzinsky T, Rumsey N. Quality of life and psychosocial adjustment to burn injury: Social functioning, body image, and health policy perspectives. International Review of Psychiatry. 2009;21(6):539-48. doi:10.3109/09540260903343901.
27. Staley M. Return to school as an outcome measure after a burn injury. Journal of Burn Care and Research. 1999;20(1):91. Doi: https://doi.org/10.1097/00004630-199901001-00023
28. Mashreky SR, Rahman A, Chowdhury SM, Giashuddin S, Svanström L, Khan TF, et al. Burn injury: economic and social impact on a family. Public Health. 2008;122(12):1418-24. doi: 10.1016/j.puhe.2008.06.007
29. Thombs BD, Notes LD, Lawrence JW, Magyar-Russell G, Bresnick MG, Fauerbach JA. From survival to socialization: A longitudinal study of body image in survivors of severe burn injury. Journal of Psychosomatic Research. 2008;64(2):205-12. doi: https://doi.org/10.1016/j.jpsychores.2007.09.003
30. Dyster-Aas J, Kildal M, Willebrand M, Gerdin B, Ekselius L. Work status and burn specific health after work-related burn injury. Burns. 2004;30(8):839-42. doi: https://doi.org/10.1016/j.burns.2004.05.010
31. Thombs BD, Haines JM, Bresnick MG, Magyar-Russell G, Fauerbach JA, Spence RJ. Depression in burn reconstruction patients: symptom prevalence and association with body image dissatisfaction and physical function. General Hospital Psychiatry. 2007;29(1):14-20. doi: https://doi.org/10.1016/j.genhosppsych.2006.09.002
32. Öster C, Willebrand M, Ekselius L. Burn-specific health 2 years to 7 years after burn injury. Journal of Trauma and Acute Care Surgery. 2013;74(4). doi: 10.1097/TA.0b013e318283cca0
33. Aschoff J. Circadian Rhythms in man. Science. 1965;148(3676):1427.
34. Pina G, Brun J, Tissot S, Claustrat B. Long-term alteration of daily melatonin, 6sulfatoxymelatonin, cortisol, and temperature profiles in burn patients: a preliminary report. Chronobiol Int. 2010;27(2):378-92. doi: 10.3109/07420520903502234
35. Masoodi Z, Ahmad I, Khurram F, Haq A. Changes in sleep architecture after burn injury: 'Waking up' to this unaddressed aspect of postburn rehabilitation in the developing world. Canadian Journal of Plastic Surgery. 2013;21(4):234-8. doi: https://doi.org/10.1177/229255031302100404
36. Mayes T, Gottschlich MM, Khoury J, McCall J, Simakajornboon N, Kagan RJ. A Pilot Review of the Long-Term Impact of Burn Injury on Sleep Architecture in Children. Journal of burn care& research. 2013; Vol.34(1), p.15-21. doi:10.1097/BCR.0b013e318272178e

37. Everson CA. Functional consequences of sustained sleep deprivation in the rat. Behavioural Brain Research. 1995;69(1):43-54. doi: 10.1016/0166-4328(95)00009-I
38. Everson CA. Sustained sleep deprivation impairs host defense. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1993;265(5):1148-R54. doi: https://doi.org/10.1152/ajpregu.1993.265.5.R1148
39. Reimer MA, Flemons WW. Quality of life in sleep disorders. Sleep Medicine Reviews. 2003;7(4):335-49. doi: https://doi.org/10.1053/smrv.2001.0220
40. Peppard PE, Young T, Palta M, Skatrud J. Prospective Study of the Association between Sleep-Disordered Breathing and Hypertension. New England Journal of Medicine. 2000;342(19):1378-84. doi: 10.1056/NEJM200005113421901
41. Shahar E, Whitney CW, Redline S, Lee ET, Newman AB, Nieto FJ, et al. Sleep-disordered Breathing and Cardiovascular Disease. American Journal of Respiratory and Critical Care Medicine. 2001;163(1):19-25. doi: https://doi.org/10.1164/ajrccm.163.1.2001008
42. Minkel J, Moreta M, Muto J, Htaik O, Jones C, Basner M, et al. Sleep deprivation potentiates HPA axis stress reactivity in healthy adults. Health Psychology. 2014;33(11):1430-4. doi: https://doi.org/10.1037/a0034219
43. Carmichael CL, Reis HT. Attachment, Sleep Quality, and Depressed Affect. Health Psychology. 2005;24(5):526-31. doi: 10.1037/0278-6133.24.5.526
44. Hobson KG, Havel PJ, McMurtry AL, Lawless MB, Palmieri TL, Greenhalgh DD. Circulating leptin and cortisol after burn injury: loss of diurnal pattern. 2004;25(6):491-99. doi: 10.1097/01.BCR.0000144532.02792.6E
45. Molteni A, Warpeha R, Brizio-Molteni L, Albertson D, Kaurs R. Circadian rhythms of serum aldosterone, cortisol and plasma renin activity in burn injuries. Annals of Clinical & Laboratory Science. 1979;9(6):518-23.
46. Berg AH, Scherer PE. Adipose Tissue, Inflammation, and Cardiovascular Disease. Circulation Research. 2005;96(9):939-49. doi: https://doi.org/10.1161/01.RES.0000163635.62927.34
47. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860+. Doi:10.1038/nature07205
48. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. The Journal of Clinical Investigation. 2005;115(5):1111-9. doi: https://doi.org/10.1172/JCI25102.
49. Miller AH, Maletic V, Raison CL. Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression. Biological Psychiatry. 2009;65(9):732-41. doi: https://doi.org/10.1016/j.biopsych.2008.11.029
50. Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends in Immunology. 2006;27(1):24-31. doi: https://doi.org/10.1016/j.it.2005.11.006

51. Mössner R, Mikova O, Koutsilieri E, Saoud M, Ehlis A-C, Müller N, et al. Consensus paper of the WFSBP Task Force on Biological Markers: Biological Markers in Depression. The World Journal of Biological Psychiatry. 2007;8(3):141-74. Doi: https://doi.org/10.1080/15622970701263303
52. Zorrilla EP, Luborsky L, McKay JR, Rosenthal R, Houldin A, Tax A, et al. The Relationship of Depression and Stressors to Immunological Assays: A Meta-Analytic Review. Brain, Behavior, and Immunity. 2001;15(3):199-226. Doi:10.1006/brbi.2000.0597
53. Anisman H, Merali Z, Hayley S. Neurotransmitter, peptide and cytokine processes in relation to depressive disorder: Comorbidity between depression and neurodegenerative disorders. Progress in Neurobiology. 2008;85(1):1-74. doi: https://doi.org/10.1016/j.pneurobio.2008.01.004
54. O'Connor JC, Lawson MA, André C, Moreau M, Lestage J, Castanon N, et al. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Molecular Psychiatry. 2009;14(5):511-22. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1038/sj.mp.4002148
55. Zhu C-B, Blakely RD, Hewlett WA. The Proinflammatory Cytokines Interleukin-1beta and Tumor Necrosis Factor-Alpha Activate Serotonin Transporters. Neuropsychopharmacology. 2006;31(10):2121-31. doi: https://doi org.ezproxy.library.uwa.edu.au/10.1038/sj.npp.1301029
56. Reichenberg A, Yirmiya R, Schuld A, Kraus T, Haack M, Morag A, et al. Cytokine-Associated Emotional and Cognitive Disturbances in Humans. Archives of General Psychiatry. 2001;58(5):445-52. doi: 10.1001/archpsyc.58.5.445
57. Vogelzangs N, Beekman ATF, De Jonge P, Penninx BWJH. Anxiety disorders and inflammation in a large adult cohort. Translational Psychiatry. 2013;3:8. doi: 10.1038/tp.2013.27
58. Schuld A, Schmid DA, Haack M, Holsboer F, Friess E, Pollmächer T. Hypothalamo-pituitary-adrenal function in patients with depressive disorders is correlated with baseline cytokine levels, but not with cytokine responses to hydrocortisone. Journal of Psychiatric Research. 2003;37(6):463-70. doi: https://doi.org/10.1016/S0022-3956(03)00054-2
59. Steptoe A, Kunz-Ebrecht SR, Owen N. Lack of association between depressive symptoms and markers of immune and vascular inflammation in middle-aged men and women. Psychological Medicine. 2003;33(4):667-74. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1017/S0033291702007250
60. Besedovsky HO, del Rey A. Immune-Neuro-Endocrine Interactions: Facts and Hypotheses. Endocrine Reviews. 1996;17(1):64-102. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1210/edrv-17-1-64
61. Capuron L, Raison CL, Musselman DL, Lawson DH, Nemeroff CB & Miller AH Association of Exaggerated HPA Axis Response to the Initial Injection of Interferon-Alpha With

Development of Depression During Interferon-Alpha Therapy. American Journal of Psychiatry. 2003;160(7):1342-5. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1176/appi.ajp.160.7.1342

62. Popoli M, Yan Z, McEwen BS, Sanacora G. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. Nature Reviews Neuroscience. 2012;13(1):22-37. doi: https://doi.org/10.1038/nrn3138
63. van de Goot F, Krijnen PAJ, Begieneman MPV, Ulrich MMW, Middelkoop E, Niessen HWM. Acute Inflammation is Persistent Locally in Burn Wounds: A Pivotal Role for Complement and C-Reactive Protein. Journal of Burn Care & Research. 2009;30(2):274-80.
64. Kim HS, Kim J-H, Yim H, Kim D. Changes in the Levels of Interleukins 6, 8, and 10, Tumor Necrosis Factor Alpha, and Granulocyte-colony Stimulating Factor in Korean Burn Patients: Relation to Burn Size and Postburn Time. Ann Lab Med. 2012;32(5):339-44. doi: https://doi.org/10.3343/alm.2012.32.5.339
65. Pan W, P. Stone K, Hsuchou H, K. Manda V, Zhang Y, J. Kastin A. Cytokine Signaling Modulates Blood-Brain Barrier Function. Current Pharmaceutical Design. 2011;17(33):3729-40. doi: https://doi.org/10.2174/138161211798220918
66. Reyes R, Wu Y, Lai Q, Mrizek M, Berger J, Jimenez DF, et al. Early inflammatory response in rat brain after peripheral thermal injury. Neuroscience Letters. 2006;407(1):11-5. doi: 10.1016/j.neulet.2006.07.071
67. Swann K, Berger J, Sprague SM, Wu Y, Lai Q, Jimenez DF, et al. Peripheral thermal injury causes blood–brain barrier dysfunction and matrix metalloproteinase (MMP) expression in rat. Brain Research. 2007;1129:26-33.
68. Pariante CM, Lightman SL. The HPA axis in major depression: classical theories and new developments. Trends in Neurosciences. 2008;31(9):464-8. doi: https://doi.org/10.1016/j.tins.2008.06.006
69. Yehuda R, Seckl J. Minireview: Stress-Related Psychiatric Disorders with Low Cortisol Levels: A Metabolic Hypothesis. Endocrinology. 2011;152(12):4496-503. Doi: https://doi.org/10.1210/en.2011-1218
70. Watson S, Mackin P. HPA axis function in mood disorders. Psychiatry. 2006;5(5):166-70. doi: https://doi.org/10.1383/psyt.2006.5.5.166
71. Pariante CM, Papadopoulos AS, Poon L, Cleare AJ, Checkley SA, English J, et al. Four days of citalopram increase suppression of cortisol secretion by prednisolone in healthy volunteers. Psychopharmacology. 2004;177(1-2):200-6. doi: https://doi.org/10.1007/s00213-004-1925-4
72. Dinan TG, Lavelle E, Cooney J, Burnett F, Scott L, Dash A, et al. Dexamethasone augmentation in treatment-resistant depression. Acta Psychiatrica Scandinavica. 1997;95(1):58-61. doi:10.1111/j.1600-0447.1997.tb00374.x

73. Bouwer C, Claassen J, Dinan TG, Nemeroff CB. Prednisone augmentation in treatment-resistant depression with fatigue and hypocortisolaemia: A case series. Depression and Anxiety. 2000;12(1):44-50. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1002/1520-6394(2000)12:1<44
74. Charles DeBattista, D.M.H., M.D. ,, Joel A. Posener, M.D. ,, B. Michelle Kalehzan, Ph.D. , and, Alan F. Schatzberg, M.D. Acute Antidepressant Effects of Intravenous Hydrocortisone and CRH in Depressed Patients: A Double-Blind, Placebo-Controlled Study. American Journal of Psychiatry. 2000;157(8):1334-7. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1176/appi.ajp.157.8.1334
75. Pileri D, Accardo-Palumbo A, D'Amelio L, Arnone G, Grisaffi C, et al. Serum levels of cortisol, immunoglobulin, and C-reactive protein in burn patients. Annals of Burns and Fires Disasters. 2009;22(1):3-5.
76. Palmieri TL, Levine S, Schonfeld-Warden N, O'Mara MS, Greenhalgh DG. Hypothalamic–Pituitary–Adrenal Axis Response to Sustained Stress after Major Burn Injury in Children. Journal of Burn Care & Research. 2006;27(5):742-8. doi: 10.1097/01.BCR.0000238098.43888.07
77. Cohen SI. Cushing's Syndrome: A Psychiatric Study of 29 Patients. British Journal of Psychiatry. 1980;136(2):120-4. doi: 10.1192/bjp.136.2.120
78. Sonino N, Fava GA, Belluardo P, Girelli ME, Boscaro M. Course of depression in Cushing's syndrome: response to treatment and comparison with Graves' disease. Hormone Research in Paediatrics. 1993;39(5-6):202-6.
79. KLING MA, ROY A, DORAN R, CALABRESE JR, RUBINOW DR, DAVID HJ, JR, et al. Cerebrospinal Fluid Immunoreactive Corticotropin-Releasing Hormone and Adrenocorticotropin Secretion in Cushing's Disease and Major Depression: Potential Clinical Implications. The Journal of Clinical Endocrinology & Metabolism. 1991;72(2):260-71. doi: 10.1210/jcem-72-2-260
80. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. Trends in Cell Biology. 2014;24(2):90-9. doi: https://doi.org/10.1016/j.tcb.2013.07.002
81. Dibner C, Schibler U, Albrecht U. lik. 2010;72(1):517-49. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1146/annurev-physiol-021909-135821
82. Hoyle NP, Seinkmane E, Putker M, Feeney KA, Krogager TP, Chesham JE, et al. Circadian actin dynamics drive rhythmic fibroblast mobilization during wound healing. Science Translational Medicine. 2017;9(415):2774 doi: 10.1126/scitranslmed.aal2774
83. Wulff KG, Silvia Wettstein, Joseph G Foster, Russell G. Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. Nature Reviews: Neuroscience.2010;11(8):589-99. doi: 10.1038/nrn2868

84. Li J, Lu W-Q, Beesley S, Loudon ASI, Meng Q-J. Lithium impacts on the amplitude and period of the molecular circadian clockwork. PloS one. 2012;7(3):33292. Doi: 10.1371/journal.pone.0033292
85. Jones SG, Benca RM. Circadian Disruption in Psychiatric Disorders. Sleep Medicine Clinics. 2015;10(4):481-93. doi: https://doi.org/10.1016/j.jsmc.2015.07.004
86. Sawamura N, Ando T, Maruyama Y, Fujimuro M, Mochizuki H, Honjo K, et al. Nuclear DISC1 regulates CRE-mediated gene transcription and sleep homeostasis in the fruit fly. Molecular Psychiatry. 2008;13(12):1138-48. doi: https://doi.org/10.1038/mp.2008.101
87. Hofstra WA, de Weerd AW. How to assess circadian rhythm in humans: A review of literature. Epilepsy & Behavior. 2008;13(3):438-44.. doi: https://doi.org/10.1016/j.yebeh.2008.06.002
88. Satlin A, Volicer L, Stopa EG, Harper D. Circadian locomotor activity and core-body temperature rhythms in Alzheimer's disease. Neurobiology of Aging. 1995;16(5):765-71.doi: https://doi.org/10.1016/0197-4580(95)00059-N
89. O'Dell LE, Chen SA, Smith RT, Specio SE, Balster RL, Paterson NE, et al. Extended Access to Nicotine Self-Administration Leads to Dependence: Circadian Measures, Withdrawal Measures, and Extinction Behavior in Rats. Journal of Pharmacology and Experimental Therapeutics. 2007;320(1):180-93doi:10.1124/jpet.106.105270
90. Murray G. Diurnal mood variation in depression: A signal of disturbed circadian function? Journal of Affective Disorders. 2007;102(1):47-53 doi: https://doi.org/10.1016/j.jad.2006.12.001
91. LINKOWSKI P, MENDLEWICZ J, KERKHOFS M, LECLERCQ R, GOLSTEIN J, BRASSEUR M, et al. 24-Hour Profiles of Adrenocorticotropin, Cortisol, and Growth Hormone in Major Depressive Illness: Effect of Antidepressant Treatment*. The Journal of Clinical Endocrinology & Metabolism. 1987;65(1):141-52. Doi: 10.1210/jcem-61-3-429
92. Deuschle M, Schweiger U, Weber B, Gotthardt U, Körner A, Schmider J, et al. Diurnal Activity and Pulsatility of the Hypothalamus-Pituitary-Adrenal System in Male Depressed Patients and Healthy Controls. The Journal of Clinical Endocrinology & Metabolism. 1997;82(1):234-8. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1210/jcem.82.1.3689
93. Pandi-Perumal SR, Smits M, Spence W, Srinivasan V, Cardinali DP, Lowe AD, et al. Dim light melatonin onset (DLMO): A tool for the analysis of circadian phase in human sleep and chronobiological disorders. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2007;31(1):1-11. doi: https://doi.org/10.1016/j.pnpbp.2006.06.020
94. Emens J, Lewy A, Kinzie JM, Arntz D, Rough J. Circadian misalignment in major depressive disorder. Psychiatry Research. 2009;168(3):259-61. doi: https://doi.org/10.1016/j.psychres.2009.04.009

95. Hasler BP, Buysse DJ, Kupfer DJ, Germain A. Phase relationships between core body temperature, melatonin, and sleep are associated with depression severity: Further evidence for circadian misalignment in non-seasonal depression. Psychiatry Research. 2010;178(1):205-7. doi: https://doi.org/10.1016/j.psychres.2010.04.027
96. Benca RM, Obermeyer WH, Thisted RA, Gillin JC. Sleep and Psychiatric Disorders: A Meta-analysis. Archives of General Psychiatry. 1992;49(8):651-68. doi:10.1001/archpsyc.1992.01820080059010
97. Cameron OG, Lee MA, Kotun J, McPhee KM. Circadian symptom fluctuations in people with anxiety disorders. Journal of Affective Disorders. 1986;11(3):213-8. doi: https://doi.org/10.1016/0165-0327(86)90072-8
98. Sipilä T, Kananen L, Greco D, Donner J, Silander K, Terwilliger JD, et al. An Association Analysis of Circadian Genes in Anxiety Disorders. Biological Psychiatry. 2010;67(12):1163-70. doi: https://doi.org/10.1016/j.biopsych.2009.12.011
99. Altier N, Malenfant A, Forget R, ChoiniÈRe M. Long-term adjustment in burn victims: a matched-control study. Psychological Medicine. 2002;32(4):677-85. doi: https://doi-org.ezproxy.library.uwa.edu.au/10.1017/S0033291702005354
100. McKibben JBA, Bresnick MG, Wiechman Askay SA, Fauerbach JA. Acute stress disorder and posttraumatic stress disorder: a prospective study of prevalence, course, and predictors in a sample with major burn injuries 2008;29(1):22-25. doi: 10.1097/BCR.0b013e31815f59c4
101. Gorwood P. Review: Restoring circadian rhythms: a new way to successfully manage depression. Journal of Psychopharmacology. 2010;24(2_suppl):15-9. doi: https://doi.org/10.1177/1359786810372981
102. Burkholder T, Foltz C, Karlsson E, Linton CG, Smith JM. Health Evaluation of Experimental Laboratory Mice. Curr Protoc Mouse Biol. 2012;2:145-65. doi: 10.1002/9780470942390.mo110217
103. DST nano-T [Internet]. Star Oddi: Logging Life Science. 2017[cited 21/10/202]. Available from https://www.star-oddi.com/products/data-loggers/small-thermo-logger.
104. Sessler DI, Lee KA, McGuire J. Isoflurane anesthesia and circadian temperature cycles in humans. Anesthesiology: The Journal of the American Society of Anesthesiologists. 1991;75(6):985-9.
105. Gogenur, I. (2010). Postoperative circadian disturbances. Dan Med Bull, 57(12), B4205.
106. Ram E, Vishne TH, Weinstein T, Beilin B, Dreznik Z. General anesthesia for surgery influences melatonin and cortisol levels. World journal of surgery. 2005;29(7):826. doi: 10.1007/s00268-005-7724-1
107. Vaughan GM, Taylor TJ, Pruitt Jr. BA, Mason Jr. AD. Pineal Function in Burns: Melatonin Is Not a Marker for General Sympathetic Activity. Journal of Pineal Research. 1985;2(1):1-12. doi:10.1111/j.1600-079X.1985.tb00623.x

108. Wilmore DW, Mason Jr AD, Johnson DW, Pruitt Jr B. Effect of ambient temperature on heat production and heat loss in burn patients. Journal of applied physiology. 1975;38(4):593-7.. doi:10.1152/jappl.1975.38.4.593
109. Wolfe RR, Herndon DN, Jahoor F, Miyoshi H, Wolfe M. Effect of Severe Burn Injury on Substrate Cycling by Glucose and Fatty Acids. New England Journal of Medicine. 1987;317(7):403-8. doi:10.1056/nejm198708133170702
110. Caldwell FT, Graves DB, Wallace BH. Vagotomy modifies but does not eliminate the increase in body temperature following burn injury in rats. Burns. 1999;25(4):295-305. doi:https://doi.org/10.1016/S0305-4179(99)00006-6
111. Knauert MP, Haspel JA, Pisani MA. Sleep loss and circadian rhythm disruption in the intensive care unit. Clinics in chest medicine. 2015;36(3):419-29. doi: https://doi.org/10.1016/j.ccm.2015.05.008
112. Harrison, J., & Steel, D. Burns and scalds [Internet]. South Australia Research Centre for Injury Studies: Flinders University. Australian Institute of Health and Welfare; 2006 [cited 2020 Nov 19]. Available from https://www.aihw.gov.au/getmedia/5b226df3-175e-4410-92ba-23a27974272c/injcat92.pdf.aspx?inline=true.
113. Olofsson K, Alling C, Lundberg D, Malmros C. Abolished circadian rhythm of melatonin secretion in sedated and artificially ventilated intensive care patients. Acta anaesthesiologica scandinavica. 2004;48(6):679-84.
114. Gazendam JA, Van Dongen HP, Grant DA, Freedman NS, Zwaveling JH, Schwab RJ. Altered circadian rhythmicity in patients in the ICU. Chest. 2013;144(2):483-9
115. Truong KK, Lam MT, Grandner MA, Sassoon CS, Malhotra A. Timing matters: circadian rhythm in sepsis, obstructive lung disease, obstructive sleep apnea, and cancer. Annals of the American Thoracic Society. 2016;13(7):1144-54.
116. MCINTOSH TK, LOTHROP DA, LEE A, JACKSON BT, NABSETH D, EGDAHL RH. Circadian rhythm of cortisol is altered in postsurgical patients. The Journal of Clinical Endocrinology & Metabolism. 1981;53(1):117-22. doi: https://doi.org/10.1210/jcem-53-1-117
117. Gögenur I, Ocak U, Altunpinar Ö, Middleton B, Skene DJ, Rosenberg J. Disturbances in melatonin, cortisol and core body temperature rhythms after major surgery. World journal of surgery. 2007;31(2):290-8. doi: /10.1007/s00268-006-0256-5
118. Yuwiler A. Effects of steroids on serotonin-N-acetyltransferase activity of pineals in organ culture. Journal of neurochemistry. 1989;52(1):46-53.
119. Cagnacci A, Kräuchi K, Wirz-Justice A, Volpe A. Homeostatic versus circadian effects of melatonin on core body temperature in humans. Journal of Biological Rhythms. 1997;12(6):509-17.

120. Cain SW, Dennison CF, Zeitzer JM, Guzik AM, Khalsa SBS, Santhi N, et al. Sex differences in phase angle of entrainment and melatonin amplitude in humans. Journal of biological rhythms. 2010;25(4):288-96.
121. Duffy JF, Cain SW, Chang A-M, Phillips AJ, Münch MY, Gronfier C, et al. Sex difference in the near-24-hour intrinsic period of the human circadian timing system. Proceedings of the National Academy of Sciences. 2011;108(Supplement 3):15602-8. doi: https://doi.org/10.1073/pnas.1010666108
122. Kuljis DA, Loh DH, Truong D, Vosko AM, Ong ML, McClusky R, et al. Gonadal-and sex-chromosome-dependent sex differences in the circadian system. Endocrinology. 2013;154(4):1501-12. doi: https://doi.org/10.1210/en.2012-1921
123. Zethof TJ, Van Der Heyden JA, Tolboom JT, Olivier B. Stress-induced hyperthermia in mice: a methodological study. Physiology & behavior. 1994;55(1):109-15. doi: https://doi.org/10.1016/0031-9384(94)90017-5
124. Jagannath A, Peirson SN, Foster RG. Sleep and circadian rhythm disruption in neuropsychiatric illness. Current Opinion in Neurobiology. 2013;23(5):888-94. doi: https://doi.org/10.1016/j.conb.2013.03.008
125. Arendt J, Skene DJ, Middleton B, Lockley SW, Deacon S. Efficacy of melatonin treatment in jet lag, shift work, and blindness. Journal of biological rhythms. 1997;12(6):604-17. Doi: https://doi.org/10.1177/074873049701200616
126. Lockley S, Skene D, James K, Thapan K, Wright J, Arendt J. Melatonin administration can entrain the free-running circadian system of blind subjects. J Endocrinol. 2000;164(1):R1-6.
127. Jilg A, Lesny S, Peruzki N, Schwegler H, Selbach O, Dehghani F, et al. Temporal dynamics of mouse hippocampal clock gene expression support memory processing. Hippocampus. 2010;20(3):377-88.
128. Shi S-q, Ansari TS, McGuinness Owen P, Wasserman David H, Johnson Carl H. Circadian Disruption Leads to Insulin Resistance and Obesity. Current Biology. 2013;23(5):372-81.
129. Varni JW, Burwinkle TM, Seid M, Skarr D. The PedsQL™* 4.0 as a Pediatric Population Health Measure: Feasibility, Reliability, and Validity. Ambulatory Pediatrics. 2003;3(6):329-41.
130. Löwe B, Wahl I, Rose M, Spitzer C, Glaesmer H, Wingenfeld K, et al. A 4-item measure of depression and anxiety: Validation and standardization of the Patient Health Questionnaire-4 (PHQ-4) in the general population. Journal of Affective Disorders. 2010;122(1):86-95.