Monitoring the physiological response to exercise is important for both competitive athletes and the general population. Cortisol is a steroid hormone that demonstrates a distinct diurnal and circadian rhythm and responds to acute exercise. A distinct portion of the circadian cortisol profile is observed immediately after nocturnal sleep offset, with cortisol concentrations increasing rapidly within 30-45 minutes from waking. Termed the cortisol awakening response (CAR), this distinct period has been studied extensively within psychoneuroendocrinology and is becoming increasingly popular within the exercise sciences. The CAR may be a uniquely important biological marker for monitoring physiological responses to exercise since it reflects both basal concentrations and responsiveness of the hypothalamic-pituitary-adrenal axis.

Neurophysiological control of heart rate variability (HRV) demonstrates considerable cross-over with the neural structures that regulate the CAR and thus may be a useful non-invasive substitute or supplement to monitoring the CAR. Therefore, the purpose of this study was to investigate the effects of acute exercise on the endocrine (cortisol, cortisone) and HRV responses to awakening. Participants reported to the laboratory for screening, fitness testing, and body composition measures. Within 7-10 days, participants returned to the laboratory in the evening (18:00) and an intravenous catheter was placed for blood sampling every 15 minutes, concluding one hour after nocturnal sleep offset. In a randomized fashion, separated by eight weeks, participants completed either a one-hour exercise protocol (70-75% of maximal power output) on the cycle ergometer or a resting
protocol within the environmental chamber and then stayed in the laboratory overnight. HRV was collected continuously, and saliva samples were collected through the evening until bedtime (22:00) and immediately after waking. Blood and saliva samples were assayed for cortisol, and post-waking saliva samples were assayed for cortisone (E). HRV was analyzed (high-frequency power) for the entire post-waking period and in 5-minute epochs immediately before each blood sample during the one-hour waking period.

Mixed-effects models were used to determine the effect of exercise on the cortisol response post-waking in the blood (and associated indices: maximal change from waking $\text{CAR}_{\text{ba}}$; area under the curve (AUC) relative to a zero concentration $\text{CAR}_{\text{baucg}}$; AUC relative to the increase $\text{CAR}_{\text{bauci}}$), in saliva (and associated indices: maximal change from waking $\text{CAR}_{\text{s}}$; AUC relative to a zero concentration $\text{CAR}_{\text{sucg}}$; AUC relative to the increase $\text{CAR}_{\text{sci}}$), and the cortisone response post-waking (EAR; and associated indices: maximal change from waking $\text{EAR}_{\text{s}}$; AUC relative to a zero concentration $\text{EAR}_{\text{scg}}$; AUC relative to the increase $\text{EAR}_{\text{sci}}$), and HRV response to awakening. In addition, models assessed the relation between cortisol responses to exercise and the CAR/EAR indices and compared the awakening response indices between biological compartments and hormones. Participants ($N = 12$, mean (SD): age = 23 (4.22) years; mass = 76.82 (8.67) kg; height = 175.57 (4.96) cm; $\text{VO}_{2\text{max}} = 48.94$ (7.49) ml.kg$^{-1}$.min$^{-1}$) demonstrated an average exercise-induced increase in cortisol of 477.33%. Results demonstrated a negative effect for exercise condition when modeling the serum and salivary cortisol responses to awakening via a quadratic growth model (serum: $\beta_{\text{condition}} = -42.26 [95\%\text{CI} = -64.52 \text{ to } -20.01]$, $p < 0.001$; saliva: $\beta_{\text{condition}} = -11.55$).
[95%CI = -15.52 to -7.57], p < 0.001). Cortisone derivatives EAR\textsubscript{AUCG} (β\textsubscript{EARS\textsubscript{AUCG}} = 3.78 [95%CI = 2.61 to 4.95], p < 0.001) and EAR\textsubscript{AUCI} (β\textsubscript{EARS\textsubscript{AUCI}} = 2.32 [95%CI = 0.22 to 4.42], p = 0.030) were significantly associated with their blood cortisol counterpart, but none were related to the area under the curve of the cortisol response to exercise. Salivary cortisone demonstrated a response to awakening that had both initial concentration and linear change across time negatively affected by exercise (β\textsubscript{Condition} = -11.07 [95%CI = -15.70 to -6.45], p < 0.001; β\textsubscript{Linear*Condition} = -53.45 [95%CI = -103.14 to -3.76], p < 0.001). There was no observed change in HRV across the waking period, but the log-transformed high-frequency power was significantly lowered on the morning after exercise (β\textsubscript{Condition} = -0.24, [95%CI = -0.45 to -0.03], p = 0.028). The HRV response to awakening was not associated with CAR or EAR derivatives. These results suggest that cortisol concentrations in saliva and blood and cortisone concentrations in saliva are significantly lower the morning following a prior evening exercise session. These reduced concentrations may result from increased cellular uptake of cortisol or a physiological decrease in adrenal output to conserve resources for the following day. Moreover, these results demonstrate that cortisone indices may be a better indicator of blood cortisol indices than salivary cortisol. Thus, the EAR should be considered as an alternative to the CAR in future work.
THE IMPACT OF PRIOR EVENING EXERCISE ON
ENDOCRINE AWAKENING RESPONSES

by

Travis Anderson

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Approved by

_____________________________
Committee Chair
I dedicate this dissertation to Mum and Dad, who always instilled in me a willingness to ask questions and find the answers. Your unwavering support and encouragement are foundational to me facing every new challenge, wherever in the world that happens to be.
This dissertation written by Travis Anderson has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I
INTRODUCTION

The importance of exercise for the maintenance of health and prevention or treatment of chronic diseases has been well documented (F. W. Booth et al., 2011). Currently, the United States Department of Health and Human Services recommends a combination of resistance and aerobic exercise, with 150-300 mins of activity per week of moderate intensity activity, or 75-150 minutes of high intensity exercise (Piercy et al., 2018). Moreover, athletes, whether considered amateur or professional, also engage in exercise training in an effort to increase physical conditioning and improve performance in competition. In both cases, individuals partake in structured exercise sessions to achieve a predefined goal. In order to discern whether an individual is making progress towards this goal, these exercise sessions should be systematically structured and designed to achieve the desired outcome. Among athletes, this process is referred to as periodization, but can be simply and colloquially discussed as an ‘exercise training plan’.

To induce phenotypical changes in response to exercise, one must engage in activity that significantly strains the relevant physiological systems. This overload then activates specific signaling pathways that lead to both intra- and extra-cellular adaptations that improve the physiological systems’ capacity to complete the required work. Therefore, in order to receive the benefit of exercise, and continue to induce beneficial adaptations, one must continuously introduce workloads to which one is
unaccustomed – an exercise training concept referred to as progressive overload. Whilst introducing overload is a necessary component of exercise training, too great of an overload for too long can result in a process known as overreaching or overtraining, which can result in a large and diverse array of negative health consequences (Cadegiani & Kater, 2019b). It is therefore imperative that an exercise training program have some degree of individual monitoring, to ensure the correct overload (i.e. dosage) is prescribed at the appropriate time.

One method of monitoring the physiological response to exercise is through the quantification of exercise-related hormones or other biomarkers. Many resting biomarkers have been proposed as useful for monitoring overreaching and overtraining, such as heart rate (Achten et al., 2004; Bellenger, Karavirta, et al., 2016; Dressendorfer et al., 1985; Fry et al., 1992; Jeukendrup et al., 1992; Le Meur et al., 2013; Li et al., 2013; Wishnitzer et al., 1986), heart rate variability (Bellenger et al., 2017; Bellenger, Karavirta, et al., 2016; Le Meur et al., 2013; Mourot et al., 2004), blood glucose (Gastmann et al., 1998; Ishigaki et al., 2005; W. J. Kraemer et al., 2006; Lehmann et al., 1991a), cytokines (C. K. Booth et al., 2006; Fry et al., 1992; Nieman et al., 2014; L. L. Smith, 2000), catecholamines (Lehmann et al., 1992; Mackinnon, 1996; Mackinnon et al., 1997), growth hormone (Cadegiani & Kater, 2018; W. J. Kraemer et al., 2006), IGF-1 (Alves Souza et al., 2014), insulin (W. J. Kraemer et al., 2006; Volek et al., 2004), and testosterone (Chicharro et al., 1998; Coutts et al., 2007; Griffith et al., 1990; Gustafsson et al., 2008; Hug et al., 2003; Ishigaki et al., 2005; W. J. Kraemer et al., 2004; Li et al., 2013; Moore & Fry, 2007). Some of these biomarkers are more invasive and difficult to
measure than others. Blood biomarkers, for example, allow for the monitoring of hormonal systems, but have traditionally required phlebotomy and biochemical analytic skills to quantify. Such blood-based biomarkers, however, are poised to become more accessible and relevant to the broader public. Although not presently available to the general population, recent technological advancements (Hogenelst et al., 2018; Rice et al., 2019; Sekar et al., 2019) may allow simple, accurate, and non-invasive hormone quantification.

In 1950, Dr. Edward C. Kendall, Dr. Tadeus Reichstein, and Dr. Philip S. Hench were awarded the Nobel Prize in Physiology or Medicine for their discovery and isolation of adrenal hormones (Reichstein, 1951). This discovery led to the use of these hormones, named glucocorticoids, in the treatment of chronic illnesses such as rheumatoid arthritis (Burns, 2016). Since then, the structure and function of the glucocorticoid hormones have received much attention, both for therapeutic uses and to differentiate healthy physiological function.

The hypothalamic-pituitary-adrenal axis (HPA-axis) is a neuroendocrine axis that is central to and critical for human physiological functioning. The neuroendocrine cascade begins with the release of corticotropin releasing hormone (CRH) from neurosecretory cells in the hypothalamus, a small sub-cortical structure in the mammalian brain approximately 1cm³ in volume in humans (Gabery et al., 2015). CRH will then stimulate the synthesis and secretion of adrenocorticotropin hormone (ACTH) from the corticotroph cells in the anterior pituitary gland into the portal vein and systemic circulation. ACTH can then act on the adrenal cortex to stimulate the synthesis and
eventual secretion of the predominant glucocorticoid hormone – in humans, cortisol. Cortisol is then able to freely circulate and induce varying effects throughout the body, by binding to intracellular glucocorticoid receptors, which are found almost ubiquitously in human nucleated cells.

Cortisol is derived from cholesterol, conserving the 4-ringed core steroid structure. This lipophilic hormone cannot readily dissolve in blood plasma, and so cortisol is primarily transported in the blood bound to soluble carrier proteins. Approximately 80% of plasma cortisol is bound to corticosteroid binding globulin (CBG) – a protein with high-affinity for cortisol (J. F. Dunn et al., 1981) that can bind one cortisol molecule. Another 10% of cortisol is bound to the large protein albumin, with affinity for cortisol approximately 1300 times lower than CBG (Levine et al., 2007). The remaining ~10% of cortisol is considered unbound or free (El-Farhan et al., 2017). Cortisol molecules can bind and unbind to these carrier proteins as dictated by the affinity between the ligand and binding protein. The free hormone hypothesis posits that bound cortisol cannot freely cross cell and nucleic membranes and thus it is the free portion that is considered biologically active (Mendel, 1989). This free portion can readily diffuse into salivary glands and be measured within saliva.

The HPA-axis is one component of the physiological stress-response system. As such, both physiological and psychological inputs to the hypothalamus will initiate the neuroendocrine cascade ultimately resulting in an increase in cortisol concentrations. Exercise is considered a physiological stressor, as it induces many perturbations to biological homeostasis, including alterations in metabolic requirements,
thermoregulation, and cardiovascular adjustments. An increase in total cortisol concentration in response to acute exercise is well documented (Fahrner & Hackney, 1998; Gozansky et al., 2005; Hill et al., 2008; Inder et al., 1998; Lutoslawska et al., 1991; Starkie et al., 2005). The increase in cortisol concentration in response to exercise tends to be proportional to the volume of exercise completed, requiring both a minimum intensity (Hill et al., 2008; Jacks et al., 2002) and duration (Duclos et al., 1997; Tremblay et al., 2005; Viru et al., 1992).

Cortisol has also been assessed during resting or basal states, in an effort to describe recovery from exercise sessions. Typically, elevated basal cortisol levels are considered to show a “catabolic” state, and poor recovery. Basal state cortisol has been assessed in overtraining and overreaching research with mixed findings, although the balance of evidence suggests that if changes are observed it is typically characterized by reduced basal/resting concentrations and reduced HPA-axis reactivity (Cadegiani & Kater, 2017a). It has been suggested that overtraining may be better distinguished by monitoring the endocrine responses to a maximal exercise bolus, as it is the capacity of the neuroendocrine systems to respond or react to the stimulus that demonstrates poor adaptability. Ideally, a biomarker that can be assessed at rest and can indicate the reactive capacity of the HPA-axis would be preferable, as an individual could modify the exercise training plan in a proactive manner and avoid the adverse consequences of overtraining. Alternatively, the biomarker may indicate an unexpected high degree of physiological readiness, and the individual can complete a higher workload and accelerate their exercise training program.
Cortisol concentrations show a robust circadian and diurnal profile, with a distinct acrophase early in the morning and decreasing to a nadir in the late evening. In addition to this well characterized pattern, cortisol tends to present a marked increase within the hour after waking from nocturnal sleep. This increase in cortisol concentration has been termed the cortisol awakening response (CAR) and has received considerable attention in the last two decades, particularly in the field of psychoneuroendocrinology. The etiology of the CAR has yet to be fully described, although it is understood to be, and utilized as, a marker of general HPA-axis activity. Although not all studies concur (Carnegie et al., 2014; Mommersteeg et al., 2006), the CAR has been implicated as a biomarker associated with a number of psychoneuroendocrine conditions, including depression (Adam et al., 2010; Dedovic & Ngiam, 2015; van Santen et al., 2011; Vrshek-Schallhorn et al., 2013), anxiety (Greaves-Lord et al., 2007; S. Walker et al., 2011), chronic fatigue syndrome (Hall et al., 2014; Heim et al., 2009; Nater et al., 2008; Roberts et al., 2004), and burnout (Grossi et al., 2005; Oosterholt et al., 2015; J. C. Pruessner et al., 1999).

Since salivary cortisol consists of only the free portion of the hormone, assessing the CAR using saliva compared to serum will introduce biological confounders. Saliva typically shows strong positive correlations with serum or plasma cortisol (Kirschbaum & Hellhammer, 1994), and serum cortisol has been shown to have a similar response to awakening compared to saliva (Wilhelm et al., 2007). However, using salivary CAR as a surrogate for blood biomarker changes may neglect critical variance that may lead to yet undetermined relations between various biomarkers. Most critically, enzymes present in the salivary gland endothelium readily convert cortisol to an inactive steroid, cortisone.
Secondly, at high concentrations of total cortisol in the blood, the free portion of cortisol increases non-linearly, as the binding proteins become saturated. Therefore, analyzing the salivary cortisone response to awakening (EAR) may more closely resemble the hormonal changes occurring in the blood.

In order to maintain cardiac output, the heart utilizes both intrinsic mechanisms and external regulatory control to modulate both stroke volume and heart rate. The rate of contraction maintains an intrinsic rhythm – referred to as cardiac autorhythmicity – but can be influenced through both neural and humoral external factors. The regions of the internal conduction system with the majority of the influence over heart rate - the sinoatrial (SA) and atroventricular (AV) nodes - are directly innervated by the autonomic nervous system (ANS), that can provide external control over the depolarization rate. The variability in time between consecutive cardiac contractions primarily caused by these ANS inputs is known as heart rate variability (HRV).

Due to the influence of the ANS on heart rate, HRV is commonly used as a non-invasive marker of ANS activity. The parasympathetic branch (PNS) of the ANS regulates heart rate on a beat-to-beat time scale, as opposed to the much longer period of sympathetic influence (5-10 seconds). Therefore, HRV metrics, particularly within the high frequency domain (0.4 –0.15Hz), are reflective of PNS activity. The degree of parasympathetic outflow to the heart is controlled through a complex integration of many neurophysiological structures in the heart and CNS. These heart-brain connections and control of HRV have been described by multiple HRV control models. However, the most recent and comprehensive model depicts multiple levels of integration between the
heart and the brain. First described by Thayer and Lane (2000), and later revised and updated by Smith et al. (2017), the Neurovisceral Integration Theory describes a hierarchy of control for HRV, wherein HRV is influenced by local intrinsic factors (Level 1) up to overarching cerebral processes (Level 7). Within this model there exists multiple possible neurophysiological pathways common to both HRV control and HPA-axis cascades. As such, HRV may be a useful non-invasive marker for characterizing HPA-axis activity.

Overtraining, burnout and chronic fatigue syndrome share symptomology. Given the relations between the CAR and these conditions, and the shared symptomology between overtraining and burnout and chronic fatigue syndrome, it is plausible that the CAR may be valuable in identifying states of overtraining. However, very few studies have assessed the effect of acute exercise on the CAR. Of these studies, no study has a) had a laboratory-controlled exercise stimulus; b) had a supervised CAR sample collection; c) investigated the entire nocturnal cortisol profile, including the cortisol response to exercise; d) simultaneously monitored HRV to assess the utility of non-invasive biomarkers to reflect or complement HPA-axis functioning; or e) assessed both salivary and blood biomarkers. Therefore, the primary purposes of this study were to examine the effects of a late-evening acute exercise bout on the CAR, EAR, and HRV and secondarily to examine the relationships between HRV and the CAR and EAR. Delimitations and Limitations

In addressing these purposes, the current study design required several delimitations. First, the current study delimited participants to only young males of an
above-average aerobic fitness capacity and relatively low body fat percentage. This delimitation was introduced to avoid the known influence of sex on the CAR, permitting a smaller sample size and to ensure that participants would be likely to complete the exercise protocol without excessive physiological strain. This study was also delimited to individuals with no known sleep disorders or non-traditional sleep schedules to avoid any confounding effects on the cortisol output during nocturnal sleep. This study also delimits the salivary collection and analyses to awakening time points only to avoid unnecessary disruptions to the sleep period. Lastly, this study delimits the exercise protocol to a cycling modality only to allow for direct quantification of the workload and limit the effects of mechanical efficiency during the evening exercise session. Lastly, steroid quantification in this study was delimited to immunoassay techniques due to time and cost factors. Other analytical techniques may avoid some issues with immunoassays, such as cross-reactivity with other steroids, and should be considered in future work.

Despite the study design attempting to control many factors known to influence the CAR and be potential confounders in the analyses, this study has several limitations to acknowledge. First, the extensive scope of this research project did not permit a period of sleep habituation before the first or second set of visits, possibly influencing participant sleep quality. However, this study did employ a randomized cross-over design, which may reduce the extent of this limitation. Similarly, blood sampling continued throughout the sleep period, further negatively impacting participants' sleep quality. However, wrist-based accelerometry was used to measure sleep quality metrics and thus may be able to be used in post-hoc analyses to address this limitation. Second,
the exercise protocol was standardized across participants, but the capacity to complete the protocol varied across the participants, requiring an individualized approach to modulating the power output to ensure that all exercise interventions lasted one hour. Therefore, this study is limited in making inferences to the effect of an exact power output on the CAR. However, because of the direct quantification of workload throughout the exercise protocol, the individualized average power output and variance in power and cadence could be statistically controlled for in post-hoc analyses. The current study design standardizes food intake for each participant between trials. However, it is limited in making inferences regarding the effect of the caloric deficit because each participant was permitted to select their food intake (type and quantity of food) based on dietary preferences. Moreover, fluid intake was permitted *ad libitum* following the second nude body mass assessment, resulting in some degree of intra- and inter-individual variance in hydration factors on these results. Assessment of hydration factors beyond nude body mass may be able to partially address this limitation.
CHAPTER II
REVIEW OF LITERATURE

General Summary

The following review of literature is organized into multiple subsections. First, a general overview of the hypothalamic-pituitary-adrenal axis is described, including regulation and control of each major hormone in the cascade, the interconversion of glucocorticoids in various tissues, and the role of various nuclear receptors regulating cortisol effects. The review then describes the cortisol response to acute exercise, with a special focus on endurance exercise. Next, the circadian control of cortisol and the cortisol response to the post-waking period is discussed, including methodological considerations for the measurement of cortisol during this specific period. The published evidence suggesting a potential effect of exercise on the cortisol response to awakening is then reviewed. Lastly, the potential for non-invasive cardiac control metrics (i.e. heart rate variability) to similarly reflect responses to both exercise and the process of waking from nocturnal sleep are described.

The Hypothalamic-Pituitary-Adrenal Axis

Cortisol secretion at the adrenal gland is regulated, principally, by the hormonal cascade occurring within the hypothalamic-pituitary-adrenal axis (HPA-axis). Hormonal synthesis and secretion at the hypothalamus and pituitary gland result in endocrine
signals that stimulate the synthesis and secretion of glucocorticoids at the adrenal gland. The axis is regulated through short and long negative feedback loops, controlling the signaling hormones in the cascade. There are also numerous regulatory elements at multiple levels of the glucocorticoid system, including neural inputs, intracellular control at target cells, and humoral conditions that can modify and regulate the eventual effects of glucocorticoids.

_Hypothalamic Control of Cortisol Release_

The hypothalamus is an approximately 4 gram structure in the human brain (Saper & Lowell, 2014) consisting of distinct neuronal nuclei including the paraventricular nucleus (PVN), the supraoptic nucleus (SON) and the arcuate nucleus (Alvarez-Bolado, 2019). The hypothalamus is also anatomically proximal to the suprachiasmatic nucleus (SCN), a neuronal structure critical for circadian rhythm regulation.

The hypothalamus communicates with other endocrine glands through ANS innervation and via neurosecretory cells: the magnocellular and parvocellular neurons (Saper & Lowell, 2014). The magnocellular nuclei are located primarily in the PVN and SON (Alvarez-Bolado, 2019; Møller et al., 2018; Saper & Lowell, 2014) and synthesize primarily arginine vasopressin (AVP) and oxytocin, which can move through the hypophyseal tract and be secreted directly into the systematic circulation. Conversely, the parvocellular neurosecretory cells primarily produce and secrete the releasing factors and hormones that will act on various cell types in the anterior pituitary gland to promote the synthesis and release of specific hormones. The parvocellular cells synthesize a number of neuropeptides, including corticotropin releasing hormone (CRH), which is considered
the first hormone in the HPA-axis cascade (Whitnall, 1993). This 41 amino acid protein (Vale et al., 1981) can be synthesized and stored in vesicles within the axon terminal end bulb (Ohtani et al., 1987), and be secreted upon PVN stimulation from a variety of axonal inputs, including the hippocampus, amygdala, subfornical organ, organum vasculosum lamina terminus, the SCN, the arcuate nucleus, and the locus coeruleus, (Palkovits, 1987; Whitnall, 1993). Over half of the parvocellular neurons also co-localize and secrete AVP (Mouri et al., 1993). When acting alone, AVP is described as a weak secretagogue (Whitnall, 1993), but will act synergistically with CRH at the pituitary gland to stimulate adrenocorticotropic hormone (ACTH) release (DeBold et al., 1984; Orth, 1992; Torpy et al., 1994).

**Pituitary Control of Cortisol Release**

The pituitary gland is a 0.5-0.6 gram endocrine gland at the base of the brain (Amar & Weiss, 2003). The gland is anatomically divided into the anterior, intermediate, and posterior lobes which synthesize and secrete specific peptide and glycopeptide hormones (Amar & Weiss, 2003). The anterior pituitary is comprised of a variety of hormone producing cells: somatotrophs (growth hormone producing) lactotrophs (prolactin), corticotrophs (ACTH and proopiomelanocortin (POMC)), gonadotrophs (follicle stimulating hormone and luteinizing hormone), and thyrotrophs (thyroid stimulating hormone).

Following its release from neurosecretory cells, CRH will bind to the G-protein coupled CRH receptor (CHR1) on corticotroph cells within the anterior pituitary. CHR1 activation results in stimulation of adenylate cyclase activity and through this second
messenger pathway will ultimately activate voltage gated calcium channels (Abou-Samra et al., 1987, p.). CRH will stimulate adrenocorticotropic hormone (ACTH) release from the corticotrophs into the systemic circulation and stimulate the synthesis of POMC (Aguilera, 1998). This large peptide is the precursor for ACTH and several other ligands, including melanocortin stimulating hormone, β-lipotrophin, and β-endorphin. CRH therefore stimulates not only the secretion of ACTH, but also the synthesis of POMC to allow rapid secretion of ACTH when next stimulated (Aguilera, 1998; Autelitano et al., 1989). In addition to direct CRH activity, there is a short portal connection between the anterior and posterior pituitary gland (Daniel, 1976). While most AVP is synthesized by magnocellular neurons, released into the posterior pituitary and then into circulation, it has been suggested that AVP can travel through this intra-pituitary portal and also contribute to ACTH release (Plotsky, 1991).

*Adrenal Production of Glucocorticoids*

The adrenal gland is located on the superior aspect of the kidneys and consists of both an inner layer termed the medulla, and a series of outer layers known as the adrenal cortex. The adrenal cortex itself is comprised of several zones: the zona glomerulosa, zona reticularis, and zona fasciculata. Each zone is comprised of not only distinct morphology, but also different structural features (Pignatti et al., 2017), that functionally permits the synthesis and secretion of specific steroid hormones.

Once in the circulation, ACTH can act on the G-protein coupled melanocortin-2 receptors (MC2R) at the adrenal cortex, primarily on cells residing in the zona fasciculata and zona reticularis (Ruggiero & Lalli, 2016). Importantly, the MC2R is localized to the
internal aspect of the cell, bound to the endoplasmic reticulum. It has been shown that additional proteins called the melanocortin-2 accessory protein are required to bind to MC2R and localize it to the cell surface membrane (Fridmanis et al., 2017), which may also modulate the sensitivity and responsiveness of ACTH signaling (Sebag & Hinkle, 2010). Upon ligand binding, MC2R activation promotes the upregulation of cyclic AMP and protein kinase A (PKA), but in contrast to CRH effects at the anterior pituitary, it also promotes cAMP response element binding protein (CREB) activity and subsequent genomic effects within the adrenal cortex cells (Lefrancois-Martinez et al., 2011).

Steroids are considered a class of hormones that all share a common core termed a gonane. This tetracyclic hydrocarbon is derived from cholesterol and can undergo many alterations to produce a variety of steroids, each with their own specific functions and interactions. The first step of steroidogenesis typically occurs in the mitochondria of the neurosecretory cell, regulated by the enzyme P450-side chain cleavage (P450scc). This enzyme cleaves the side chain from cholesterol to form pregnenolone and isocaproic aldehyde. Further modifications to pregnenolone will then form molecules separated into five major classes: progestogens, estrogens, androgens, mineralocorticoids, and glucocorticoids. The major glucocorticoid (GC) in humans is cortisol. This 21-carbon hormone is synthesized through the action of multiple enzymes within the adrenal cortex, where the final step is the conversion of 11-deoxycortisol to cortisol via the action 11β-hydroxylase.

Intracellularly, CREB promotes several key processes necessary for eventual cortisol secretion. Firstly, and in contrast to the rapid release of CRH and ACTH, cortisol
cannot be produced and stored in intracellular vesicles due to its lipophilic structure. Consequently, cortisol must be synthesized in an on-demand manner, which typically results in at least a several minute lag between activation of the HPA-axis and eventual cortisol increases in the circulation (Horrocks et al., 1990). ACTH promotes the synthesis of cortisol through several mechanisms. First, it will upregulate the translation of the P450scc enzyme. Second, CREB also upregulates the production of the protein steroidal acute regulatory protein (StAR) that promotes the movement of cholesterol to the mitochondria to undergo steroidogenesis. This appears to be the rate-limiting step in the pathway, at least during low or brief ACTH exposure (Stocco, 2001). cAMP/PKA second messengers will also activate and mobilize StAR proteins already present. There also appears to be evidence the ACTH will upregulate the activity of hormone sensitive lipase, also likely through its cAMP activity, which promotes the delivery of cholesterol to the cell and increases the availability to the mitochondria (F. B. Kraemer & Shen, 2002).

Following its biosynthesis, cortisol can be secreted to the circulation.

*Cortisol and Nuclear Receptors*

As a steroid with a hydrophobic nature, cortisol is found bound to carrier proteins. Approximately 90% of circulating cortisol is bound to a protein, with 75-85% being bound to the low-capacity, high-affinity protein corticosteroid binding globulin (CBG) and 10-20% bound to the high-capacity, low-affinity protein albumin (Coolens et al., 1987; J. F. Dunn et al., 1981). The free, unbound portion is considered to be the biologically active portion, according to the free hormone hypothesis (Mendel, 1989,
However, because of the relatively low affinity between cortisol and albumin, this portion of cortisol is also often considered available to target tissues.

Once in the target cell, cortisol will act on intracellular receptors to exert effects. Classically, cortisol binds to the glucocorticoid receptor (GR) which exists in the cytosol bound to heat shock proteins (Pratt et al., 2004) and regulatory proteins (Oakley & Cidlowski, 2013; Petta et al., 2016). Once cortisol binds to the GR, these regulatory components detach and permit the translocation of the hormone/receptor complex into the cell nucleus. Once in the nucleus, the binding of the complex on glucocorticoid response elements (GRE) will initiate the attraction and binding of transcription factors required for the regulation of gene transcription (Freedman, 1992). These intracellular and genomic effects are considered the traditional mechanism through which steroids affect target cells. However, there have been substantial advancements made in describing glucocorticoid specific, rapid, and non-genomic effects, mediated through membrane bound G-protein receptors (Löwenberg et al., 2007; Maier et al., 2005).

The GR is approximately 777 amino acids in length, although a number of isoforms exist as a result of alternative mRNA splicing of the nine exons (Petta et al., 2016). The receptor generally consists of the N-terminus domain, a DNA binding domain, the hinge region, and a ligand binding domain. The most common isoforms, the GRα and GRβ isoforms, are distinct in their amino acid sequences near the C-terminus, within the ligand binding domain (Oakley & Cidlowski, 2013). The GRβ is 50 amino acids shorter and thus exhibits specific properties, distinct from GRα. Most consequentially, the GRβ is antagonistic to the actions of GRα, via the competition for
binding to response elements and the formation of receptor heterodimers (Oakley & Cidlowski, 2013). This receptor isoform interaction thus provides an additional level of cortisol regulation. In fact, the innumerable interactions between these intracellular receptors between varying cell types under varying conditions to permit specific glucocorticoid actions have led some authors to refer to the GR as “molecular swiss army knives” (Spencer et al., 2018).

Importantly, the mineralocorticoid receptor (MR), associated with the binding and facilitation of activity for the steroid class of mineralocorticoids (e.g. aldosterone) has a 5 to 10-fold higher affinity for cortisol than the GR (Reul et al., 2000). This difference in affinity between receptors provides a base-level mechanism for the regulation of cortisol biological activity - cortisol will exert effects only when intracellular concentrations are great enough to bind to the lower affinity GR receptors (Spencer et al., 2018). It has been proposed that an improper or poorly regulated balance of GR and MR receptors will result in undesirable glucocorticoid effects (de Kloet, 2014). In fact, the role of receptor balance in regulating glucocorticoids effect on mood, cognitive performance, psychological resilience, and depression have been reviewed at length (de Kloet et al., 1998, 2016). The intracellular regulation of receptors and receptor ratios therefore adds an additional level of control within the HPA-axis and its effectors (Funder, 1993).

Hundreds of genes are known to be responsive to glucocorticoid administration (John et al., 2009; Reddy et al., 2009). As such, cortisol is considered to be a pleiotropic hormone, stimulating and regulating a variety of effects throughout the body. The term ‘glucocorticoid’ derives from the apparent effects of this group of steroids on glucose
metabolism and metabolic processes in general. However, the metabolic control regulated by cortisol is highly varied and determined by a number of factors including tissue type, metabolic state of the organism, and extent of GC exposure (Magomedova & Cummins, 2015). Genome wide studies have suggested that the modulation of GR responsive genes should not be viewed as an activation or repression of select genes, but rather a dynamic, continuous, and time-dependent expression of a wide variety of genes (John et al., 2009). Moreover, recent reviews have described a rich epigenetic influence on GR function and subsequent glucocorticoid effects (Bartlett et al., 2019).

Intracellular Cortisol Regulation

Prior to binding to a nuclear receptor, cortisol can be rapidly converted to the inactive GC cortisone through the action of 11β hydroxysteroid dehydrogenase (11β-HSD). Two isoforms of 11β-HSD exist, 11β-HSD1 and 11β-HSD2, to convert cortisone to cortisol, and cortisol to cortisone, respectively (Figure 1).

Figure 1. 11β-hydroxysteroid dehydrogenase converts active cortisol to inactive cortisone (11β-HSD2) in the salivary gland.

It is believed that mineralocorticoid sensitive tissues require the colocalization of MR and 11β-HSD2 to readily inactivate glucocorticoids and permit binding of
mineralocorticoids to MR. Immunolocalization studies have demonstrated this colocalization and activity in many tissues, including the submandibular and parotid salivary glands (Hirasawa et al., 1997).

**Feedback Loops Involved in the Control of Cortisol Release**

The HPA-axis is regulated through a series of positive and negative feedback loops. Cortisol can inhibit HPA-axis activation at the level of the anterior pituitary and the hypothalamus. The biological half-life of circulating cortisol is approximately 66 minutes (Weitzman et al., 1971), although the half-life of free cortisol is much shorter at only ~2 minutes (Dorin et al., 2012). The half-life of the peptide hormone ACTH is similar to other pituitary hormones and considerably shorter than circulating cortisol, at approximately 15-20 minutes (Dorin et al., 1996; Veldhuis et al., 1987). The negative feedback of cortisol on ACTH results in an underlying ultradian rhythm with a pulsatile length of approximately 76 minutes (Henley et al., 2009). The feedback to the anterior pituitary via circulating cortisol is perhaps the most prominent contributor to the ultradian rhythm of cortisol secretion, as opposed to CRH oscillations (J. J. Walker et al., 2012). This negative feedback is a result of cortisol acting on the corticotrophs of the pituitary and operating through traditional genomic actions. Since the POMC promotor has an associated negative GRE, cortisol can directly inhibit its synthesis (Jenks, 2009) and thereby inhibit the production and release of ACTH.

The negative feedback loop of cortisol on the hypothalamus is less well understood. It appears that cortisol regulation of CRH synthesis and secretion may be under both genomic and non-genomic control (Herman & Tasker, 2016). Recently,
surface G-protein cortisol receptors have been identified, which may help to explain the rather rapid (on the order of minutes) inhibition of parvocellular neuronal activity with administration of glucocorticoids (Tasker et al., 2006). Although these receptor mechanisms are not well explained, this inhibition is thought to occur through the control of glutamate and calcium in the neuronal terminal (Di et al., 2003). Cortisol can also provide negative feedback to the adrenal gland. Due in part to the ubiquity of GR throughout cell types, including at the adrenal cortex (Briassoulis et al., 2011), some researchers have proposed intra-adrenal feedback loops, where cortisol can influence cortisol production (Spiga & Lightman, 2015).

In these negative feedback loops, cortisol can inhibit ACTH and CRH release (rapid negative inhibition) as well as downregulate the expression of CRH and POMC and thus provide a mechanism for longer-term negative feedback (Gjerstad et al., 2018). An often undiscussed positive feedback mechanism has also been described (Herman et al., 2012) wherein glucocorticoids may augment the stress response, possibly through feedback to stress-excitative neurons in the amygdala. This positive-feedback may be implicated in some of the chronic HPA-axis conditions and disorders. In addition, other physiological feedback loops exist to regulate the HPA-axis, external to the HPA-axis itself. For example, cytokines, produced and secreted by both immune and non-immune cells, are able to stimulate the HPA-axis to ultimately secrete glucocorticoids (A. J. Dunn, 2000; Gądek-Michalska et al., 2013). Glucocorticoids have well documented immunosuppressive effects, thus regulating this cytokine-HPA-axis loop.
Cortisol Responses to Exercise

Exercise Is A Physiological Stressor

In the early-20th century, Dr. Hans Selye used noxious agents to induce a homeostatic disruption to physiological function (Selye, 1936) and termed this stress. To differentiate general stress responses from those that led to disease, the terms distress and eustress were introduced (Selye, 1956). Later, Selye described stress as a non-specific response of the body (i.e. physiological systems) to a demand (Selye, 1976), where the non-specificity required more than one “biological unit” to be affected (Selye, 1956) - impacting, simultaneously, many physiological systems (Everly & Lating, 2019). Since the stress was the response variable, the term stressor was introduced to describe the event or action that caused the stress. From its initial description, this terminology has been adopted broadly, across many disciplines.

The concept, definition, and use of the stress terminology has been debated since its inception. For example, researchers may not differentiate between the stress response and the stress exposure (Koolhaas et al., 2011). Others have argued that the common usage of the term stress is too wide-ranging and encompassing, and thus should be limited to only life-threatening events (Kagan, 2016). Despite agreement on the historical context of the term, McEwen and McEwen have argued that the terms stress and stressor be placed in the context of allostatic load (McEwen & McEwen, 2016). Here, allostasis refers to a dynamic system, where many physiological systems interact, adjust, and adapt (i.e. change), in an effort to maintain stability of the organism as a whole. The cumulative
deterioration to the body that accompanies such variations in these systems is termed the allostatic load (McEwen & Wingfield, 2003).

Agreement on the use of these terms in the scientific literature is unlikely to be resolved in the near future, but in order to discuss these phenomena, descriptors must be chosen, regardless of the controversy surrounding their use. Thus, herein, the term stressor is used to denote the external factor that disrupts homeostasis, stress refers to the generalized physiological response, and stressful qualitatively describes the set of environmental conditions the organism is in. Any greater level of specificity or context will be provided where necessary.

As described, it is well known that cortisol concentrations will tend to increase in response to stressful situations, whether these be psychological (Biondi & Picardi, 1999; Kemeny, 2003) or physiological (Viru & Viru, 2004). Since physical exercise is defined as a significant challenge to homeostasis and allostasis, it too demonstrates a robust cortisol response. In this regard, one can consider both the acute effects of engaging in exercise, as well as the change in resting concentrations or acute responses to exercise following an exercise training program (i.e. chronic exercise effects).

The hypothalamus acts as a sensor for a variety of homeostatic markers. A major role of a subset of hypothalamic neurons is to detect alterations in blood nutrient concentrations and initiate or regulate the neuroendocrine cascade in response (Blouet & Schwartz, 2010). It is well known that glucocorticoids are a potent stimulator and mobilizer of metabolic substrates, including glucose, fatty acids (Xu et al., 2009), and amino acids (Brillon et al., 1995).
Cortisol Response to Acute Exercise

Exercise-induced alterations in cortisol concentrations vary widely and are highly dependent on the context of the exercise and activity. Early research typically showed cortisol to be unchanged or show a transient decrease with physical activity. For example, researchers reported a slight decrease in plasma cortisol when cycling at 100W for 20 mins (Cornil et al., 1965), while others reported no change in plasma cortisol following a 1-mile run (Rose et al., 1970).

It later became evident however, that higher intensity exercise resulted in an increase in cortisol concentration. In 1973, Davies and Few (1973) hypothesized that exercise intensity influenced cortisol responses to exercise and thus they recruited participants to exercise at 60-90% of VO\(_{2}\text{max}\) (“heavy”) or <50% VO\(_{2}\text{max}\) (“light”) while monitoring the plasma cortisol concentrations. Results clearly demonstrated that circulating cortisol significantly increased when engaged in high intensity activity. These results were confirmed the following year, when researchers demonstrated a decrease in plasma cortisol when walking at 6.4 km/h for 1 hour, but an increase in plasma cortisol when exercising at the greatest workload they could maintain for the same duration (Few, 1974). Thus, it appeared that cortisol responses to exercise were dependent on the intensity of the exercise and were summarized as such in a review that same decade (Shephard & Sidney, 1975).

In the most-cited study on this apparent intensity threshold, Hill et al. (2008) demonstrated a clear intensity dependent effect on cortisol production and secretion with exercise. In this study, 12 male runners completed 30 minutes of running at 40%, 60%,...
and 80% of VO$_{2\text{max}}$. There was a clear increase in the cortisol response at 60% and 80% of VO$_{2\text{max}}$, with no change in cortisol concentration observed when exercising at 40% of VO$_{2\text{max}}$. The authors postulated that these increases were not a result of decreased metabolic clearance rate and were therefore attributable to metabolic demands of the activity. The results of this study agree with observations related to an intensity threshold and converge on an intensity of at least 60% of VO$_{2\text{max}}$ being necessary to cause increases in cortisol concentration. It has been suggested that the decrease in cortisol observed with low intensity exercise is likely and conversely due to increased metabolic clearance (Davies & Few, 1973).

Cortisol has been demonstrated to have a critical role in regulating plasma glucose conditions. For instance, an exquisite study design utilizing a pancreatic-adrenocortical-pituitary clamp has demonstrated that cortisol aids in the maintenance of blood glucose following insulin-induced hypoglycemia (De Feo et al., 1989). In response to exercise specifically, a central function of cortisol synthesis and secretion is to mobilize metabolic substrates and maintain blood glucose concentrations to aid in exercise performance. For example, cortisol can directly stimulate and enhance gluconeogenesis in hepatic tissue, have permissive effects on the metabolic function of hormones such as norepinephrine, and inhibit blood glucose uptake and thus spare glycogen stores (for a review and additional details, see: Hackney et al., 2008). This crucial role of cortisol in regulating blood glucose partially explains the increase in circulating cortisol concentrations during high-intensity exercise, where metabolic demands require a shift towards glycolysis and glycogenolysis.
However, although relatively robust this threshold-effect is not absolute and independent of other factors. For example, Dourida et al. (2019) reported a decrease in serum cortisol in subjects performing treadmill exercise at 70% of VO$_{2\text{max}}$ for 30 minutes. However, it must be noted that these subjects were exercising at intensities that were only estimated from standardized formulae – the participants were “walking” at 4.8 km.h$^{-1}$ and thus could have been exercising at significantly lower intensities. Similarly, 11 otherwise sedentary participants completed intermittent and continuous 40 minute exercise protocols, at 60% HRR (Ahmadi et al., 2018), which would typically be greater than the established threshold. Researchers reported a decrease in cortisol concentration from pre-to post-samples under both conditions.

The magnitude of the increase in cortisol concentration is therefore dictated by additional factors beyond the intensity of exercise. One such factor is the duration of the activity. Early studies demonstrated that when cycling at lactate threshold, cortisol concentrations increased only after 90 minutes of exercise (Schwarz & Kindermann, 1989). This effect was again observed in a more contemporary study, where participants ran at 50-55% of VO$_{2\text{max}}$. In this instance, cortisol concentrations decreased when running for 40 and 80 mins, but increased when the exercise duration was extended to 120 minutes (Tremblay et al., 2005).

Cortisol can also be significantly elevated by resistance exercise, and has been considered to be a factor in the remodeling of skeletal muscle (W. J. Kraemer & Ratamess, 2005). Total muscular work appears to modulate the cortisol response, as resistance training protocols with greater volume (Ahtiainen et al., 2003) or less time
between sets (W. J. Kraemer et al., 1996) both demonstrate larger cortisol responses. This was further exemplified in a study by Wahl et al. (2014) who, despite no change in cortisol concentration with 60 mins of cycling at 70% of peak power output, demonstrated an increase in serum cortisol when researchers applied electromyostimulation to the thigh, calf, and buttocks.

Return to Baseline Cortisol Concentrations

Both the exercise intensity and duration effects reflect the tendency for neuroendocrine responses to be related to the volume of exercise, typically characterized as the interaction between exercise intensity and duration (Hackney, 2006). Depending on the duration and magnitude of the cortisol increase, resting concentrations may take up to 48 hours to return to baseline, demonstrated by researchers employing a high-intensity and exhaustive exercise bolus (Anderson et al., 2016). This continued activation of the HPA-axis post-exercise is likely to disrupt and affect other HPA-axis functioning and phenomena, including those related to the typical circadian variation. Recently, Hooper et al. (2019) studied 22 males who competed in the Ironman World Championships and observed an elevated cortisol concentration immediately following race completion. Although cortisol concentrations appeared to return to baseline 24 hours later, concentrations increased again 48 hours after the race. Clearly cortisol responses to exercise are not isolated to laboratory environments. An extensive road cycling race during high ambient temperature conditions produced a robust, 7-fold increase in cortisol concentrations (Vingren et al., 2016). Interestingly, the cortisol increase was greater in those athletes that completed the 164km distance faster.
Moderators of Cortisol Response to Exercise

Other factors are also well known to blunt or augment the cortisol response to exercise. For instance, the time of day in which the activity occurs will potentially alter the cortisol response. Bonato et al. (2017) demonstrated a 26.1% increase in salivary cortisol when completing a high-intensity exercise session in the morning, compared to a 33.4% increase when exercising in the evening. These results were mirrored in subjects completing a submaximal treadmill exercise session for 30 minutes, with serum cortisol increasing 150% when exercising at 7am, compared to 200% at 7pm, and 600% at midnight (Kanaley et al., 2001). Furthermore, it appears that the effect of time of day may interact with an individual’s chronotype, as ‘evening’ chronotypes had a greater increase in salivary cortisol in the morning exercise condition compared to ‘morning’ chronotypes (Bonato et al., 2017). The body composition of an individual may also contribute to the magnitude of the cortisol response, as demonstrated by the greater cortisol area under the curve in response to a resistance exercise paradigm in obese participants (G. A. Thomas et al., 2012).

Acute exercise in a competitive environment has been demonstrated to induce a greater cortisol response to the activity compared to a non-competitive environment (Viru et al., 2007), which the authors credit to the additional psychological influences on HPA-axis activity. This also appears to interact with the level of competition and player status, as professional athletes and amateurs demonstrate disparate cortisol response to training vs. competitive soccer matches (Jiménez et al., 2020). In addition, hot/humid environments tend to exacerbate the cortisol response (Brenner et al., 1997; Laing et al.,
2005), although this is not a consistent finding (Hoffman et al., 1996). Relatedly, hypohydration also tends to modulate the cortisol response to exercise (Maresh et al., 2006). In sum, it appears that to maximize the cortisol response to exercise, participants should exercise 1) at an intensity >60% of VO$_{2\text{max}}$; 2) for a duration of 30-60 minutes; 3) in a hot and humid environment; and 4) in the late evening.

**Chronic Exercise Effects on Cortisol Response to Exercise**

It has been reported that the hypothalamus demonstrates substantial neuroplasticity in response to chronic stress (Herman & Tasker, 2016). As such, the responsiveness of the HPA-axis to acute stressors can be modulated by the chronic exposure of the organism to previous stressors. It is therefore worthwhile highlighting the impact of chronic exercise training on the acute cortisol response to exercise. It is generally accepted that following a chronic exercise program cortisol responses to a stress of the same absolute work are lower (Viru, 1992). Sato et al. (2016) recently demonstrated this effect in male endurance runners and age matched controls. The non-athletes demonstrated an increase in serum cortisol concentrations at running intensities of 40% and 70% of peak VO$_2$, whereas the athletes presented significant elevations only after completing the 90% of peak VO$_2$ workload. However, if the same relative exercise stress is applied, chronic exercise training can actually induce a more robust cortisol response to the activity (Viru, 1992). It is believed that this increase may be due to adrenal gland hypertrophy which accompanies chronic exercise training (Bartalucci et al., 2012; Tharp, 1975). This enhanced cortisol response in trained athletes was recently emphasized in a study that utilized an insulin tolerance test to remove the impact of other
physiological systems; cortisol responsiveness to the hypoglycemic condition was 53.3% greater than non-athlete controls, which the authors described being a result of a “hormonal conditioning” process (Cadegiani & Kater, 2019a). Importantly, this cortisol response would be dependent on the state of the athlete post-training, as intensified periods of training can result in a blunted cortisol response (Hough et al., 2013).

In summary, the greater the stressor on the individual, the greater the cortisol response to the activity, whether this be increases in intensity, duration, or both. This HPA-axis activity is in response to the combined total of metabolic, thermoregulatory, and psychological perturbations to the individual, requiring significant responses to maintain homeostasis and allostasis. Here, the term stressor necessitates both real and perceived threats to homeostasis, such that independent psychological effects are included.

Glucocorticoids and Circadian Regulation

Neuroendocrine Control of Circadian Rhythms

The suprachiasmatic nucleus (SCN) in the mammalian brain is considered to be the “master pacemaker” due to its intrinsic transcriptional/translational clock mechanism. This clock mechanism is primarily controlled by four proteins, operating in two, self-oscillating negative feedback loops (Nicolaides et al., 2017; Partch et al., 2014). The circadian rhythm maintained by the SCN is definitionally on the order of approximately a complete light/dark cycle (i.e. ~24 hours). However, the phase and amplitude of these rhythms are able to be adjusted and offset by both photic and arousal-related stimuli (Rosenwasser & Turek, 2015). This clock mechanism contains both a positive and
negative arm that acts in a negative feedback loop to control clock-related genes and neuronal efferent activity. The positive arm consists of the *clock* and *bmal* genes and their paralogs CLOCK and BMAL1; these proteins form a heterodimer and will bind to their DNA response element to promote the synthesis of period and cryptochrome proteins, Per 1/2/3 and Cry 1/2 (Nader et al., 2010). These proteins are considered to be the negative arm of the transcription/translational loop and in humans are most abundant during the nocturnal or sleep period. These proteins will then form heterodimers (i.e. Per/Cry dimers) and inhibit the binding of the CLOCK/BMAL dimer and hence inhibit their own transcription and synthesis (Nader et al., 2010). Many control mechanisms exist for this relatively simple negative feedback loop. For example, Per proteins are phosphorylated and inhibited through casein kinase activity, allowing for their removal from the nucleus and a restarting of the cycle. In addition, REV-Erb and ROR proteins are known to be clock-regulated and can actually bind to the ROR response element and inhibit the binding of the BMAL protein, hence adding a further level of control. In fact, it is speculated that the REV-Erb/ROR loop will elongate an otherwise relatively short loop, and permits the stability of the 24-hour rhythmicity (Oster et al., 2016).

The SCN is entrained to external light signals. The SCN sits anatomically proximal to the optic nerve, and thus can receive signals directly following the signal being received by the optic nerve. Retinal ganglion cells in the eyes contain the photopigment melanopsin that becomes activated when these cells are exposed to light (Hattar et al., 2002). The entrainment of the SCN is thought to occur through CREB signaling at the SCN (B. Lee et al., 2010), likely through Per regulation (Tischkau et al.,
The robustness of this clock mechanism is essential for the circadian rhythm observed in many systems in the body, including the endocrine system.

**Circadian Rhythm of Cortisol**

Cortisol concentrations over a 24-hour period demonstrate a relatively stable diurnal profile, characterized by increasing concentrations in the early morning, peaking soon after waking, and then decreasing across the course of the day reaching a nadir in the late evening (Hellman et al., 1970; Peters et al., 2013; Weitzman et al., 1971). This circadian pattern has been described as being the result of a number of complex interactions between intracellular CLOCK proteins in the SCN, direct neural activation of the adrenal gland, and local intracellular circadian regulators in the adrenal gland itself (Chung et al., 2011). At all points across the 24-hour period, stress-inducing events can super-impose an acute increase in cortisol concentration on top of this circadian pattern. In addition, some researchers have also reported seasonal differences in these circadian profiles (Kanikowska et al., 2019), although the research was completed on a relatively small sample size.

Because of this circadian profile, there exist several indices for characterizing the cortisol output across a 24-hour period. One of the most common is the diurnal cortisol slope, that typically measures cortisol at several points throughout the 24-hour period and calculates linear slopes for the profile. These slopes are therefore highly dependent on the timepoints that are selected for analysis, as differences in these selections alters the factors contributing to the cortisol profile. The most common form of circadian slope is...
calculated from immediately after waking to the point just before going to sleep (Adam et al., 2017). It is also possible to calculate the peak (i.e. ~30-45 mins after waking) to sleep slopes, peak to trough slopes, and fixed time point slopes. Typically, all of these slopes are calculated as either change scores, change over time, or multilevel regressions that can be fit to the data and coefficients extracted (Adam et al., 2017).

Aside from these linear trends, some have also fit non-linear trends to the data (i.e. cosine waves) and extracted amplitude characteristics (Baird et al., 2012). Each of these has distinct advantages and disadvantages, and so researchers must select the approach that most adequately addresses their research question. Current recommendations are to calculate these diurnal indices without the inclusion or consideration of the immediate waking period, as this sub-component of the diurnal profile may be under distinct and independent control (Adam et al., 2017).

The Role of Cortisol as A Circadian Regulator

Circadian oscillating clock genes are found almost ubiquitously in mammalian cells (Balsalobre, 2002; Stratmann & Schibler, 2006). It is believed that the SCN sits as the conductor atop a hierarchy of clock mechanisms (Buhr & Takahashi, 2013), and although it is not thought to be able to dictate the rhythmicity of the clocks per se, it does provide entrainment clues from the environment and provide tuning signals to the clocks in the peripheral tissues. In this role, the SCN is more appropriately considered a master synchronizer, rather than a pacemaker (Buhr & Takahashi, 2013). It is believed that one method of synchronizing the SCN with these peripheral clocks is via the endocrine system. Although melatonin has been considered as a possible circadian synchronizing
hormone, it has been argued that cortisol is most well suited to performing this function. (Spencer et al., 2018).

The anatomical proximity of the PVN and SCN has been suggested to indicate a co-evolutionary development. It has been argued that diurnal cortisol variation and the classic cortisol ‘stress’ response are not physiologically distinct events, but rather are reflective of the same underlying physiology (Spencer et al., 2018). In this model, a ‘stress’ that induces an increase in cortisol concentration, could be considered an ‘exaggerated awakening’ wherein the organism has moved from an unconscious state to a conscious one. The co-evolution of cortisol serving in both a circadian and stress response role has also been interpreted through this model. It is believed that any environmentally imposed stressor on an organism is most likely to occur during the movement of the organism from the secure home/nest. In fact, circadian disruption, particularly that which affects the HPA-axis, has itself been considered a stressor that may increase allostatic load (McEwen et al., 2015). Since the peripheral clock proteins are also a target of cortisol action, there exist many complex bidirectional feedback loops between circadian regulated processes and the HPA-axis, including stress responses (Rao & Androulakis, 2019).

It is known that peripheral tissues, including skeletal muscle, also secrete a number of molecules that cross-talk with the brain (Delezie & Handschin, 2018; Kim et al., 2019; Pedersen, 2019) and may be involved in circadian synchronization (Delezie & Handschin, 2018; Gabriel & Zierath, 2019). Although this feedback from the skeletal muscle to the brain does not involve cortisol directly and is primarily concerned with
signaling proteins such as cytokines, the feedback to the brain has the potential to regulate HPA-axis function. Therefore, exercise may affect the circadian regulation of cortisol via the direct activation of the HPA-axis and through feedback mechanisms from the contracting skeletal muscle to the brain. As a result, exercise has been suggested as a behavioral mechanism to improve circadian alignment in peripheral tissues (E. E. Schmitt et al., 2019). As an additional layer of complexity, it has recently been proposed that regular exercise may also affect the permeability of the blood brain barrier (Małkiewicz et al., 2019), making the potency and effectiveness of these various feedback mechanisms adaptable over time.

The Quantification of Glucocorticoids

Glucocorticoids, including cortisol, can be readily quantified in the blood, both in serum and plasma. However, these assays require a venipuncture for sample collection, and thus increase participant burden and makes home-based cortisol assessment logistically difficult. The assessment of cortisol in saliva has become a common practice in both exercise (Gatti & De Palo, 2011; Lindsay & Costello, 2017; Papacosta & Nassis, 2011) and psychoneuroendocrine research (C. G. Engeland et al., 2019; Kirschbaum & Hellhammer, 1994; Smyth, Hucklebridge, et al., 2013). Free cortisol can passively diffuse from the blood into salivary glands and be expressed in the resulting whole saliva. Therefore, the quantification of salivary cortisol is thought to be representative of the free portion of the hormone in the blood (Umeda et al., 1981; Vining et al., 1983).

The delay between salivary and serum cortisol elevations were directly tested in a well-designed study examining the cross-correlations between HPA-axis biomarkers. In
In one of the first studies to demonstrate the efficaciousness of saliva for the monitoring of free cortisol in serum, researchers demonstrated a correlation coefficient between the two compartments of $r = 0.893$ (Umeda et al., 1981). Administration of a low dose of ovine CRH produced an increase in both serum and salivary cortisol (Kumar et al., 2005). Over the 90 mins of sample collection, these biological compartments showed a similar profile, peaking and decreasing at approximately the same time. It is important to note that samples were collected only every 15 minutes, and thus the delay between salivary cortisol reflecting blood concentrations cannot be determined. Even so, the correlation between salivary and serum cortisol ranged from $r = 0.87$ at baseline to $r = 0.7$, 90 minutes later (Kumar et al., 2005), suggesting variability between concentrations in these two biological compartments. Moreover the initial increases for serum and salivary cortisol, 30 minutes after administration of CRH, were 78.8% and 803.3% respectively (Kumar et al., 2005) – a clear dissociation. In regards to the circadian variation in the hormone, Dorn et al. (2007) demonstrated good synchronicity between salivary and serum cortisol, with the amplitude, frequencies, and peak and trough times between the two compartments occurring at near equivalent times.
The strength of the relationship between salivary and blood cortisol concentrations can be altered by exercise. A resistance training protocol demonstrated significant relations between salivary and serum cortisol prior to exercise ($r = 0.52$), but an even stronger relationship following exercise ($r = 0.62$), when serum cortisol increased from 27.82 to 34.75 ug/dl (Cadore et al., 2008). The relationship between salivary and serum cortisol can also improve with endurance exercise. Early studies demonstrated significant correlations between serum and salivary cortisol at all timepoints during 30 minutes of cycling at 70% $\text{VO}_{2\text{max}}$, but coefficients were greatest 15 mins after exercise ($r = 0.93$) (O’Connor & Corrigan, 1987). Others have demonstrated a similar result, with correlations increasing from baseline ($r = 0.76$) to the end of exercise ($r = 0.81$) (J. Powell et al., 2015). However, in this study, salivary cortisol peaked immediately post-exercise, whereas serum cortisol peaked 15 mins later. The authors speculated that this was likely due to CBG saturation or activity of $11\beta$-HSD2 at the salivary gland (J. Powell et al., 2015). Similarly, in a study by VanBruggen et al. (2011), participants exercised at 40%, 60%, and 80% of $\text{VO}_{2\text{max}}$ for 30 minutes, and cortisol was measured in both blood and saliva. Results demonstrated the typical intensity-threshold effect; however, cortisol in serum was increased after both moderate and high-intensity exercise, whereas salivary cortisol demonstrated an increased concentration following only the high-intensity condition. What is more germane however, is that the authors noted the robustness of the relation between salivary and serum cortisol decreased at high concentrations (i.e. concentrations achieved following exercise-induced cortisol increases). The threshold of cortisol concentrations at which the salivary and serum cortisol is no longer proportional
also tends to be the upper range of concentrations observed during a healthy male circadian rhythm, early in the morning (Wilhelm et al., 2007).

It is clear that salivary and serum cortisol are highly correlated (Kirschbaum & Hellhammer, 1994), including within elite athletes (Lippi et al., 2016), from adolescents to adults (Adebero et al., 2020). However, the proportion of salivary to total (i.e. blood based) cortisol varies between 1-9% (D. H. Hellhammer et al., 2009). Moreover, the relation between cortisol concentrations in the two compartments show a non-linear relation with increasing concentrations of the hormone (Vining et al., 1983). This curvilinear association is primarily due to the saturation of CBG at high concentrations of cortisol (450-500 nmol/l), which may occur during stressful events that stimulate HPA-axis activity (D. H. Hellhammer et al., 2009). Moreover, because of the presence of 11β-HSD2 in the epithelia of the salivary glands (Hirasawa et al., 1997; R. E. Smith et al., 1996), serum free cortisol is rapidly converted to cortisone in saliva. Therefore, salivary cortisone is greater than salivary cortisol, and has been shown to have a linear relationship with serum cortisol, even at high concentrations (Perogamvros et al., 2009). Recently, Bae et al. (2019) demonstrated that salivary cortisone is responsive to a psychological stress test, and exhibits a response peak at the same time as serum cortisol. Moreover, salivary cortisone demonstrated the highest discriminatory power (differentiating between experimental and control groups), and had a higher sensitivity than salivary cortisol at a level of 95% specificity (Bae, Reinelt, et al., 2019).

The effects of CBG saturation and 11β-HSD2 activity may also be complicated by the biochemical characteristics of CBG itself. As described by Cameron et al. (2010), a
tight thermo-couple exists between CBG and cortisol, such that at increased temperature, more cortisol will disassociate from CBG and become biologically active. This is an important quality to consider when assessing the role of exercise on cortisol dynamics, as exercise tends to increase core body temperature, and the blood itself acts as the major heat dispersion medium. Core body temperature also demonstrates circadian variation. Resting core body temperature will fluctuate from 0.8-1.2 °C, rise rapidly after waking and reach its acrophase between 8-10am (Refinetti, 2010). CBG itself also demonstrates a circadian rhythm, with concentrations being lowest in the early morning period (Debono et al., 2016; Melin et al., 2019). Therefore, both exercise-induced and circadian variation in core body temperature may affect the free portion of the hormone, in addition to the changes in the total hormonal concentration.

**The Cortisol Awakening Response**

**Neurophysiology of Awakening**

There are many neuroanatomical substructures that are either directly implicated in the sleep-to-wake transition or are indirectly activated or inhibited by the process. Two branches of the ascending arousal system have been characterized and each activates subcortical structures that act to relay signals to the cortex for the full attainment of consciousness (Saper et al., 2005). Within these branches, several structures have been implicated in the formation of a so-called ‘flip-flop’ switch, that stimulates the waking process. Recent functional connectivity MRI data suggests that both the sleep- and wake-promoting region of the anterior and posterior hypothalamus, respectively, have “unique and complimentary” brain networks (Boes et al., 2018).
During sleep, the ventrolateral preoptic nucleus (VLPO) inhibits neurons associated with the arousal system; namely the locus coeruleus, tuberomammillary nucleus, and the Raphe neurons; collectively termed monoaminergic cell groups. During periods of wakefulness, these same arousing neural bodies will inhibit the VLPO neurons, thus creating a bidirectional inhibition system. However, the direct inhibition of each area creates a somewhat unstable system, as activation of one body could result in immediate sleep or waking to be initiated. Orexin containing neurons have been described as a stabilizer in this system – activated during wakefulness and directly stimulating the monoaminergic neurons whilst being inactivated by the VLPO during sleep. This creates a more stable system, since the VLPO does not have orexin receptors (see Saper et al., 2005 for review).

**HPA-Axis Activation During Waking**

There are clear connections between the HPA-axis and the process of awakening. Specifically, CRH is known to activate the locus coeruleus, and the norepinephrine containing neurons of the locus coeruleus stimulate both parvocellular neurons and the amygdala (Buckley & Schatzberg, 2005; Tsigos & Chrousos, 2002) and some have suggested that CRH may be an initiating factor in spontaneous waking (Chang & Opp, 2001). The process of waking therefore stimulates the HPA-axis (and related structures), leading to alterations in circulating hormones.

**Cortisol Response to Awakening**

The cortisol awakening response (CAR) was first described by Dr. Jens Pruessner, Dr. Clemens Kirshbaum, and Dr. Kirk Hellhammer (1995). These researchers
observed a marked rise in cortisol concentrations in the immediate post-waking period. In 1997, Pruessner and colleagues published the results from three distinct studies and populations who all presented an increase (5-9.5 nmol/l) in free cortisol upon awakening (J. C. Pruessner et al., 1997). Given the relatively high correlation between the multiple days of salivary collection (r = 0.39 – 0.67), the authors proposed this cortisol increase that accompanied awakening to be a relatively stable marker of HPA-axis activity (J. C. Pruessner et al., 1997). In the two decades since, many researchers have studied the details of this awakening response and have shown relations with a multitude of psychological and physical conditions.

A year following this initial observation, Scholtz et al. (1998) presented data from a study of university students that suggested the CAR was greater in those who report higher scores on chronic stress surveys. This was in comparison to the analysis of teachers, wherein those scoring high on a ‘burnout’ scale had lower CAR values than the group reporting low burnout scores (J. C. Pruessner et al., 1999). This study was also one of the first to show that dexamethasone treatment the prior evening (0.5 mg) could significantly blunt the CAR (J. C. Pruessner et al., 1999). Burnout in white-collar workers has also been demonstrated to produce a high CAR as measured by AUC (Grossi et al., 2005). Early research also implicated the CAR in psychological disorders; Pruessner et al. (2003) observed differences in the CAR between groups selected by self-reported symptomology, with a greater CAR occurring in those who scored higher on the Hamilton Depression Inventory.
The relative increase in cortisol concentration is typically 50-100%, with concentrations peaking within 30-45 minutes following awakening. The absolute change in concentration is dependent on the fluid used to measure cortisol, as saliva consists of only the free, unbound portion of cortisol, while cortisol concentration determinants from blood are often calculated as only total cortisol, which includes the bound and unbound portions. Typically, when assessed in saliva, the CAR is about a 9.5 nmol/L increase in concentration, while serum concentration increases are about 110 nmol/L. This increase can be detected in both saliva and in blood analysis (Wilhelm et al., 2007), suggesting that the awakening response is not simply an emergent construct of cortisol movement between various bodily fluids (i.e. blood to saliva). Rather, the CAR is a physiological phenomenon that is regulated by several key physiological interactions.

As discussed, cortisol follows a strong circadian diurnal rhythm, with nadirs being observed in the late evening and into the night, and the acrophase occurring in the morning, soon after waking. This is not observed in all mammals, as the pattern is inversed in rodents to synchronize with the sleep (day) and wake/activity (night) cycles specific to those species. As such, it appears that cortisol (corticosterone in rodents) peaks at the time of the 24-hour cycle in which the organism has greater activity requirements, and is decreased during periods of rest and sleep, when mobilization is expected to be low.

The CAR appears to be distinct from this typical diurnal rhythm, as demonstrated by Wilhelm at el. (2007). In this study, researchers profiled (blood samples) subjects across a 12-hour period and tested whether the CAR was simply a continuation of the rise
in cortisol that begins during the early morning time prior to awakening. The researchers demonstrated that the cortisol increase following awakening was distinct from the circadian rhythm and was therefore superimposed over the circadian pattern. The superimposition of cortisol increases is not novel, as stressors applied to an individual throughout the 24-hour period can result in elevations in cortisol concentrations (Spencer & Deak, 2017), as in the case with exercise.

Regulation of the Cortisol Awakening Response

The mechanisms related to this increased cortisol concentration after waking are not fully understood. It has been demonstrated that the administration of (0.5mg) dexamethasone is sufficient for the complete blunting of the CAR (Ebrecht et al., 2000; Mommersteeg et al., 2006), suggesting the CAR is pituitary driven. However, there is also evidence to suggest cortisol concentrations become decoupled from the ACTH concentrations around this morning peak (Fehm et al., 1984). As such, it has been speculated that the adrenal cortex is modulated in its affinity for ACTH, primarily through sympathetic neural drive directly to the adrenal cortex (W. C. Engeland & Arnhold, 2005).

The most direct evidence for this suggestion is found within the animal literature. Firstly, animal models have been used to demonstrate the anatomical and physiological interactions, at multiple levels, between the HPA-axis and the sympathoadrenal axis in response to stress (Kvetňanský et al., 1995). A study in rats demonstrated that the transection of the splanchnic nerve modified the responsiveness of the adrenal cortex to ACTH administration (Ulrich-Lai et al., 2006). This study extended the findings of
earlier studies that used transneural viral tracing to elucidate multisynaptic pathways from the SCN to the adrenal cortex (Buijs et al., 1999). Supporting evidence for the adrenal sensitization of the adrenal gland by sympathetic innervation has been described in clinical populations (W. C. Engeland & Arnhold, 2005). It has also been suggested that local CLOCK proteins at the adrenal gland regulate the responsiveness of the gland to ACTH, since mutant animals without these functional proteins showed a diminished corticosterone response to ACTH treatment (Oster et al., 2006). It should be highlighted that although these observations have occurred in primarily rodents, there is compelling evidence suggesting that these direct neural pathways to the adrenal cortex also exist in humans (Buijs et al., 2003).

The SCN has been demonstrated to be an instrumental structure in relaying light-information and induced glucocorticoid secretion at the adrenal gland (Ishida et al., 2005). As such, it would follow that light exposure upon waking would significantly alter cortisol secretion following waking. Indeed, this effect was demonstrated soon after waking effects on cortisol were described (Scheer & Buijs, 1999). Since then, two major studies have assessed the role of artificial dawn simulators, but they report disparate results. Thorn et al. (2004) demonstrated an increased CAR following exposure to an artificially created dawn environment (gradual increase to 250 lux), whereas no effect was found in a later study (Van de Werken et al., 2010). Importantly, Van de Werken and colleagues exposed subjects to light in the control condition post-waking in an effort to isolate the independent effects of the simulated dawn. Thus the differences in these studies, as noted by the authors, can be attributable to the post-waking light exposure,
which can greatly influence the CAR (Van de Werken et al., 2010). The influence of nocturnal light exposure showed that cortisol concentration will increase acutely in response to continuous bright light exposure before a slow decrease is observed (S. A. Rahman et al., 2019).

*Methodological Considerations of the Cortisol Awakening Response*

Similar to the characterization of diurnal variation, there are multiple metrics for the measurement and interpretation of the CAR. The least complicated measure is the method used in the initial reporting of the CAR (J. C. Pruessner et al., 1995), and is calculated as a simple change score ($\Delta$) in cortisol concentrations between waking and some later time (usually +30-45 mins). Other methods for assessing the CAR are relative changes (e.g. % increases above the initial value) or mean increases, calculated as the change from the waking sample to the average of multiple post-waking samples.

As Fekedulegn et al. (2007) describes, repeated measures of a hormone contain two primary types of information: the amplitude of the response (i.e. concentration of the hormone), and the distance between successive measures (i.e. time). Calculating the area under the curve (AUC) is a method of representing both of these components within a single value. Following the publication of simple AUC calculations by Pruessner and colleagues (J. C. Pruessner et al., 2003), two AUC metrics are commonly calculated and reported for the CAR (for examples please see Appendix Q). First, AUC can be calculated in reference to the initial value (AUCI), in which case the AUC is representative of the total cortisol output in response to awakening over a given time interval (usually 1 hour). This calculation captures information regarding the degree of
increase and decrease over time. Alternatively, the AUC can be calculated in reference to a concentration of zero, or the ‘ground’ (AUCG). In this calculation, the AUCG is representative of the total adrenal output of cortisol, rather than just strictly the response to awakening. It has been argued that because it reflects only the response to awakening, AUCI should be considered the best marker of the CAR (Stalder et al., 2016).

More recently, researchers have experimented with other CAR metrics. For example, Evans et al. (2019) recently demonstrated the utility of the CAR ‘salience’ metric which the authors suggest is representative of the pulsatility of the HPA-axis, as classically characterized by deconvolution analysis (Trifonova et al., 2013). The salience metric is calculated from only three samples, by first finding the slope between the first and second sample, and the slope between the second and third sample, and then calculating the difference between these two slopes. The CAR salience metric was shown to be highly related to peak-to-trough amplitude calculated from Q5 sampling, and was a better metric for detecting individuals with seasonal affective disorder than AUCI.

In 2016, a coalition of the most prominent CAR academics collaborated on outlining expert recommendations for the measurement, analysis, and interpretation of the CAR (Stalder et al., 2016). Within this review, several methodological considerations were highlighted. One large concern in the measurement of the CAR is related to both the timing of samples and compliance with the protocol. Indeed, the authors state that “the validity of CAR data critically relies on the temporal accuracy of saliva sampling across the post-awakening period” (Stalder et al., 2016). Within the timing of sample collection,
one can consider both the timing of the initial sample (relative to waking), and then the
timing of subsequent samples, relative to the first time point.

In regards to the first issue, early research suggested a 10 minute latent period
post-waking, in which no increase in cortisol was observed (Smyth, Clow, et al., 2013).
Some studies have reported a high proportion (26%) of the population that are negative
CAR responders, even in a relatively healthy cohort (Eek et al., 2006). These negative
responses have been suggested to be a result of non-compliance due to the home-based
sample collection (Wüst, Wolf, et al., 2000a). Indeed, close inspection of these data show
the negative-responders to have a much greater mean delay in sampling after waking (9
mins on workdays and 17 mins on weekend), even though this was not a statistically
significant difference (Eek et al., 2006).

Data presented by Smyth et al. (2016) suggests a delay in the collection of the
first sample of more than 7 minutes results in an underestimated CAR, whilst short delays
of only 4-6 minutes can produce an overestimated value. The authors suggested the
prevalence of sampling delays post-awakening may be a result of sleep inertia and not
necessarily intentioned non-compliance, and so objective verification of waking time is
recommended (Stalder et al., 2016). There can also be substantial deviations from the
sampling protocol and inaccuracies in the collection of the additional post-waking sample
collections (reviewed in Stalder et al., 2016). However, these stated methodological
issues around timing are relevant only to the self-collection of samples by subjects in
real-world environments where the time of each sample cannot be verified by a third-
party (i.e. researcher). The current project aims to limit these issues by critically monitoring the timing of waking and time since waking during sample collection.

Since most CAR investigations occur in natural settings (i.e. the home environment), it can be difficult to control the movement of participants following the awakening event. As such, researchers have postulated that the CAR may be influenced by the posture of the individual after waking – Hucklebridge et al. empirically tested this speculation with a counterbalanced cross-over design, wherein subjects were required to complete both a supine only and supine to standing condition. The results from this study indicate that the CAR is not affected by posture (Hucklebridge et al., 2002).

In order to assess whether the CAR is in fact a response to the process of waking as opposed to the expected circadian rhythm of cortisol secretion, Wilhelm and colleagues (2007) sampled blood at Q90 from 2300h – 0200h, Q30 from 0200h until waking at 0700h, and then Q15 for an hour. During the hour of waking, saliva samples were also collected. The researchers assessed if the slope of cortisol increase from early morning until waking was similar to the slope of the awakening response via a mixed-effects growth curve model (Wilhelm et al., 2007). The results demonstrated that awakening itself was an independent contributor to the cortisol profile. Interestingly, the results demonstrated a similar pattern in ACTH, suggesting that the CAR is at least to some degree influenced by an ACTH response to awakening (Wilhelm et al., 2007).

The time of awakening is also worth considering when assessing the CAR. In a study of nurses working various shifts, researchers demonstrated a clear effect of the time of awakening, with earlier waking times presenting larger CARs (Federenko et al., 2004).
Although this study showed no evidence for a CAR after a nap in the evening, waking between 18:45 and 20:30 (Federenko et al., 2004), previous studies demonstrated a muted CAR relative to morning awakening when waking in the evening, four hours before the scheduled waking time (Hucklebridge et al., 2000). Others have found a small CAR after both morning and afternoon naps, although sleep duration appears to need to be >90 mins to experience a CAR upon waking (Devine & Wolf, 2016). Others have failed to demonstrate strong relationships between CAR and sleep quality metrics (Eek et al., 2012). Although Elder et al. (2016) have reported relationships between Stage 2 sleep and the CAR as measured by the mean increase (change from first waking sample to the average of all other post-waking samples), the relationship between CAR and specific sleep parameters, including both duration and architecture, remain to be fully elucidated (Elder et al., 2014).

**Purpose of the Cortisol Awakening Response**

The teleological role of the CAR is currently unknown, however hypotheses have been posited and tested. Early CAR researchers speculated that since cortisol has a robust substrate mobilization role, the CAR may be an effort to mobilize substrates upon awakening. Hucklebridge et al. tested this notion in 1999, by studying the relationship between the CAR and blood glucose concentrations. Researchers found no correlation between glucose and CAR values, nor did they observe individuals with low blood glucose values to have a more or less robust CAR (Hucklebridge et al., 1999). The authors concluded that the CAR does not seem to be a substrate mobilizing phenomena.
Others have suggested that the CAR may be representative of the maximal adrenal capacity of the individual. One study utilized pharmacological stimulation of the adrenal gland and demonstrated a significant correlation \( r = 0.63 \) between the cortisol response to administration of 0.25 mg ACTH and the CAR (Schmidt-Reinwald et al., 1999). It is unclear at this time whether the CAR would also be significantly related to maximal adrenal output following a non-pharmacological intervention (i.e. exercise). Further, given the known effects of cortisol in modulating peripheral cell biological CLOCK mechanisms (Spencer et al., 2018), the CAR may serve as a single large secretory burst that acts to entrain peripheral clocks to not only the circadian rhythm and light-dark cycle, but also the conscious state or locomotive period of the organism that is expected to coincide with awakening.

Relatedly, the CAR may also be associated with the circadian regulation of the immune system. Cortisol plays a critical role in immune function and inflammatory profiles, particularly potential interactions with exercise produced myokines (Fernandes et al., 2018). Evidence suggests that cortisol is capable of modulating the cytokine production and release from particular immune cells (Yeager et al., 2004), which in turn modulate coordination between interrelated immune cells. Petrovsky and Harrison (1997) described an apparent switch from type 1 to type 2 T-helper cells that follows a circadian rhythm and correlate with plasma cortisol values. In turn, some have speculated that the CAR may serve a physiological function in providing circadian balance or “switching” within the cell-mediated immune system (Clow et al., 2004, 2010; Hucklebridge et al., 1999).
Some researchers have also speculated that the CAR may be an effort to overcome sleep inertia in the post-waking period. In one of the few studies on this topic, the authors note that the CAR seems to be negatively associated with the dissipation of sleep inertia, as measured by self-reported “activation” (Van de Werken et al., 2010). Thorn et al. (2009) have also reported data indicating that the CAR may be associated with levels of post-awakening arousal; although, in contrast, this was a positive relation. The CAR has also been suggested to assist in cognitive capacities and restoring full cognitive capacities, as demonstrated by the relations between reaction time tasks and the CAR. Moreover, time-lagged analysis of cortisol and positive and negative affect have demonstrated a relation between cortisol and feelings of activeness, alertness and relaxation across the day (Hoyt et al., 2016).

A functional interpretation of the available evidence has been proposed by Dr. Emma Adam (2006), based in part on the results of salivary CAR assessments in 156 older adults. In this study, researchers showed that emotional affects such as sadness and loneliness on the day prior to sample collection, and feelings of tiredness throughout the day following sample collection, were related to the CAR and awakening sample, respectively (Adam et al., 2006). As such, the authors suggested that the CAR, and indeed absolute cortisol concentrations, may contribute to a system that provides a “boost” to the individual, in anticipation of the upcoming demands of the day and has thus been described as the “boost hypothesis” of the CAR. This hypothesis includes a role for cortisol in priming and maintaining psychological and physiological systems for activity and reactivity to potential stressors during the active (i.e. awake) period.
Conversely, the hypothesis would predict a low or blunted CAR to provide an insufficient preparation for the upcoming day, and thus be related to feelings of tiredness and fatigue. Indeed, this was observed in a study by Powell et al. (2012), who showed a lower AUCI to be associated with greater distress responses to daily life, after adjusting for covariates, and monitoring responses over two consecutive days.

Although this framing of the CAR is inherently prospective in nature (i.e. the CAR is preparatory for events yet to occur), it also includes and reflects the physiological and psychological residue from the previous day, whether overt or perceived. Recently, Elder et al. (2018) demonstrated the effect of a prior day (the evening prior to sample collection) psychological stress on the CAR. The researchers informed participants of a stressful cognitive event on the following morning, prior to the participants sleeping. This intervention was sufficient in inducing a larger CAR the following morning and demonstrates the effect of prior events modulating the CAR (Elder et al., 2018). This mirrors the results of Doane and Adam (2010), who reported a positive relation between the CAR and state feelings of sadness and loneliness of the previous day.

This hypothesis is also congruent with a series of studies that have demonstrated a central role of the hippocampus in the regulation of the CAR (Fries et al., 2009). The hippocampus is a bilateral sub-cortical structure that is primarily associated with both spatial and recognition memory (Broadbent et al., 2004). Researchers found an absent CAR in patients with hippocampal damage, regardless of the etiology of the damage (Buchanan et al., 2004). Interestingly, aside from the lack of a cortisol response to awakening, these patients presented a relatively normal circadian cortisol profile
Similarly, a small group of amnesic patients \((n = 6)\) also failed to present a discernable increase in response to awakening relative to controls (Wolf et al., 2005). Although confounded by other endocrine dysfunction, a sample of Type 2 diabetics also had a blunted (absent) CAR, which was coincidently correlated to hippocampal volume (Bruehl et al., 2009). Even after controlling for insulin concentrations, the CAR appeared to be associated with hippocampal volume in both groups (Bruehl et al., 2009). This is in agreement with prior investigations, which also found positive relations between the CAR and hippocampal volume (M. Pruessner et al., 2007).

The “boost hypothesis” is further supported by additional lines of evidence. For example, early investigations of the CAR showed a tendency for CAR to be greater on weekdays than on weekend days (Kunz-Ebrecht et al., 2004; Schlotz et al., 2004; Weidenauer et al., 2019). These results have been interpreted to indicate the perceived stress of the workday being greater than that of the weekend. However, it must be noted that this interpretation is speculative and may be influenced by a number of the methodological issues.

State and Trait Influences on the Cortisol Awakening Response

Early in the study of the CAR, in an attempt to understand the factors influencing the magnitude and shape of the HPA-axis reactivity to waking, researchers investigated the impact of state and trait factors on the CAR. In 2000, Wust et al. studied the CAR in sets of monozygotic and dizygotic twins and concluded that the CAR was under considerable genetic control, whilst the general diurnal pattern was not (Wüst, Federenko,
et al., 2000). In a separate paper that same year, the same research team collated results from four separate studies and reported a day-to-day intraindividual correlation of the AUC metric as $r = 0.69$ and suggested this demonstrates high stability and thus trait factors such as personality measures (Wüst, Wolf, et al., 2000b). Importantly however, even this relatively high correlation accounts for less than 50% of CAR variability. Others have confirmed a high correlation ($r = 0.82$) between successive day AUCG measures (Elder et al., 2016). However, these authors also reported a poor test-retest relation for the mean increase metric (Elder et al., 2016), suggesting that AUCG may be more stable (i.e. total adrenal output) than the specific assessment of the CAR.

There is also the suggestion that trait-like factors such as ones chronotype may be related to the CAR (Kudielka et al., 2006; Randler & Schaal, 2010). In a well-controlled sleep laboratory study, researchers showed a moderate positive correlation between the initial awakening sample, as well as the AUCG, with scores on the Morningness-Eveningness Questionnaire (Petrowski et al., 2020). However, when investigating differences in the CAR between three distinct chronotypes (evening-types, morning-types, and neither), researchers recently found no effect of chronotype (Weidenauer et al., 2019). Therefore, the effect of chronotype should be considered, but currently remains unclear. Other gene-related factors have also been suggested to be associated with the CAR. For example, Van Leeuwen and colleagues presented evidence of differential suppression of the CAR with dexamethasone between individuals with different mineralocorticoid receptor single nucleotide polymorphism variants (Van Leeuwen et al., 2010). While this study found no difference in the CAR between these single-nucleotide
polymorphic groups without dexamethasone suppression, this does suggest a potential role for the MR in modulating the CAR. A substantial influence of sex on the CAR has also been reported in several investigations (Van Leeuwen et al., 2010).

In perhaps the most rigorous investigations of state and trait contributions to the CAR, Hellhammer and colleagues (2007) employed a structural equation modeling approach to test the contributions of both state and trait factors. This was modeled according to the Latent State-Trait Theory, which posits that human responses to a stimulus (including physiological responses to, for example, waking) are a function of the characteristics of the individual (trait factors), the situation (state factors), and the interaction of these two factors; moreover, the theory imagines that the relative contributions of each factor changes across time (Steyer et al., 1999). These researchers demonstrated that the CAR could not be fit by a trait only model, and should most appropriately be considered to be comprised of state variables superimposed over latent traits (J. Hellhammer et al., 2007). This model confirmed that occasion specific factors that are variable on a day-to-day basis, determined a large portion of the CAR. Indeed, this was also confirmed in the reporting of a collation of three studies, in which researchers presented evidence that CAR was the least stable (i.e. least trait-like) cortisol index, when compared to total daily output and diurnal slope (Ross et al., 2014).

As demonstrated, there are a multitude of potential roles and functions the CAR may have within human physiology. One must also consider that a single definitive role likely does not exist across all contexts. Although a single evolutionary force may have initially selected for the development of the CAR, there may currently be several
functions served by the CAR, which would align well with the pleiotropic nature of cortisol. A comprehensive review of the literature suggests that the CAR shows large inter-day variability and is thus determined in a large part by state factors (Law et al., 2013). A number of state factors were discussed as affecting the CAR, including light exposure, sleep quantity and quality, prior day experiences and anticipated demands of the upcoming day, alcohol consumption, and the menstrual cycle; however, the authors also noted that “there has not been any research to investigate the effects of acute exercise during the prior day…on the CAR” (Law et al., 2013).

The Utility of the Awakening Period to Monitor Exercise Responses

For researchers and practitioners wanting to monitor cortisol levels in response to exercise training, it is tempting to monitor a single baseline value for changes across a training period. Indeed, the use of a single cortisol measure, and changes in such a baseline measure, have been previously associated with overtraining (Atlaoui et al., 2004; Barron et al., 1985; Chicharro et al., 1998; Fry et al., 1992; Gouarné et al., 2005; Gustafsson et al., 2008; Hedelin et al., 2000; Ishigaki et al., 2005; W. J. Kraemer et al., 2004; Lehmann et al., 1991b; Li et al., 2013; Mackinnon et al., 1997; Meeusen, 2010; Minetto et al., 2008; O’Connor et al., 1989; Schmikli et al., 2011; Snyder et al., 1995; Tanskanen et al., 2011; Volek et al., 2004). Evidence suggests that baseline cortisol levels in athletes may be tiered, such that athletes may present low, moderate, or high cortisol values when a single sample is taken in the morning (Rist & Pearce, 2019). An additional issue with a single morning value for monitoring exercise responses is that this does not reflect HPA-axis reactivity, and rather represents only basal activity. As such, the
collection and interpretation of several serial cortisol values may be more appropriate to monitor intraindividual alterations to HPA-axis functioning. Previously, these serial cortisol measures have been in response to some exercise stress test, in order to characterize adrenal reactivity. For example, Nicoll et al. (2019) demonstrated a difference in cortisol responses to an anaerobic power test in overreached athletes relative to controls.

*The Effect of Exercise on the Cortisol Awakening Response*

Since the CAR has been implicated in conditions such as burnout and chronic fatigue syndrome, characterized by periods of intense stress and poor adaptability, one research group considered whether the CAR would also be affected by a period of intensified exercise. Fifteen elite soccer players underwent 7 days of intensive training (a total of ~21 hours), as would be typical of a preseason camp environment, and provided awakening saliva samples for 2 days before and after this training period (Minetto et al., 2008). While the two days of sampling showed high stability, the post-training CAR was significantly greater compared to before training (AUC; 12.4±2.4 vs. 16.43.3 nmol.l⁻¹), as was the first sample after waking (Minetto et al., 2008). These researchers also demonstrated relations between the change in CAR and change in performance variables such as 20m speed and counter-movement jump (Minetto et al., 2008). Prior to this, Gouarné et al. (2005) presented data suggesting that triathlon athletes (n = 2) diagnosed with overtraining syndrome had a substantially lowered CAR (36.4% and 0% increase 30 mins post-waking) at the end of the season, compared to 151% and 64% at the start of the triathlon season. Comparatively, the non-overtrained athletes had a progressively
increasing CAR across the season. Of course, this is a small sample size under extreme exercise training conditions and thus should be extrapolated with caution.

Few studies have investigated whether exercise in the evening would modulate the CAR. However on the morning after late-night soccer match (Ucar et al., 2018) or netball competition (Juliff et al., 2018) researchers found no difference in the CAR. However, it must be noted that a) these interventions were real-world activities and thus the work completed for each subject cannot be known nor controlled for; and b) the saliva samples were collected in the individual’s homes, introducing possible sources of sampling error. A study in endurance runners demonstrated a significant and positive relation between the training load completed the previous day and the CAR measured the following morning (Anderson et al., 2018). Again, this study employed a quite heterogenous convenience sample and relied on self-report and self-collection of samples, without controlling for many of the covariates discussed above.

Recently, Fekedulegn and colleagues reported a significant interaction between self-reported sleep quality and whether individuals met minimum leisure-time physical activity in predicting the CAR in law enforcement officers (Fekedulegn et al., 2018). After controlling for relevant factors, for those officers that did not complete at least 150 minutes of physical activity per week, poor sleep resulted in a significantly lower CAR (Fekedulegn et al., 2018). A possible interpretation of these results is that sufficient habitual exercise may provide a protective role, permitting disturbances in other health behaviors without disrupting the CAR. This study requires, at a minimum, additional research that assesses objective markers of exercise and sleep.
A 12-week exercise program in patients with depression resulted in an increase in morning cortisol concentrations, both immediately after waking and 40 minutes post-waking (and thus, deductively, AUCI and AUCG) as well as an alteration in the diurnal cortisol slope (M. S. Rahman et al., 2018). A study of school-aged children demonstrated divergent effects on CAR following a 10-week exercise training intervention. In this study, children engaged in either a cardiovascular fitness program, which required running and ballgames, whilst a separate group engaged in games that challenged their motor control skills. The reported results suggested an increase in CAR (AUCG) for the cardiovascular training group, but a decrease in CAR for those children that completed the lower-intensity motor control program (Wegner et al., 2019). Interestingly, these group predictors were only significant when interacting with the change in either cardiovascular or motor fitness in the participants (Wegner et al., 2019).

A recent study in professional Rugby players completing a pre-season intensive training camp demonstrated a blunted salivary cortisol response to awakening on the 4th and final day of the training camp (Serpell et al., 2019), although common CAR metrics were not calculated or assessed. Results from the endocrine and metabolic responses to overtraining study demonstrated a lowered CAR in the overtrained athletes compared to the healthy athletes (Cadegiani & Kater, 2017b). Follow-up analyses of this data demonstrated this blunting to be statistically significant (Anderson, Wideman, et al., 2021) and are highly suggestive of CAR being a useful metric in identifying HPA-axis disruptions that occur in overtrained athletes.
Heart Rate Variability

Neurophysiological Control of Heart Rate

As the tenth cranial nerve, the vagus nerve is responsible for parasympathetic innervation on the heart, to both the sinoatrial (SA) and atrioventricular (AV) nodes and the myocardium. Vagal input to the heart has both chronotropic and inotropic effects. Innervation to the SA node activates cholinergic muscarinic G-protein coupled receptors (GPCR) that have inhibitory activity on adenylate cyclase. Adenylate cyclase increases intracellular cAMP, and thus the inhibition of adenylate cyclase by cholinergic activation lowers cAMP concentration. This has downstream effects on the phosphorylation of other proteins and ultimately results in reduced intracellular calcium, thus decreasing contractile force (inotropic effects). Further, inward rectifying potassium channels become activated with increased parasympathetic input, allowing greater potassium to enter the cell, hyperpolarizing the membrane and requiring a greater amount of time for the SA node cells to undergo spontaneous depolarization (chronotropic effects). Moreover, the vagus will cross talk with sympathetic innervation prior to receptor activation, inhibiting the sympathetic effects on the cell. Because the vagus will act directly on potassium channels and does not require second messengers, the effect of increased vagal stimulation is extremely rapid, and can exhibit effects within the same beat as the stimulation occurs. Although the heart receives both parasympathetic and sympathetic input at all times, the rapidity of vagal effects when compared to the sympathetic stimulation (5-10 seconds) necessitate that beat to beat variability be dictated
by vagal inputs. This variation in time between cardiac contractions is termed heart rate variability (HRV).

Heart Rate Variability Metrics

One can measure the time between these beats (RR-intervals) and then mathematically described the variability in that time-series. One method of doing this is through the calculation of the root mean square of successive differences (RMSSD) - a very common time domain metric. The RMSSD is representative of the beat to beat changes in HR. Given that these beat to beat differences are regulated by the vagus nerve (parasympathetic input), and that high frequency power (see below) also represents vagus input, RMSSD and HF are highly correlated. RMSSD is the primary metric utilized by Dr. Julian Thayer when connecting HRV to cognitive processes and executive functioning in the Neurovisceral Integration Model.

Aside from the time-domain, one can also consider HRV metrics within the frequency-domain. In order to assess frequency domain metrics, one must first convert the RR time interval sequence into the frequency domain. This can be accomplished using either a discrete Fourier or fast Fourier transformation of the time series. In short, a Fourier transformation decomposes a time series of RR intervals into its sinusoidal components. The spectral power (integral) of the frequency domain can then be calculated for various frequency ranges. In order to assess various components of this frequency domain, researchers have established a number of defined frequency bands. Typically, high frequencies are considered between 0.15 and 0.4 Hz, and the integral of this spectrum is called high-frequency power (HF) (Camm et al., 1996). Therefore, HF
represents variations in RR intervals ranging from 2.5 to 7 seconds and thus, is influenced most directly and uniquely by the parasympathetic system.

In terms of assessing HRV, it has been recommended to utilize the “three R’s” structure, that assesses Resting, Reactivity, and Recovery of HRV (Laborde et al., 2017). In this fashion, one can test for basal HRV, the response to a stimulus (e.g. waking), and the post-event recovery (e.g. time following waking). It has also been recommended to utilize 5-min recordings, or 5 minute epoch lengths within larger recording windows (Laborde et al., 2017).

*Heart Rate Variability and Monitoring Exercise Responses*

HRV has been suggested to be useful for the monitoring of training load and recovery in athletes (Dong, 2016). Professional soccer players showed a 0.3 - 26.1% depressed RMSSD on the day following a match (Rabbani et al., 2019), suggesting HRV metrics the following day can be responsive to acute exercise perturbations. Since some have reported an interaction between nocturnal HRV (HF power) and sleep-wake regularity in predicting poor mood (Gao et al., 2019), HRV across the sleep-wake period may provide unique insight into these exercise-related perturbations.

In addition, the ANS disruptions that have been observed in overtrained athletes have also been suggested to be reflected in HRV metrics (Aubert et al., 2003; Makivić et al., 2013). Moreover, some authors have suggested that HRV may be a useful indicator of cardiovascular training effectiveness (Makivić et al., 2013) - with time domain indices (e.g. the standard deviation of the time series) demonstrating the largest exercise training induced effect size (da Silva et al., 2015). Others have advocated for the use of frequency
domain metrics following an orthostatic challenge to distinguish between various fatigue states (L. Schmitt et al., 2015). This may be especially pertinent, given a meta-analysis found that both overreaching and positive adaptation to exercise training can cause an increase in resting RMSSD (Bellenger, Fuller, et al., 2016) and thus, RMSSD does not appear capable of distinguishing between positive and negative adaptations to exercise training.

Heart Rate Variability and the Neurovisceral Integration Model

Various models of vagal control over heart rate and thus HRV have been described and discussed. However, in 2000, Dr. Julian F. Thayer put forth a model termed the neurovisceral integration perspective that attempted to find commonalities in neural networks within the central nervous system that regulate HRV. From this perspective, Thayer posited a neurovisceral integration model (NVI) that links changes in cognitive function and cognitive performance with HRV and described both direct and indirect pathways that link the prefrontal cortex with bodies associated with regulating parasympathetic activity. The integration between cognitive functioning and cardiac control stem from the overlapping structures described in the critical autonomic network (CAN) and the rostral limbic system. Since the output layer of the CAN is through parasympathetic and sympathetic innervation, Thayer contends that HRV is a representation of the CAN-ANS function. A precept of the NVI model is that the prefrontal cortex is an inhibitor of some or all components of the limbic system, such that cognitive processes can regulate the limbic structures which in turn modulates ANS output. When, however, the prefrontal cortex activity is decreased, limbic structures (e.g.
the amygdala) are less inhibited and can activate the paraventricular nucleus of the hypothalamus and other regulatory structures to increase heart rate and decrease heart rate variability. Thus, the neurovisceral integration model was originally proposed to explain the observed links between cognitive outcomes, mental health, HRV, and peripheral physiology. This model attempted to synthesize correlational data and provide an underlying mechanistic explanation for these observations.

The NVI model was revised in 2009, and then updated again in 2017. The original model appealed to a dynamic systems model to explain the integration between these systems. This was both abstract and complex. The original model used mathematical language to describe a high-dimensional state-space, where movement through the CAN is dependent on the state of each CAN region. Movement of signals through the CAN was then dictated by modulatory influences on the network, dictating the specific phase-space. Some states may be more stable than others and considered attractors, which may be reflected in the emotional/cognitive state of the individual. The updated model structures this network in a hierarchical fashion. Lower levels function to modulate peripheral energy needs (present) while the higher order functions to modulate present and future demands. Therefore, higher order functions are dependent on lower order functions.

The current version of NVI suggests that the model should be expanded to include a multi-level dimension—from basic to complex—and also includes multiple NVI loops (R. Smith et al., 2017). The range and complexity of functions increases at each level of this
physiological hierarchy. New types of information are integrated at each level, and so more flexibility is required at each level.

Within the first and second levels of the hierarchy, intrinsic and direct cardiac reflexes are described. The baroreflex is dually innervated but is tends to be largely under vagal control, and thus there tends to be a relationship between indices of baroreflex and vagally mediated HRV. When blood pressure increases, vagal activity increases, and inhibits efferent $\alpha$-adrenergic outflow (and vice versa). The second level expands on these reflexes to incorporate brain stem structures, such as the dorsal motor nucleus of the vagus (DMNV), the nucleus ambiguus (NA) and the nucleus tractus solitarius (NTS).

The third level involves the same structures as the second level (NA, DMNV, and NTS), but instead of short loops to the cardiac tissue, vasculature and myocardium, the third level projects to other, external organ systems such as the ventilatory system and possibly renal and inflammatory control, through vagal withdrawal to specific organs and organ-systems. It is through this third level that the well-known respiratory sinus arrhythmia patterns are controlled. Circumventricular organs (CVO) are structures that lack a blood-brain barrier and permit direct peripheral communication such as the organum vasculosum laminae terminalis (OVLT) and subfornical organ (SFO) structures. These detect peripheral changes (osmolality, metabolic status etc.) and can communicate with other brain regions to initiate behavioral change (e.g. the cingulate cortex, hypothalamus, amygdala, periaqueductal gray, and NTS).

The fourth level involves hypothalamus and periaqueductal gray (PAG) integration, that have bidirectional connections with lower brainstem nuclei. Importantly,
the hypothalamus has upward projections to the cortex and major projections from the prefrontal cortex, hippocampus, and amygdala back to the hypothalamus. The fifth level incorporates signals from the amygdala and basal forebrain. These structures also have significant downward projections to the hypothalamus, PAG and brainstem nuclei and are highly connected to the entire cortex; which appear to facilitate autonomic attention to emotional stimuli. These bilateral prefrontal cortex connections allow the amygdala to the have strong effects on attention and focus but also allows the prefrontal cortex to modulate the amygdala. The amygdala may also have direct connections to the basal forebrain, which projects to the cortex and hippocampus, as well as projections from the locus coeruleus to the basal forebrain. Note the role of the amygdala and hippocampus in regulating HRV at Level 4 and 5 in particular – these neural structures are also implicated in the regulation of the CAR (as described above).

The sixth level of the hierarchy is thought to regulate lower levels of the hierarchy based on the perception of the current state, while the seventh level involves regulation of the lower levels based on conceptual interpretations of the meaning of perceptual input (based on past experience). There is considerable overlap between the brain regions associated with each of these levels when considering the gross neuroanatomy. Level 6 is best characterized by the anterior insula, which receives somatosensory information from the periphery, and thus its influence on cardiac control is considered representative of the whole-body state. There are also links to the cingulate gyrus and anterior cingulate cortex. In contrast, Level 7 is best understood to modulate autonomic function through its connections to higher order cortical regions involved in conceptual processing. Structures
on this level are most likely to be influential over long time scales and are less sensitive to the internal milieu.

Lastly, the eighth level involves the frontal-parietal networks and an executive control network that is thought to allow goal-relevant information to be held in working memory and connect to many of the Level 6 and 7 structures. The top down approach suggests that Level 8 can control how information flows between these structures to coordinate conscious goal-directed behavior. By holding a representation in memory, one can modulate the activity of the conceptual interpretation and perception of the state and therefore adjust the manner in which it is semantically represented.

Thayer’s neurovisceral integration hierarchy provides a model through which researchers may test the effects of, or interactions with, various layers of the hierarchy, and interactions with other related systems that integrate with various levels of the hierarchy (R. Smith et al., 2017).

*Heart Rate Variability and the Cortisol Awakening Response*

The neural circuitry associated with HPA-axis activation/inhibition (Herman & Cullinan, 1997; Ziegler et al., 1999) share many commonalities with the extrahypothalamic structures described by Smith et al. (2017). There are distinct physiological and neuroanatomical links between the ANS and the HPA-axis. In particular, the amygdala and hippocampus and associated neural pathways (Charmandari et al., 2005) have been implicated in glucocorticoid output (Feldman et al., 1995). Fluorescent tracer studies in rats have confirmed projections from the nucleus ambiguous to the cardiac control plexuses and the dorsal motor nucleus of the vagus nerve (Cheng &
Powley, 2000). Rotenburg et al. (2016) have recently described these relationships and established a sound neurophysiological rationale to hypothesize parallel activation of these systems that can be monitored through HRV and cortisol concentrations. Further, animal models have revealed hypothalamic projections from the PVN to the sympathetic neural bodies in the locus coeruleus (Reyes et al., 2005) and from the locus coeruleus to the PVN (Sawchenko & Swanson, 1982). Specifically related to the HPA-axis regulation, CRH is a known agonist and modulator of locus coeruleus activity and is likely the major neuropeptide/hormone that links the ANS and HPA-axis activity (Valentino & Van Bockstaele, 2008). Increased activity of the sympathetic nervous system would drive a concomitant decrease in parasympathetic outflow to the heart. Within this context, the parasympathetic nervous system (represented by HF power) and HPA-axis would be inversely related and demonstrate opposite responses to stimuli.

The interconnective nature of these systems suggests that one should not be defining each of these as a distinct system. Rather, there are complex interactions at a variety of levels within each of these systems. The locus coeruleus appears to be central to these interconnections and is intricately involved in the ascending arousal system during sleep to wake transitions (described above). Figure 2 illustrates the overlapping neural structures (Figure 2H) that control awakening processes (Figure 2A), the CAR (Figure 2B), Levels 2-5 of HRV regulation as described above (Figure 2C-F), and HPA-axis control of cortisol secretion during exercise (Figure 2G). See Appendix A for abbreviations of neural structures and Appendix B for individual and larger images.
If the shared or common neurophysiology results in activation of the ANS in a proportional manner to the HPA-axis during awakening, then HRV may be a useful, non-invasive tool for the assessment of HPA-axis reactivity to awakening. If these metrics are indeed distinct and represent separate physiological phenomena, then the measurement of both CAR and HRV could permit insight into both physiological stress-reactivity systems and may provide additive information above either measured independently.
Figure 2. The shared neural structures between the ascending arousal system (A), regulation of CAR (B), HRV regulation according to the Neurovisceral Integration Model Level 2 (C), Level 3 (D), Level 4 (E), and Level 5 (F), and HPA-axis response to exercise (G). The overlap between these areas is shown in H.
In one of the only studies that has assessed the relationship between CAR and HRV variables collected concurrently, Stalder et al. (2011) monitored 36 healthy young adults and found an awakening response in both cortisol (i.e. the typical CAR) and a decrease in HRV. In this study, participants were permitted to ambulate 15 minutes after waking, and the HRV parameters were pooled across the entire 45-minute sampling period post-waking. Even so, the researchers were able to find weak independent relations between the CAR (AUCI) and HF in the pre-awakening period and in the post-awakening period (Stalder et al., 2011). These data and methodology provide a basis for a more robust investigation of the relationship between HRV and the CAR. A recently published thesis project on elite cross-country skiers demonstrated a negative relationship between nocturnal HRV measures (RMSSD) with a single cortisol sample collected immediately after waking (Kuorelahti, 2019).

A study in children demonstrated a relation between HRV collected at a separate time under resting conditions, to be associated with the CAR (Michels et al., 2013). In this study, low frequency power (LF) and LF/HF were significantly related to the CAR, while HF and the proportion of RR intervals > 50ms (pNN50) were related to the diurnal cortisol slope (Michels et al., 2013). Similarly, in a separate study in children aged 7-12, it was reported that greater LF and LF/HF whilst awake (night prior), and LF during stage 2 sleep and REM sleep were associated with AUCG (Urfer-Maurer et al., 2018). Higher LF and total power during stage 2 sleep were negatively associated with AUCI (Urfer-Maurer et al., 2018). Importantly, none of these studies collected HRV and awakening cortisol samples simultaneously, and thus they are testing primarily for trait-like relations.
between the ANS and HPA-axis. Rotenburg and McGrath (2016) reported that HRV measures and the CAR interact in predicting perceived stress in children. Specifically, they showed the interaction of LF and LF/HF with AUCG and AUCI, respectively, were associated with subjects rating of perceived stress (Rotenberg & McGrath, 2016).

Summary of the Review of Literature

In summary, this literature review has described 1) The complexity of HPA-axis control, multiple feedback loops operating on varying time scales, and the intricate intracellular control of glucocorticoid effects, including receptor isoforms and enzymatic control; 2) the cortisol responses to acute exercise, including an intensity threshold for increases in circulating cortisol concentrations; 3) the distinct cortisol increase that accompanies waking from nocturnal sleep, including potential confounding methodological issues such as biological fluid being sampled; 4) the potential for exercise, particularly acute and/or excessive exercise, to influence the CAR; and 5) the utility of HRV during the waking period to reflect the CAR, or act in unison to represent acute responses to exercise from the previous day. Based on the available literature and critical interpretation, the following research questions remain:

1) Is the CAR affected by acute exercise or reflective of the cortisol response to exercise?

2) Does salivary cortisone present an awakening response, and is this more closely related to blood-based CAR assessments than salivary CAR?

3) Does HRV demonstrate a response to awakening, and is this related to the CAR or effected by acute exercise?
To address these questions, this project had the following specific aims and research hypotheses:

**Specific Aim 1:** To assess the degree to which acute exercise impacts the CAR on the following morning, the relation between HPA-axis responses to exercise and the CAR, and the association between blood and salivary metrics of the CAR.

**Research Hypothesis (RH) 1.1:** Serum and salivary cortisol will demonstrate a distinct rise during the awakening period and be significantly decreased following exercise on the prior night. The CAR (CAR, AUCI, AUCG) in serum and saliva will be decreased following acute exercise on the preceding evening.

**RH 1.2:** The CAR (CAR, AUCI, AUCG) will demonstrate a significant negative association with the cortisol response to exercise, as assessed from the start of exercise through 30 minutes post-exercise.

**RH 1.3:** All serum CAR indices will be positively associated with the salivary CAR counterpart.

**Specific Aim 2:** To assess the awakening responses of salivary cortisone and the effect of exercise on the cortisone awakening response, the relation of EAR indices to the cortisol response to exercise, and the association between EAR and blood-based indices of the CAR.

**Research Hypothesis 2.1:** Salivary cortisone will demonstrate a distinct rise during the awakening period and be significantly decreased following exercise on the prior night. The EAR will be decreased (EAR, AUCI, AUCG) following acute exercise on the preceding evening.
**Research Hypothesis 2.2:** The EAR (EAR, AUCI, AUCG) will demonstrate a significant negative relation with the cortisol response to exercise, as assessed from the start of exercise through 30 minutes post-exercise.

**Research Hypothesis 2.3:** All salivary cortisone response to awakening (EAR) indices will be positively associated with the blood CAR counterpart.

**Specific Aim 3:** To assess the awakening responses of HRV, the effect of exercise on the HRV awakening response, and the relation between HRV and CAR and EAR.

**Research Hypothesis 3.1:** HRV (HF) will demonstrate a distinct and decreasing response to awakening.

**Research Hypothesis 3.2:** The HRV awakening response will be decreased following acute exercise the preceding evening, relative to the control condition.

**Research Hypothesis 3.3:** The HRV awakening response will demonstrate a significant negative relation with all indices of the CAR and EAR.

**Research Hypothesis 3.4:** The HRV awakening response, CARb_{AUCI}, and EARs_{AUCI} will be independently related to the cortisol response to exercise.
CHAPTER III
METHODOLOGY

Introduction

The cortisol awakening response (CAR) demonstrates potential as a biomarker for the monitoring of exercise responses and adaptation to an exercise program. However, it is currently unknown whether the CAR will be affected by acute exercise. Further, given the requisite biochemical interactions that occur with salivary cortisol assessment, it is unclear whether the CAR, in the context of exercise monitoring, is best assessed via blood or saliva or whether alternative potential endocrine awakening responses (e.g., cortisone) might be better suited for monitoring acute exercise responses. Lastly, HRV during the waking period may be a useful, non-invasive biomarker that could be used either as an alternative to the CAR or in concert with these blood-biomarkers.

Research Design

This study was approved by the Institutional Review Board at the University of North Carolina at Greensboro (#20-0244). All participants had the study protocols described verbally and in writing, were permitted to ask any questions, and provided signed written informed consent before participating. This study was a randomized, cross-over design experiment. All participants completed both an exercise condition and control condition in a randomized order. The study protocol required participants to report to the exercise physiology laboratory at the University of North Carolina at
Greensboro for 2 sets of visits, with 2 visits per set for a total of 4 separate visits. Each set of visits required a *Screening* visit and an *Experimental* visit, separated by approximately 7-10 days. The two sets of visits were separated by at least 8 weeks (Figure 3).

**Figure 3. Study visit timeline.**

*Participants*

Participants were recruited from the University of North Carolina at Greensboro and surrounding areas. Recruitment was conducted via word-of-mouth and the use of flyers. Participant renumeration was $75 upon completion of all study requirements and return of all equipment.

*Demographics*

Participants were delimited to males, between 18-30 years of age, who were not taking any medications that would disrupt any aspect of the endocrine or autonomic nervous systems, including but not limited to non-steroidal anti-inflammatory pharmaceuticals and centrally acting ANS agents such as Ritalin. Participants were required to self-report a regular sleep cycle, and not engage in any shift work that
requires either a delay in the onset of nocturnal sleep or a shortening of sleep duration. Despite the role of obesity and body mass index on the CAR being uncertain, multiple studies have reported CAR modulation based on these demographic variables (Rodriguez et al., 2015). Therefore, participants were also restricted to < 25% body fat, as measured by air displacement plethysmography. Participants were also excluded from further participation if the recorded VO$_{2\text{max}}$ was <35 ml.kg$^{-1}$.min$^{-1}$, or if the score on the Beck Depression Inventory was >20. Lastly, participants were required to restrict participation in moderate-vigorous exercise and have typical sleep for the 24 hours prior to the experimental visit. Efforts were made to schedule sessions to assist in this requirement, but visits were rescheduled if the experimental screening suggest this guideline was not adhered to.

**Power Analysis**

There is a paucity of research investigating the effect of acute exercise on the CAR. As such, simulations were used to estimate the required sample size to detect a significant main effect of exercise on the CAR. In short, data were simulated for a regular CAR response in blood samples, based on the results of Wilhelm et al. (2007). Data were simulated for both a control and exercise condition, but given the intraindividual stability of the CAR, the starting value in each condition for each individual (wake + 0 minutes) was modelled with a correlation of $r = 0.6$. Given these distribution parameters, 10000 participants data were simulated. The effect of exercise was then simulated where exercise had no effect, a small to moderate effect, and a large effect on the CAR values. Each of these effects was then randomly sampled with replacement, 20 times each, with
the number of subjects equal to 2 through 20. For each sample and sample size, data were modelled according to the growth model in Research Hypothesis 1.1 (see below), and the main effect of condition was tested for statistical power via the *powerSim* function in the *simr* package (Green & MacLeod, 2016), with simulations = 20. The average power for each sample size and effect size was then computed. Refer to Appendix C for the results of the simulation, including a depiction of the zero, small, and large effect models. The Cohen’s d statistic was then computed for the average difference in AUCG between conditions to confirm the appropriate estimate of effect size. The results of the simulation suggest an N = 15-16 to achieve adequate power (1- β > 0.8) to detect a main effect of condition, given a small to medium effect size (Cohen’s d = 0.42). For a large effect (Cohen’s d = 0.85), the simulations suggest a sample size of N = 9 to achieve adequate power (1- β > 0.8). These sample sizes are in line with another study that employed an exercise intervention and assessed the CAR response (N = 15; Minetto et al., 2008). In order to limit Type II error in the context of an unknown effect size, an *a priori* sample size was set at N = 15 for this study.

*Screening Visit*

Participants arrived at the laboratory in the morning, between 7am and 10am. On the first visit to the laboratory, the study protocol was described to participants and all participants were asked to read and sign a written informed consent. Participants then answered a series of medical history questions (Appendix D) and completed a depression risk questionnaire (Beck Depression Inventory II [BDI-II]; Appendix E). Additional questionnaires were then completed by the participant to assess chronotype (Munich...
ChronoType Questionnaire; Appendix F), self-reported physical activity (International Physical Activity Questionnaire; Appendix G), self-reported sleep quality (Pittsburg Sleep Quality Index; Appendix H) and socioeconomic background (Hollingshead Index; Appendix I).

Following the completion of these questionnaires, participants had their body composition assessed by 7-site skinfold assessment (Jackson & Pollock, 1978) and air displacement plethysmography (BodPod; COSMED USA, Concord, CA).

Participants were then prepped for a heart rate variability orthostatic challenge assessment. Participants had three electrodes placed on their torso, connected by three wires to a receiver, and wore a respiratory chest strap. These were connected to a receiver (MP160; BioPac Systems, Goleta, CA) to detect the electrocardiogram and respiratory signal and data was collected via the AcqKnowledge software (BioPac Systems, Goleta, CA). Participants were asked to lie in a supine position for 5.5 minutes, followed by upright sitting for 5.5 minutes, and finally 5.5 minutes of standing. In each postural condition, participants were asked to minimize movement as much as possible. Participants continued wearing the electrodes but were disconnected from the wires and straps. Participants then had a heart rate chest strap and receiver placed around their torso (Polar H10 Heart Rate Sensor; Polar Electro Inc., Bethpage, NY), engineered to measure HRV signals. This chest strap was synchronized with the accompanying watch (Polar V800; Polar Electro Inc., Bethpage, NY). The collection of HRV data started immediately after the chest strap was placed and the start time of recording was noted.
A resting blood sample was then obtained from a forearm vein. All blood collection was completed by a trained research technician and occurred within the exercise physiology laboratory. A total of 10cc of mixed venous blood was drawn into serum tubes. Blood was allowed to clot at room temperature for >20 minutes, and then centrifuged for 12 minutes at 3000rpm. Serum was pipetted into cryovials in 0.5mL aliquots and stored at -80°C. Simultaneously, participants also provided a saliva sample via passive drool. Participants were required to provide at least 0.5 mL of liquid saliva.

Immediately following the blood draw and saliva collection, participants were appropriately fitted to the cycle ergometer (Velotron RacerMate; Quarq Technology, Spearfish, SD) and mouthpiece for gas collection during the maximal exercise test. Participants completed a 5 minute warm up, consisting of cycling at a self-selected cadence at 75W. Then, starting at 50W, subjects were asked to maintain their cadence as close as possible to 90 rpm. Resistance (i.e. wattage) was electronically increased by 1W every 2 seconds. The test continued until the subject could no longer maintain a cadence >50 rpm. At each minute of the test, the rating of perceived exertion (RPE) was assessed via the Borg 6-20 scale. Expired gas data was collected and analyzed via indirect calorimetry (TrueOne 2400 Metabolic Measurement System; ParvoMedics, Murray, UT), and included VO₂, VCO₂, and respiratory exchange ratio. HR was collected using the chest strap HR monitor synchronized to the ParvoMedics system. The purpose of this test was to calculate the desired power range for use during the experimental trial, and to account for any changes in aerobic capacity between experimental trials.
At the conclusion of the maximal exercise test, participants immediately had an additional 10cc of mixed venous blood drawn, and provided another saliva sample, following the same protocol as the pre-test procedure. Samples were processed and stored in the same manner. Participants then completed a second HRV orthostatic challenge as previously described.

At the conclusion of the initial session, participants were provided with a wrist-worn accelerometer (GT9X, ActiGraph, Pensacola, FL) initialized to begin data collection that morning at 11:59h. Participants were instructed to wear the accelerometer continuously until they returned for their first experimental visit (approximately 7-10 days). Participants were provided with a log (Appendix J) to record any periods of non-wear. Participants were also provided with dietary (Appendix K) and Liq.In7 fluid logs (Johnson et al., 2017; Appendix L), which they were asked to complete each day they wore the accelerometer.

Lastly, participants were provided with four cryovials, and four short plastic straws, which were used for saliva collection prior to their first experimental visit. Participants were instructed on the proper saliva collection protocol. Participants were asked to provide a saliva sample at 19:00h the evening prior to their experimental visit, immediately after waking on the morning of their experimental visit, and again 30 and 45 minutes after waking on the morning of their experimental visit. Participants were instructed to take nothing by mouth during this 45-minute post-waking period. In addition, participants were asked to refrain from teeth brushing or significant physical activity until after the last sample was collected. Participants were asked to return the
samples they reported for the experimental visit at the exercise physiology laboratory at UNCG. Upon receipt, samples were immediately stored at -80°C.

**Experimental Visit**

Participants reported to the exercise physiology laboratory at UNCG by 6pm. Participants first answered a series of questions to ensure compliance with the research protocol (Appendix M). Failure to adhere to the exercise restriction guidelines (i.e. not refraining from moderate to vigorous exercise within 24 hours of the visit) resulted in the visit being rescheduled. Visits were also rescheduled if the participant reported an abnormal sleep schedule prior to the visit.

Participants first provided a small urine sample, which was tested for urine specific gravity and osmolality. Participants then had nude body mass recorded, and were fitted with a chest strap for the monitoring of HRV throughout the entirety of the visit. HRV recordings were started immediately and the start time of recording was noted.

Participants then had an indwelling intravenous (i.v.) catheter placed in the forearm, secured by transparent adhesive covering. A keep-vein-open (KVO) protocol was then initiated. The KVO included the i.v. catheter being connected to a gravity-fed tubing system. This administered saline at a drip rate of 20-30 ml.hour⁻¹ to maintain line patency without the use of heparin. The i.v. tubing included a dual valve system that permitted the collection of mixed venous blood via luer-lock syringe.

Blood collection began at 18:45h and continued for the remainder of the visit. 5 ml of whole blood was collected every 15 minutes throughout the entire visit until waking, either directly into BD Vacutainer serum tubes or via syringe before being
transferred to the serum tube, followed by a 5 ml i.v. line saline flush. Immediately upon volitional nocturnal sleep offset, participants indicated they were awake by ringing a bell at the bedside. Independent of the time since the last sample, blood was again sampled every 15 minutes, with the final sample collected at 1-hour post-awakening. Blood was permitted to clot at room temperature and then centrifuged at 4°C at 3000 rpm. Serum was pipetted into cryovials in 0.5 ml aliquots and stored at -80°C until analysis. Beginning at 19:00h, subjects produced a saliva sample via passive drool every waking hour, prior to retiring to bed at 22:30h, and immediately and every 15 minutes after waking to align with the post-waking blood samples.

Participants were randomized to either the exercise or control condition in their first visit. Participants then completed the alternate condition at the second visit. If assigned to the control condition, following catheter placement, participants entered the environmental chamber (Cantrol Environmental Systems, Markham, ON, Canada) by 18:55h and remained seated in the environmental chamber until 20:30h.

If assigned to the exercise condition, following the catheter placement participants entered the environmental chamber were seated on the cycle ergometer by 18:55h. Starting at 19:00h, participants began the exercise intervention. The purpose of this exercise intervention was to induce a robust and near-maximal cortisol response to exercise. Therefore, a combination of moderate-high exercise intensity and moderately prolonged exercise in a hot and humid environment was used, with an acknowledgment that there was an inherent need to balance exercise intensity and duration to create a protocol that participants could complete. The exercise protocol specifically required
participants to complete a warm-up that consisted of cycling for 5 minutes with increasing intensity every 30 seconds to a power output that corresponded to 70-75% of the maximal power obtained in the VO$_{2\text{max}}$ assessment from the screening session. Participants then maintained this power output range for 55 mins (1 hour total duration). If cadence decreased below 60 rpm, the power output was reduced in 5W increments. If cadence increased above 90 rpm, power output was increased in 5W increments, with the goal of maximizing the total workload across the 55 minute period. Heart rate and RPE was recorded every 5 minutes. No fluid intake was permitted throughout the exercise session, and environmental chamber conditions were standardized for all experimental sessions at 30°C, 50% humidity. This temperature and humidity was selected based on the results of Hoffman et al. (1994) who demonstrated an elevation in cortisol concentration using only a walking protocol at 33°C and 55.6% humidity (although in the statistical analyses – a 4 [treatment] by 5 [time] ANOVA – did not reveal statistically significant differences in their sample of 10 males). It was therefore proposed here that the combination of temperature, humidity and restriction of fluid intake, coupled with the higher exercise intensity will be adequate to induce a large cortisol response to exercise. Following completion of the 1 hour protocol, and following the 20:00h saliva sample, participants rested in a seated position for another 30 minutes within environmental chamber. Immediately after exiting the chamber, participants had a second nude body mass recorded, after which participants were permitted to drink water ad libitum.

Participants were provided a meal of their choosing from list of options. The same meal was replicated in both experimental conditions. Food was consumed between
21:00h and 21:30h. Participants were permitted to engage in quiet, non-physical activities (e.g. reading, watching television or movies) until 22:30h. At this time, participants provided a saliva sample and were then required to lie in bed, in a private room, protected from excessive external light. Participants were not permitted any electronic devices or distracting materials in the sleep room and were encouraged to sleep. Blood samples continued to be collected every 15 minutes, making efforts to minimally disturb the participant.

Participants were instructed upon waking the following morning to ring a bell located by the bedside. This alerted the researcher to begin awakening sample collection. Participants were asked to remain in the dark and remain supine throughout the waking period. Immediately after awakening, the first saliva sample was collected and resynchronized with blood collection. Saliva and blood was collected every 15 mins until 1 hour post-awakening (i.e. 5 samples: 0, +15, +30, +45, and +60 minutes). Participants were asked every two minutes (aside from salivary collection periods) to count backwards by 3 from a random 3 digit number to ensure they stay awake throughout testing (Appendix N). Participants were assured that neither time nor accuracy was recorded for this task, and this task was simply to ensure the participant was awake.

After the collection of the awakening samples, the i.v. catheter was removed. The participant was then scheduled for the second set of visits at a date more than 8 weeks from the first visit to allow adequate time for full replacement of blood volume. The participant was provided with breakfast and monitored for 30 minutes prior to the leaving the laboratory. Figure 4 depicts the experimental session timeline, for a participant that
woke at 04:30h. These protocols will be completed in exactly the same manner for the second set of visits. The data collection forms for the screening visits and experimental visits can be found in Appendices O and P respectively.

Figure 4. Experimental visit timeline. Figure illustrates the collection of blood samples every 15 minutes (red), saliva samples every hour, immediately before sleep, and every 15 minutes after waking (blue), HRV collection continuously from 18:30h to one hour post-waking (green), exercise from 19:00h to 20:00h (yellow), and sleep from 22:30h to 04:30h (purple).

Biochemistry

Select blood and saliva samples collected during both overnight visits, were assayed for cortisol using commercial competitive enzyme-linked immunoassay (IBL-America, Minneapolis, MN). Awakening saliva samples were first thawed and centrifuged at 14,000 rpm for 15 minutes prior to assay. All samples were assayed in duplicate and were reanalyzed if any individual intra-replicate coefficient of variation was >20%. All participant samples from a single overnight visit were analyzed on the same day.

Saliva samples from the post-awakening period (5 samples per participant per visit) were assayed for cortisone via enzyme immunoassay (Arbor Assays; Ann Arbor, MI). Saliva samples were first thawed and centrifuged at 14,000 rpm for 15 minutes prior to assay. Lastly, blood glucose was quantified by colorimetric assay immediately pre- and
post-exercise, and in the post-awakening +0 minute and post-awakening +60 minute samples. All time points for a single participant were assayed on the same plate to reduce variability. Samples were assayed in duplicate and were reanalyzed if any individual coefficient of variation was >20%.

Data Reduction

Awakening Responses

The determination of the area under the curve (AUC) typically requires somewhat complex calculus. However, in 2003, Pruessner et al. published recommendations for the calculation of AUC with respect to the ground (or a “0” concentration; AUCG) and AUC with respect to the initial value (or increase; AUCI) (J. C. Pruessner et al., 2003). These methods were published in an effort to standardize the metrics utilized in CAR research, and for ease of comparison across studies. As such, in the present study, AUCG and AUCI were calculated via the trapezoidal method as described by Pruessner et al. (2003). Because 5 samples with constant time intervals between samples were used for these calculations, the reduced versions of the formulas were implemented (see Appendix Q for sample calculations). For cortisol (blood and saliva; CAR), cortisone (saliva only; EAR) during the awakening period and for each condition, the following metrics were calculated: AUCI, AUCG, and Δ. The Δ score was calculated as the difference between the first awakening sample (+0 minutes) and the greatest concentration during the awakening period. Refer to Appendix Q for visual demonstration of these metrics. These metrics were computed for blood cortisol (CARbΔ), salivary cortisol (CARsΔ), and salivary cortisone (EARsΔ). The AUCG was calculated for blood cortisol (CARbAUCG),
salivary cortisol (CARs\textsubscript{AUCG}), and salivary cortisone (EARs\textsubscript{AUCG}). The AUCI was calculated for blood cortisol (CARb\textsubscript{AUCI}), salivary cortisol (CARs\textsubscript{AUCI}), and salivary cortisone (EARs\textsubscript{AUCI}). In addition, the exercise period cortisol values (6 samples per subject) will be used to calculate an exercise AUCG (AUCG\textsubscript{exercise}).

**Heart Rate Variability**

Raw R-to-R interval (RR) files were imported to Kubios HRV Premium (Version 3.1.0) for analysis. The RR time series was first corrected for artifact via the inbuilt auto-correction. Then, five-minute epochs prior to each post-awakening sample were isolated, standardized to each individual waking time and not to clock time. For example, if the participant woke at 06:53h, the first HRV epoch to be analyzed was 06:48h to 06:53h, the second epoch to be analyzed was 07:03h to 07:08h and so forth. Each epoch was detrended via a smoothing priors algorithm ($\lambda = 500$) and transformed to frequency domain via fast Fourier transformation. HF was then calculated for each epoch based on a frequency band of 0.15 – 0.4 Hz. A quadratic trend was then fit to the inverse of these values, and the area under the curve with respect to the ground was calculated using the quadratic curve fit to these five HF values (HF\textsubscript{AUCG}). See Appendix R for more details on this data reduction and analysis approach.

**Statistical Approach**

All data analysis was completed via the statistical software R (R Core Team, 2020). Ordinary least squares linear regressions were computed using \textit{lm} function from the \textit{stats} package, and fixed-effect models were fit using the \textit{lmer} function in the \textit{lme4} package (Bates et al., 2015, p. 4). For all mixed-effects models, varying random effects
structures were tested and the best fitting model, as assessed by Akaike Information Criterion (AIC), -2loglikelihood (-2LL), and $\chi^2$, and the most parsimonious model was selected for further analysis and interpretation of parameter estimates. All inferential statistical analyses were set at $\alpha = 0.05$.

To analyze the different hypotheses related to SA1, multiple mixed-effects and ordinary least squares linear regression models were computed.

**RH 1.1.** Separate mixed-effects conditional growth model was used to test the effect of condition (fixed effect) on serum and salivary cortisol, as a function of and interaction with time (fixed effect). Here, time included both a linear and quadratic effect, to capture the expected increase and decrease in concentration of cortisol. In addition, separate mixed-effects models were used to test the effect of condition on CARb$_A$, CARb$_{AUCG}$ and CARb$_{AUCI}$, CARs$_A$, CARs$_{AUCG}$, and CARs$_{AUCI}$.

**RH 1.2.** The associations between AUCG$_{exercise}$ and CARb$_A$, CARb$_{AUCG}$ and CARb$_{AUCI}$ were analyzed in separate ordinary least squares linear regression models.

**RH 1.3.** The independent relations between corresponding blood post-awakening samples (CARb$_A$, CARb$_{AUCG}$, and CARb$_{AUCI}$) and salivary biomarkers (CARs$_A$, CARs$_{AUCG}$, and CARs$_{AUCI}$) were analyzed in separate mixed-effects models.

To analyze the different hypotheses related to SA2, multiple mixed-effects and ordinary least squares linear regression models were computed.

**RH2.1.** A mixed-effects conditional growth model was used to test the effect of condition (fixed effect) on cortisone, as a function of and interaction with time (fixed effect). Here, time was again included as both a linear and quadratic effect, to capture the
expected increase and decrease in concentration of cortisone. In addition, separate mixed-effects models were be used to test the effect of condition on EARsΔ, EARsAUCG and EARsAUCI.

**RH2.2.** The associations between AUCGexercise and EARsAUCG, EARsAUCI and EARsΔ were analyzed in separate ordinary least squares linear regression models.

**RH2.3.** The independent relations between corresponding blood cortisol indices (CARbΔ, CARbAUCG, CARbAUCI) and salivary cortisone indices (EARsAUCG, EARsAUCI, and EARsΔ) were analyzed in separate mixed-effects models.

To analyze the different hypotheses related to SA3, multiple mixed-effects and ordinary least squares linear regression models were computed.

**RH3.1.** A mixed-effects conditional growth model was used to test the effect of condition (fixed effect) on HF, as a function of and interaction with time (fixed effect). Data were visually inspected and alternative models fit to determine whether time should be modelled as a linear or quadratic effect.

**RH3.2.** A mixed-effects model was used to test the effect of condition (fixed effect) on HF AUCG.

**RH3.3.** The independent associations of CAR indices (CARbΔ, CARbAUCG, and CARbAUCI) and EAR indices (EARsΔ, EARsAUCG, and EARsAUCI) and HF AUCG were assessed via mixed-effects model, and included a main and interaction fixed-effect of condition.
**RH3.4.** The independent effects of $HF_{AUCG}$, $CARb_{AUCI}$, and $EAR_{SAUCI}$ to predict $AUC_{exercise}$ on the morning after exercise was tested by an ordinary least squares multiple linear regression.

Appendix S shows the model formulation for a random-intercept model that could feasibly be used to answer RH1.1, RH2.1, or RH3.1, and simulated model results aligned with respective depictions of the awakening responses and effect of condition.

**Summary**

This study tested whether acute exercise significantly impacts a variety of endocrine awakening responses, tests the associations between indices of the awakening response between biological fluids, and tests for the effect of exercise on a hypothesized HRV awakening response.
CHAPTER IV
RESULTS

Participants

Eleven participants completed all components of this study. One participant (CAR012) completed only the Control condition, and thus there are 23 hormonal profiles across the two conditions (n = 12 control profiles, n = 11 exercise profiles). The participant missing the Exercise condition is thus excluded from analyses contrasting the two conditions, but included in analyses assessing relations between variables for which this subject contributes observations from the single condition. Participants did not differ (as assessed via paired-samples t-test) by age (mean (SD) = 24.13 (4.39) years), height (mean (SD) = 174.67 (5.48) cm), mass (mean (SD) = 74.81 (9.71) kg), resting heart rate (mean (SD) = 66.17 (6.98) bpm), or resting blood pressure (systolic mean (SD) = 112.61 (9.56) mmHg, diastolic mean (SD) = 75.83 (6.93) mmHg) between the two conditions (Table 1).

Table 1. Comparison of demographic data between screening visits.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24 (5)</td>
<td>24 (4)</td>
<td>24 (4)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174.1 (6.07)</td>
<td>175.28 (4.97)</td>
<td>174.67 (5.48)</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>74.16 (10.69)</td>
<td>75.53 (8.98)</td>
<td>74.81 (9.71)</td>
</tr>
<tr>
<td>Resting Heart Rate (bpm)</td>
<td>65 (7)</td>
<td>67 (7)</td>
<td>66 (7)</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>111 (6)</td>
<td>115 (12)</td>
<td>113 (10)</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>76 (6)</td>
<td>76 (8)</td>
<td>76 (7)</td>
</tr>
</tbody>
</table>
Biochemistry

Serum samples were analyzed at Q15 for the first two and last two hours of each profile (to capture the exercise time period and waking time period with the greatest resolution possible), and Q30 between these periods. Serum was assayed for cortisol using commercially available competitive binding enzyme immunoassay (IBL-America, Minneapolis, MN). The full profile for each participant, for each condition, was assayed on a single plate, with a standard curve calculated for each plate and high and low controls assayed on each plate. All values for the high and low control samples were within the manufacturer supplied reference ranges. The intraassay coefficient of variation (CV) for the serum cortisol samples was 5.28% and the inter-assay CV was 6.24%. Because assay kits were from two kit lots, to ensure quality control all samples from a single person were assayed within a single kit lot. The inter-lot CV was determined by assaying the same controls across lots and was calculated as 7.78%. Serum samples from four time points (19:00h, 20:00h, waking+0, and waking+60) were also assayed for blood glucose (Cayman Chemical, Ann Arbor, MI). Intraassay and inter-assay CV were 2.53% and 10.82%, respectively.

Saliva samples were assayed for salivary cortisol (IBL America, IBL-America, Minneapolis, MN) and salivary cortisone (Arbor Assays, Ann Arbor, MI). All samples from a single participant were assayed on the same plate. The intraassay and inter-assay CV for salivary cortisol were 5.25% and 13.11%, respectively. The intraassay and inter-assay CV for salivary cortisone were 5.11% and 5.69%, respectively.
Missing Data

Of the 839 serum samples that were scheduled to be collected and analyzed, only 4 samples were missing due to issues with blood collection. All 4 of these samples were from subject *CAR001* in the Control condition, at +15, +30, +45, and +60 minutes post waking, due to a clotted i.v. catheter. The salivary samples during this period were collected, however, and so the missing serum samples were imputed from the saliva samples via multiple imputation using chained equations (MICE). These imputations were based on the relationship between salivary cortisol, salivary cortisone, and serum cortisol analyzed from all other participants (Figure 5). An assumption of MICE is that data is missing at random which, aside from complications with the intravenous line being more probable as time elapses and the missingness being representative of a biological time series, is partially satisfied. Univariate time series imputation techniques may have been possible if a single observation were missing from the waking samples, but the number of sequential samples made this approach less suitable. Following this imputation, serum data from all 12 participants were used in analyses. In addition, 1 saliva sample was excluded for having a concentration >5.4 standard deviations above the mean, possibly indicating blood contamination. Following deletion, this saliva value was then imputed using the same methods described above.
Figure 5. Missing values were imputed based on the relation between serum cortisol and salivary cortisone (A) and salivary cortisol (B). Imputed samples are represented by blue triangles.

The relation between serum cortisol and salivary cortisone (Figure 6) was best characterized by a quadratic model ($\beta_{\text{Quadratic}} = 2.54 \times 10^{-4}$, $p < 0.001$, adjusted $R^2 = 0.660$) whereas the relation between serum cortisol and salivary cortisol was best characterized by a linear model ($\beta_{\text{Linear}} = 0.15$, $p < 0.001$, adjusted $R^2 = 0.562$).
Figure 6. The relation between serum cortisol and salivary cortisol (A) and salivary cortisone (B).

The proportion of serum cortisol represented by the two salivary biomarkers, salivary cortisol was on average 7.93% of serum cortisol, whereas salivary cortisone was on average 18.99% of serum cortisol.

**Screening Data**

The average time between screening visits was 11.7 weeks. There were no significant differences between screening visits for any body composition assessment (Table 2).
Table 2. Comparison of body composition assessment variables between screening visits.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>BodPod Fat Mass (kg)</td>
<td>9.72 (2.84)</td>
<td>10.29 (3.59)</td>
<td>9.99 (3.16)</td>
</tr>
<tr>
<td>BodPod Fat Free Mass (kg)</td>
<td>64.44 (9.57)</td>
<td>65.23 (9.35)</td>
<td>64.82 (9.25)</td>
</tr>
<tr>
<td>BodPod Fat (%)</td>
<td>13.08 (3.37)</td>
<td>13.76 (5.2)</td>
<td>13.41 (4.25)</td>
</tr>
<tr>
<td>BodPod Fat Free Mass (%)</td>
<td>86.92 (3.37)</td>
<td>86.24 (5.2)</td>
<td>86.59 (4.25)</td>
</tr>
<tr>
<td>Skinfolds Fat (%)</td>
<td>12.48 (3.98)</td>
<td>12.87 (5.18)</td>
<td>12.67 (4.49)</td>
</tr>
</tbody>
</table>

Note: comparisons between control and exercise conditions were tested via paired samples t-test.

There were no significant differences between any variable assessed in the maximal exercise test on the cycle ergometer (Table 3).

Table 3. Comparison of variables extracted from maximal exercise test performed on the cycle ergometer between screening visits.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>VO2max (ml.kg⁻¹.min⁻¹)</td>
<td>50.21 (7.83)</td>
<td>49.64 (7.5)</td>
<td>49.94 (7.5)</td>
</tr>
<tr>
<td>Maximal Heart Rate (bpm)</td>
<td>187.42 (7.61)</td>
<td>187.18 (8.92)</td>
<td>187.3 (8.07)</td>
</tr>
<tr>
<td>Respiratory Exchange Ratio</td>
<td>1.18 (0.07)</td>
<td>1.2 (0.06)</td>
<td>1.19 (0.07)</td>
</tr>
<tr>
<td>Peak Power (Watts)</td>
<td>304.08 (39.26)</td>
<td>310.73 (42.72)</td>
<td>307.26 (40.14)</td>
</tr>
</tbody>
</table>

Note: comparisons between control and exercise conditions were tested via paired samples t-test.

There were no significant differences between control and exercise conditions in chronotype, as measured by the mid-sleep time on free days derived from the MCTQ (t(10) = 1.99, p = 0.075). Similarly, there was no significant difference between control and exercise conditions for the PSQI global score (t(9) = 1.27, p = 0.236). Differences for sleep onset times, wake times, and sleep duration between work days and free days, and between experimental conditions were analyzed by two-way completely within ANOVA (Table 4). There were no significant main or interaction effects for sleep onset (all p > 0.05). There was however a main effect for day type on wake time (F(1,21) = 13.034, p =
Pairwise t-tests with Bonferroni corrections demonstrated a later wake time (00:55) on free days compared to work days ($t(21) = 4.50, p_{adj} < 0.001$). There were no significant main or interaction effects for sleep duration (all $p > 0.05$).

### Table 4. Comparison of sleep and chronotype variables between screening visits.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wake Time Work Day (HH:MM 24h)</td>
<td>07:01 (01:16)</td>
<td>07:29 (01:01)</td>
<td>07:14 (01:09)</td>
</tr>
<tr>
<td>Sleep Duration Work Day (HH:MM)</td>
<td>07:23 (00:42)</td>
<td>07:35 (00:44)</td>
<td>07:29 (00:42)</td>
</tr>
<tr>
<td>Sleep Onset Free Day (HH:MM 24h)</td>
<td>00:07 (01:09)</td>
<td>00:23 (01:10)</td>
<td>00:15 (01:08)</td>
</tr>
<tr>
<td>Wake Time Free Day (HH:MM 24h)</td>
<td>08:07 (00:55)</td>
<td>08:12 (00:58)</td>
<td>08:09 (00:56)$^\dagger$</td>
</tr>
<tr>
<td>Sleep Duration Free Day (HH:MM)</td>
<td>08:00 (01:03)</td>
<td>07:48 (01:01)</td>
<td>07:54 (01:01)</td>
</tr>
<tr>
<td>Mid-Sleep Time on Free Day (HH:MM 24h)</td>
<td>04:04 (01:00)</td>
<td>04:17 (00:56)</td>
<td>04:10 (00:57)</td>
</tr>
<tr>
<td>PSQI Global Score (A.U.)</td>
<td>5.27 (3.66)</td>
<td>4.27 (2)</td>
<td>4.77 (2.93)</td>
</tr>
</tbody>
</table>

*Note: $^\dagger$ = significantly different from Work Day. PSQI = Pittsburgh Sleep Quality Index. HH:MM 24h = clock time in 24-hour format. HH:MM = duration in hours and minutes.*

### Experimental Data

There was an average of 10.3 days (7.2) and 7.4 days (2.0) between screening and experimental visits for the Control and Exercise conditions, respectively. Seven participants were randomly assigned to the Control condition in the first set, whereas 5 participants were assigned to the Exercise condition. Subsequently, in the second set 5 participants were assigned to the Control condition, and 7 participants were assigned to the Exercise condition, although only 6 Exercise visits were completed.
There were no significant differences between conditions for urine specific gravity ($t(10) = 0.37, p = 0.72$), urine osmolality ($t(10) = 0.19, p = 0.86$), or urine color ($t(10) = -0.64, p = 0.54$) between experimental conditions. There was however a higher average perceived stress reported at the control condition compared to the exercise condition ($t(10) = 2.47, p = 0.03$).

Table 5. Comparison of urinary markers of hydration and perceived stress between control and exercise conditions.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>USG (A.U.)</td>
<td>1.010 (0.007)</td>
<td>1.009 (0.006)</td>
<td>1.010 (0.006)</td>
</tr>
<tr>
<td>Color (A.U.)</td>
<td>3.00 (1.04)</td>
<td>3.27 (1.35)</td>
<td>3.13 (1.18)</td>
</tr>
<tr>
<td>Osmolality (mOsm.kg$^{-1}$)</td>
<td>410 (277)</td>
<td>398 (259)</td>
<td>404 (263)</td>
</tr>
<tr>
<td>PSS (A.U.)</td>
<td>13.08 (7.54)</td>
<td>9.45 (8.25)$^\dagger$</td>
<td>11.35 (7.92)</td>
</tr>
</tbody>
</table>

Note: $^\dagger$ = significantly different from Control condition. = USG = Urine Specific Gravity. PSS = Perceived Stress Scale Score.

The one hour exercise session resulted in increases for objective internal (heart rate) and relative (RPE) load metrics (Table 6). Despite the target of 70-75% of peak power for the one hour duration, the exercise session resulted in an average relative power output of 51.1% of peak power. This was due to the power being reduced during the exercise session when participant cadence dropped below 60 rpm. Even so, HR as expressed relative to the maximal HR achieved during the maximal exercise test at the screening visit was on average 87.04% (Figure 7).
Specifically, peak HR ($t(10) = 17.21$, $p < 0.001$), average HR ($t(10) = 18.72$, $p < 0.001$), relative HR ($t(10) = 14.77$, $p < 0.001$), peak RPE ($t(10) = 37.68$, $p < 0.001$), and average RPE ($t(10) = 33.22$, $p < 0.001$) were all significantly elevated in the exercise condition relative to the control condition. The exercise condition also resulted in a significantly greater change in nude body mass (Table 6; $t(10) = 4.46$, $p = 0.001$).

Interestingly, mean increases in nude body mass were observed in the control condition (not statistically significant), despite fluid intake being restricted until after the post-exercise nude body mass assessment. This may have been a result of the mass of the i.v. tubing being included in the post- but not pre-measurement, or from the small volume of
saline administered over the first approximately two hours of the study visit. Lastly, plasma volume shifts were calculated according to the method of Dill and Costill (1974). Plasma volume change was significantly greater ($t(9) = 4.25, p = 0.002$) in the exercise condition (-9.07% [4.22]) compared to the control condition (-0.88% [4.87]).

Table 6. Comparison of internal and external load between control and exercise conditions.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Power (Watts)</td>
<td>0.00 (0.00)</td>
<td>225.62 (26.78)$^\dagger$</td>
</tr>
<tr>
<td>Average Power (Watts)</td>
<td>0.00 (0.00)</td>
<td>155.07 (31.62)$^\dagger$</td>
</tr>
<tr>
<td>Average Relative Power (%)</td>
<td>0.00 (0.00)</td>
<td>51.1 (3.7)$^\dagger$</td>
</tr>
<tr>
<td>Exercise Energy Expenditure (kcals)</td>
<td>-</td>
<td>658.93 (126.44)</td>
</tr>
<tr>
<td>Average Relative VO$<em>2$ (% of VO$</em>{2\text{max}}$)</td>
<td>-</td>
<td>55.52 (8.05)</td>
</tr>
<tr>
<td>Peak Heart Rate (bpm)</td>
<td>108.14 (13.54)</td>
<td>183.12 (7.47)$^\dagger$</td>
</tr>
<tr>
<td>Average Heart Rate (bpm)</td>
<td>73.60 (8.12)</td>
<td>161.48 (10.04)$^\dagger$</td>
</tr>
<tr>
<td>Average Relative Heart Rate (%)</td>
<td>39.08 (4.23)</td>
<td>87.04 (6.14)$^\dagger$</td>
</tr>
<tr>
<td>Peak RPE (A.U.)</td>
<td>6.25 (0.46)</td>
<td>19.12 (0.99)$^\dagger$</td>
</tr>
<tr>
<td>Average RPE (A.U.)</td>
<td>6.20 (0.39)</td>
<td>16.95 (0.98)$^\dagger$</td>
</tr>
<tr>
<td>NBM Change (%)</td>
<td>0.11 (0.30)</td>
<td>-0.82 (0.79)$^\dagger$</td>
</tr>
</tbody>
</table>

Note: $^\dagger$ = significantly different from Control condition. RPE = Rating of Perceived Exertion Borg 6-20 scale. NBM = nude body mass. Relative Power and Relative Heart Rate are calculated as the percentage of the average heart rate relative to the maximal value achieved during the maximal exercise test from the screening visit specific to that set of visits. Exercise energy expenditure is calculated from Velotron power profiles, assuming a cycling efficiency of 20%. Average Relative VO$_2$ is calculated by corresponding the average power output to power during the VO$_{2\text{max}}$ assessment.

The purpose of the exercise session was to stimulate the HPA-axis and maximize the cortisol response to exercise. The cortisol profiles for each individual participant, excluding the 1-hour post waking samples, are depicted in Figure 8. Cortisol concentrations increased, on average, by 477.33% during the exercise condition. Cortisol concentrations during the 1-hour exercise and 30 minute recovery period were on average 367 nmol.l$^{-1}$ greater compared to the equivalent time points during the control condition.
Interestingly, only participant CAR008 did not demonstrate a robust serum cortisol response to the exercise. In addition, please note that participant CAR012 completed only the control condition.

![Figure 8. Serum cortisol profiles for each participant, excluding the post-waking samples.](image)

Blood glucose was also assayed at four time points: pre- and post-exercise, and at waking and 60 minutes post waking (Table 7). A 2 by 2 completely within ANOVA
tested differences between time and condition for the exercise and waking samples separately. Results demonstrated no condition by time interaction for glucose concentrations during the waking period. However, there was a significant interaction effect for time and condition when analyzing serum glucose in the evening before and after the exercise or control intervention. Pairwise post-hoc analyses with Bonferroni corrections demonstrated glucose concentrations to be decreased at 20:00h (end of exercise) compared to 19:00h (pre-exercise) (t(10) = 4.58, p < 0.001), in the Exercise condition only (data not shown).

Table 7. Comparison of blood glucose concentration between control and exercise condition and between time points.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre Exercise (mg.dl⁻¹)</td>
<td>86.05 (14.09)</td>
<td>74.21 (11.95)</td>
</tr>
<tr>
<td>Post Exercise (mg.dl⁻¹)</td>
<td>87.97 (7.22)</td>
<td>62.98 (14.2)†</td>
</tr>
<tr>
<td>Waking +0 (mg.dl⁻¹)</td>
<td>82.96 (12.05)</td>
<td>79.92 (14.32)</td>
</tr>
<tr>
<td>Waking +60 (mg.dl⁻¹)</td>
<td>84.18 (14.14)</td>
<td>78.12 (10.42)</td>
</tr>
</tbody>
</table>

Note: † = significantly different from Pre Exercise.

Wrist worn accelerometry was used to quantify the quantity and quality of sleep obtained by participants during the experimental visit (Table 8). Analysis demonstrated no differences in sleep efficiency (t(9) = 2.13, p = 0.062), total sleep time (t(9) = 0.70, p = 0.502), wake time (t(9) = 0.42, p = 0.682), number of awakenings t(9) = -0.22, p = 0.829), fragmentation index (t(9) = -0.56, p = 0.59), or sleep fragmentation index (t(9) = -1.39, p = 0.197) between conditions. There was however a significant difference between conditions for waking after sleep onset (t(9) = -2.41, p = 0.039), length of awakenings (t(9) = -2.65, p = 0.027), and the movement index (t(9) = -2.44, p = 0.037). In sum it
appears that late evening exercise results in worse sleep quality compared to the control condition, although this is only reflected in a few variables assessed from sleep actigraphy.

Table 8. Comparison of accelerometry-derived quantification of sleep quantity and sleep quality

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wake Time (HH:MM 24h)</td>
<td>06:26(01:20)</td>
<td>06:34(01:07)</td>
<td>06:30(01:12)</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>73.65 (14.84)</td>
<td>66.02 (13.09)</td>
<td>69.84 (14.21)</td>
</tr>
<tr>
<td>Total Sleep Time (mins)</td>
<td>486.36 (63.26)</td>
<td>456.64 (79.56)</td>
<td>471.5 (71.77)</td>
</tr>
<tr>
<td>Wake After Sleep Onset (mins)</td>
<td>112.45 (75.43)</td>
<td>137.73 (43.13)†</td>
<td>125.09 (61.34)</td>
</tr>
<tr>
<td>Number of Awakenings (#)</td>
<td>23.64 (7.26)</td>
<td>23.55 (9.03)</td>
<td>23.59 (7.99)</td>
</tr>
<tr>
<td>Length of Awakenings (mins)</td>
<td>4.83 (3.51)</td>
<td>6.72 (3.55)†</td>
<td>5.78 (3.57)</td>
</tr>
<tr>
<td>Movement Index (A.U.)</td>
<td>20.88 (8.6)</td>
<td>26.02 (8.59)†</td>
<td>23.45 (8.79)</td>
</tr>
<tr>
<td>Fragmentation Index (A.U.)</td>
<td>10.57 (10.32)</td>
<td>11.15 (6.4)</td>
<td>10.86 (8.38)</td>
</tr>
<tr>
<td>Sleep Fragmentation Index (A.U.)</td>
<td>31.45 (17.57)</td>
<td>37.17 (11.78)</td>
<td>34.31 (14.89)</td>
</tr>
</tbody>
</table>

Note: † = significantly different from Control condition. Efficiency = sleep minutes relative to total minutes between 22:30h and wake time; Movement Index = % of epochs with y-axis counts >0; Fragmentation Index = % of 1-minute epochs scored as “sleep” relative to all periods of sleep; Sleep Fragmentation Index = Movement Index + Fragmentation Index.

Specific Aim 1

The purpose of Specific Aim 1 was to 1) test the effect of exercise on the serum and salivary cortisol awakening response; 2) test the association between the cortisol response to exercise and CAR indices; and 3) test the association between the CAR indices derived from serum and saliva.
**RH1.1: The effect of exercise on the cortisol awakening response as assessed in serum and saliva.**

*Serum*

Mixed-effects growth models were first used to model the change in serum cortisol across the 1-hour waking period and test for main and interaction effects with exercise condition. Unconditional models demonstrated an intraclass correlation (ICC) of 65%, justifying the use of random-effects models. Random intercept only, and random intercept and slope random effects structures were compared. Including the random effect of time (i.e. slope), did not improve model fit (random intercept only: AIC = 1324.9, $-2LL = -657.45$; random intercept and slope: AIC = 1327.5, $-2LL = -656.72$; $\chi^2(2) = 0.483$). Therefore, models with random intercepts only were retained and examined further. Models including only the effect of time demonstrated a significant quadratic effect of time ($\beta_{Linear} = 234.89$, [95%CI = 119.28 to 350.50], $p < 0.001$; $\beta_{Quadratic} = -245.42$, [95%CI = -356.28 to -134.56], $p < 0.001$, Conditional $R^2 = 0.705$, Marginal $R^2 = 0.050$). When included as an interaction effect with the linear and quadratic effects of time (Table 9, Model 2), there was no significant main effect of exercise condition ($\beta_{Condition} = -43.99$ [95%CI = -90.56 to 2.57], $p = 0.064$, Conditional $R^2 = 0.744$, Marginal $R^2 = 0.080$), and condition did not interact with linear or quadratic time.
Table 9. Fixed effects of serum cortisol models with condition as a main and interaction effect.

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$ Estimates</td>
<td>95%CI</td>
</tr>
<tr>
<td>Intercept</td>
<td>388.28</td>
<td>327.95 – 448.61</td>
</tr>
<tr>
<td>Time</td>
<td>234.89</td>
<td>126.21 – 343.58</td>
</tr>
<tr>
<td>Time$^2$</td>
<td>-245.42</td>
<td>-349.64 – -141.20</td>
</tr>
<tr>
<td>Condition</td>
<td>-42.26</td>
<td>-64.52 – -20.01</td>
</tr>
<tr>
<td>Time * Condition</td>
<td>29.05</td>
<td>-190.59 – 248.69</td>
</tr>
<tr>
<td>Time$^2$ * Condition</td>
<td>-34.08</td>
<td>-244.70 – 176.54</td>
</tr>
</tbody>
</table>

Note: Control condition is used as the reference category. Model 1 = Condition as a main effect; Model 2 = Condition as an interaction effect with linear and quadratic effects of time.

When included as a main effect only (Table 9, Model 1), a significant negative main effect of exercise condition was observed (Figure 9; $\beta_{\text{Condition}} = -42.26$ [95%CI = -64.52 to -20.01], p < 0.001, Conditional $R^2 = 0.747$, Marginal $R^2 = 0.080$).

![Figure 9. The effect of exercise on the serum cortisol awakening response.](image)

In comparing CARb indices between conditions, only mixed-effects models with random intercepts only converged and thus are reported here. Models demonstrated that there was no significant effect of exercise condition on CARb_{AUCG} (β_{Condition} = -153.32 [95%CI = -349.97 to 43.32], p = 0.126, Conditional R² = 0.687, Marginal R² = 0.034), CARb_{AUCI} (β_{Condition} = 21.56 [95%CI = -158.37 to 200.90], p < 0.566, Conditional R² = 0.174, Marginal R² = 0.002), or CARb_{Δ} (β_{Condition} = 10.38 [95%CI = -42.86 to 63.62], p = 0.702, Conditional R² = 0.086, Marginal R² = 0.006; Figure 10).

![Figure 10](image)

Figure 10. Comparison of CARb_{AUCG} (A), CARb_{AUCI} (B), and CARb_{Δ} (C) between conditions.

*Saliva*

Mixed-effects growth models were also used to model the change in salivary cortisol across the 1-hour waking period and test for main and interaction effects with exercise condition. Unconditional models demonstrated an ICC of 48%. Including the
random effect of time (i.e. slope), did not improve model fit \( \text{random intercept only: AIC} = 939.08, -2\text{LL} = -464.54; \text{random intercept and slope: AIC} = 938.53, -2\text{LL} = -462.26; \chi^2(2) = 4.550, p = 0.103 \). Therefore, models with random intercepts only were retained and examined further. Models including only the effect of time demonstrated a significant quadratic effect of time \( (\beta_{\text{linear}} = 52.25, [95\% CI = 29.93 \text{ to } 74.56], p < 0.001; \beta_{\text{quadratic}} = -42.04, [95\% CI = -63.44 \text{ to } -20.65], p < 0.001, \text{Conditional } R^2 = 0.576, \text{Marginal } R^2 = 0.092) \). When included as a main effect only (Table 10, Model 1), a significant negative main effect of exercise condition was observed \( (\beta_{\text{condition}} = -11.55 [95\% CI = -15.52 \text{ to } -7.57], p < 0.001, \text{Conditional } R^2 = 0.695, \text{Marginal } R^2 = 0.178; \text{Figure 11}) \).

![Figure 11. The effect of exercise on the salivary cortisol awakening response.](image-url)
When included as an interaction effect with the linear and quadratic effects of time (Table 10, Model 2), there was no longer a significant main effect of exercise condition observed ($\beta_{\text{Condition}} = -5.05$ [95%CI = -13.23 to 3.14], $p = 0.227$, Conditional $R^2 = 0.701$, Marginal $R^2 = 0.186$), or significant interactions with linear or quadratic time.

Table 10. Fixed effects of salivary cortisol models with condition as a main and interaction effect.

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta_{\text{Estimates}}$</td>
<td>95%CI</td>
<td>p</td>
<td>$\beta_{\text{Estimates}}$</td>
<td>95%CI</td>
<td>p</td>
</tr>
<tr>
<td>Intercept</td>
<td>27.06</td>
<td>18.01 – 36.11</td>
<td>&lt;0.001</td>
<td>23.95</td>
<td>14.29 – 33.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time</td>
<td>52.25</td>
<td>32.83 – 71.67</td>
<td>&lt;0.001</td>
<td>63.74</td>
<td>37.04 – 90.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time$^2$</td>
<td>-42.04</td>
<td>-60.67 – -23.42</td>
<td>&lt;0.001</td>
<td>-49.07</td>
<td>-74.67 – -23.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Condition</td>
<td>-11.55</td>
<td>-15.52 – -7.57</td>
<td>&lt;0.001</td>
<td>-5.05</td>
<td>-13.23 – 3.14</td>
<td>0.227</td>
</tr>
<tr>
<td>Time * Condition</td>
<td></td>
<td></td>
<td></td>
<td>-24.02</td>
<td>-62.63 – 14.59</td>
<td>0.223</td>
</tr>
<tr>
<td>Time$^2$ * Condition</td>
<td></td>
<td></td>
<td></td>
<td>14.69</td>
<td>-22.34 – 51.71</td>
<td>0.437</td>
</tr>
</tbody>
</table>

Note: Control condition is used as the reference category. Model 1 = Condition as a main effect; Model 2 = Condition as an interaction effect with linear and quadratic effects of time.

In comparing CARs indices between conditions, only mixed-effects models with random intercepts only converged and thus are reported here. An analysis of the effect of condition on salivary indices of the CAR demonstrated that there was a significant negative effect of exercise condition on CAR$\text{AUCG}$ ($\beta_{\text{Condition}} = -45.20$ [95%CI = -82.44 to -7.96], $p = 0.017$, Conditional $R^2 = 0.590$, Marginal $R^2 = 0.108$), CAR$\text{AUCI}$ ($\beta_{\text{Condition}} = -27.78$ [95%CI = -47.42 to -8.13], $p = 0.006$, Conditional $R^2 = 0.629$, Marginal $R^2 = 0.133$), and CAR$\text{A}$ ($\beta_{\text{Condition}} = -8.79$ [95%CI = -16.67 to -0.92], $p = 0.029$, Conditional $R^2 = 0.533$, Marginal $R^2 = 0.104$; Figure 12).
Figure 12. Comparison of CARsAUCG (A), CARsAUCI (B), and CARsΔ (C) between conditions. † = significant difference from Control condition.

RH1.2: The relation between cortisol response to exercise and cortisol awakening response.

The cortisol response to exercise (AUCG_{exercise}) was calculated by taking the area under the curve of serum cortisol from the start of exercise (19:00h) to the end of the 30-minute recovery period (20:30h). The relation between AUCG_{exercise} and CARb_Δ, CARb_{AUCG}, and CARb_{AUCI} were tested via ordinary least squares linear regression, using only the data from the exercise condition. There were no significant relations between AUCG_{exercise} and CARb_{AUCG}, (β_{AUCG_{exercise}} = 4.16×10^{-3} [95%CI = -0.02 to 0.03], p = 0.709, R^2 = 0.015), CARb_{AUCI} (β_{AUCG_{exercise}} = -0.01 [95%CI = -0.03 to 2.4×10^{-3}], p = 0.089, R^2 = 0.287), or CARb_Δ (β_{AUCG_{exercise}} = -3.6×10^{-3} [95%CI = -8.3×10^{-3} to 1.2×10^{-3}], p = 0.127, R^2 = 0.239; Figure 13).
Figure 13. The association between the cortisol response to exercise and serum indices of the cortisol awakening response: CARbAUCg (A), CARbAUCi (B), and CARbΔ (C).

RH1.3: The relation between blood and salivary indices of the cortisol awakening response.

The associations between indices of the cortisol awakening response between serum and saliva were assessed via mixed-effects models. Only models with random intercepts only converged and thus are reported here. There was no significant association between serum and saliva indices for CARAUCi (βCARsAUCi = 1.42 [95%CI = -1.21 to 4.05], p = 0.290, Conditional R^2 = 0.050, Marginal R^2 = 0.289), or CARΔ (βCARsΔ = 1.49 [95%CI = -0.56 to 3.54], p = 0.153, Conditional R^2 = 0.087, Marginal R^2 = 0.319). There was however a significant positive association between serum and saliva CARAUCg (βCARsAUCg = 4.51 [95%CI = 3.39 to 5.64], p < 0.001, Conditional R^2 = 0.900, Marginal R^2 = 0.641; Figure 14).
Figure 14. The association between $\text{CARb}_{\text{AUCG}}$ and $\text{CARs}_{\text{AUCG}}$ (A), $\text{CARb}_{\text{AUCI}}$ and $\text{CARs}_{\text{AUCI}}$ (B), and $\text{CARb}_\Delta$ and $\text{CARs}_\Delta$ (C).

Specific Aim 2

RH2.1: Effect of exercise on cortisone awakening response.

Mixed-effects growth models were first used to model the change in salivary cortisone across the 1-hour waking period and test for main and interaction effects with exercise condition. The ICC of the unconditional model was 54%. Including the random effect of time (i.e. slope), did not improve model fit (random intercept only: AIC = 970.46, -2LL = -480.23; random intercept and slope: AIC = 972.27, -2LL = -479.14; $\chi^2(2) = 2.193, p = 0.334$). Therefore, models with random intercepts only were retained and examined further. Models including only the effect of time demonstrated a significant linear and quadratic effect of time ($\beta_{\text{Linear}} = 66.61$, [95%CI = 38.89 to 94.33], $p < 0.001$; $\beta_{\text{Quadratic}} = -41.18$, [95%CI = -68.90 to -13.46], $p < 0.004$, Conditional $R^2 = 0.641$, Marginal $R^2 = 0.097$). When included as a main effect only (Table 11, Model 1), a
significant negative main effect of exercise condition was observed ($\beta_{\text{Condition}} = -11.07$ [95%CI = -15.76 to -6.37], $p < 0.001$, Conditional $R^2 = 0.717$, Marginal $R^2 = 0.147$).

Table 11. Fixed effects of salivary cortisone models with condition as a main and interaction effect.

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th></th>
<th>p</th>
<th>Model 2</th>
<th></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta_{\text{Estimates}}$</td>
<td>95%CI</td>
<td></td>
<td>$\beta_{\text{Estimates}}$</td>
<td>95%CI</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>77.98</td>
<td>67.39 – 88.57</td>
<td>&lt;0.001</td>
<td>77.98</td>
<td>67.40 – 88.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time</td>
<td>66.61</td>
<td>41.40 – 91.82</td>
<td>&lt;0.001</td>
<td>92.17</td>
<td>57.80 – 126.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time$^2$</td>
<td>-41.18</td>
<td>-66.39 – -15.98</td>
<td>0.001</td>
<td>-51.23</td>
<td>-85.59 – -16.86</td>
<td>0.003</td>
</tr>
<tr>
<td>Condition</td>
<td>-11.07</td>
<td>-15.76 – -6.37</td>
<td>&lt;0.001</td>
<td>-11.07</td>
<td>-15.70 – -6.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time * Condition</td>
<td></td>
<td></td>
<td></td>
<td>-53.45</td>
<td>-103.14 – -3.76</td>
<td>0.035</td>
</tr>
<tr>
<td>Time$^2$ * Condition</td>
<td></td>
<td></td>
<td></td>
<td>21.00</td>
<td>-28.69 – 70.69</td>
<td>0.408</td>
</tr>
</tbody>
</table>

Note: Control condition is used as the reference category. Model 1 = Condition as a main effect; Model 2 = Condition as an interaction effect with linear and quadratic effects of time.

When included as an interaction effect with the linear and quadratic effects of time (Table 11, Model 2), a significant negative main effect of exercise condition ($\beta_{\text{Condition}} = -11.07$ [95%CI = -15.70 to -6.45], $p < 0.001$) and a significant negative interaction with linear time ($\beta_{\text{Linear} \times \text{Condition}} = -53.45$ [95%CI = -103.14 to -3.76], $p < 0.001$, Conditional $R^2 = 0.727$, Marginal $R^2 = 0.159$) was observed (Figure 15).
Figure 15. The effect of exercise on the salivary cortisone awakening response.

In comparing EARs indices between conditions, only mixed-effects models with random intercepts only converged and thus are reported here. Models demonstrated that there was no significant effect of exercise condition on $\text{EARS}_{\text{AUCG}}$ ($\beta_{\text{condition}} = -42.87$ [95%CI = -88.75 to 3.01], $p = 0.067$, Conditional $R^2 = 0.561$, Marginal $R^2 = 0.068$).

However, there was a significant negative effect of condition on $\text{EARS}_{\text{AUCI}}$ ($\beta_{\text{condition}} = -39.52$ [95%CI = -69.45 to -9.58], $p = 0.010$, Conditional $R^2 = 0.283$, Marginal $R^2 = 0.219$), and $\text{EARS}_{\text{A}}$ ($\beta_{\text{condition}} = -11.40$ [95%CI = -20.67 to -2.13], $p = 0.016$, Conditional $R^2 = 0.455$, Marginal $R^2 = 0.146$; Figure 16).
Figure 16. Comparison of EARs\textsubscript{AUCG} (A), EARs\textsubscript{AUCI} (B), and EARs\textsubscript{Δ} (C) between conditions. † = significant difference from Control condition.

RH2.2: Relation between cortisol response to exercise and EAR

The associations between AUC\textsubscript{G}\textsubscript{exercise} and EAR\textsubscript{Δ}, EARs\textsubscript{AUCG}, and EARs\textsubscript{AUCI} were tested via ordinary least squares linear regression, using only the data from the exercise condition. There were no significant relations between AUC\textsubscript{G}\textsubscript{exercise} and EARs\textsubscript{AUCG}, ($\beta_{\text{AUCG}\text{exercise}} = 9.8 \times 10^{-4} [95\% \text{CI} = -4.3 \times 10^{-3} \text{ to } 6.3 \times 10^{-3}], p = 0.685, R^2 = 0.019$), EARs\textsubscript{AUCI} ($\beta_{\text{AUCG}\text{exercise}} = 6.1 \times 10^{-4} [95\% \text{CI} = -2.3 \times 10^{-3} \text{ to } 1.1 \times 10^{-3}], p = 0.441, R^2 = 0.067$), or EAR\textsubscript{Δ} ($\beta_{\text{AUCG}\text{exercise}} = 3.6 \times 10^{-4} [95\% \text{CI} = -9.9 \times 10^{-4} \text{ to } 2.7 \times 10^{-4}], p = 0.226, R^2 = 0.158$; Figure 17).
Figure 17. The association between the cortisol response to exercise and indices of the salivary cortisone awakening response: \( \text{EARS}_{\text{AUCG}} \) (A), \( \text{EARS}_{\text{AUCI}} \) (B), and \( \text{EARS}_\Delta \) (C).

**RH2.3: The relation between the indices of the serum cortisol awakening response and indices of the salivary cortisone awakening response.**

The associations between serum cortisol salivary cortisone indices of the awakening response were assessed via mixed-effects models. Only models with random intercepts only converged. There was a significant association between salivary \( \text{EARS}_{\text{AUCG}} \) and \( \text{CARb}_{\text{AUCG}} \) \( (\beta_{\text{EARS}_{\text{AUCG}}} = 3.78 \ [95\% \text{CI} = 2.61 \text{ to } 4.95], \ p < 0.001, \ \text{Conditional } R^2 = 0.834, \ \text{Marginal } R^2 = 0.611) \) and between \( \text{EARS}_{\text{AUCI}} \) and \( \text{CARb}_{\text{AUCI}} \) \( (\beta_{\text{EARS}_{\text{AUCI}}} = 2.32 \ [95\% \text{CI} = 0.22 \text{ to } 4.42], \ p = 0.030, \ \text{Conditional } R^2 = 0.293, \ \text{Marginal } R^2 = 0.170) \). The association between \( \text{CAR}_\Delta \) and \( \text{EARS}_\Delta \) did not reach statistical significance \( (\beta_{\text{EARS}_\Delta} = 1.73 \ [95\% \text{CI} = -0.02 \text{ to } 3.49], \ p = 0.053, \ \text{Conditional } R^2 = 0.240, \ \text{Marginal } R^2 = 0.146; \ \text{Figure 18}) \).
Figure 18. The association between CAR_{\text{AUCG}} (A), CAR_{\text{AUCI}} (B), and EAR (C).

Specific Aim 3

RH3.1: Effect of exercise on HRV (high frequency power) awakening response.

Research hypothesis 3.1 tested the effect of exercise condition on high frequency power during the awakening period. For this research hypothesis, five, five-minute epochs every 15 minutes during the first hour of awakening were first used to calculate HF power. These epochs were initially proposed to be centered around each blood and saliva sample. However, due to unforeseen concerns regarding the HRV signal during the sample collection (due, possibly, to substantial movement and changes to ventilation during the production of the saliva sample), these epochs were instead regressed by 2.5 minutes, so that the five minutes prior to the blood and saliva sample collection was used for each epoch. The HF power in each epoch was log transformed (natural logarithm) prior to analysis (lnHF). Mixed-effect models were used to determine the extent to which there was a linear or quadratic change in lnHF during the waking period. Including a
quadratic term did not significantly improve model fit ($\chi^2(1) = 1.858$, $p = 0.173$) and thus a linear effect of time only was included in the final model. Including the random effect of time did not improve model fit (random intercept only: AIC = 242.41, -2LL = -117.21; random intercept and slope: AIC = 246.37, -2LL = -117.18; $\chi^2(2) = 0.048$, $p = 0.976$). Results demonstrated that there was no significant main effect of time ($\beta_{\text{Time}} = 9.3 \times 10^{-4}$, [95%CI = -5.8 $\times$ 10^{-3} to 7.6 $\times$ 10^{-3}], $p = 0.786$), condition ($\beta_{\text{Condition}} = -0.16$, [95%CI = -0.51 to 0.20], $p = 0.382$), or significant interaction between time and condition ($\beta_{\text{Time*Condition}} = 3.3 \times 10^{-3}$, [95%CI = -1.3 $\times$ 10^{-2} to 6.4 $\times$ 10^{-3}], $p = 0.502$, Conditional $R^2$ = 0.726, Marginal $R^2$ = 0.016). However, when including the effect of condition as a main effect only (i.e., removing the interaction with Condition), there was a significant main effect for condition ($\beta_{\text{Condition}} = -0.26$, [95%CI = -0.47 to -0.05], $p = 0.015$, Conditional $R^2$ = 0.727, Marginal $R^2$ = 0.015), suggesting that while lnHF does not significantly change across the waking period, it is, on average, lower following an exercise session during the prior evening (Figure 19).
RH3.2: The effect of exercise on the HRV response to awakening (HF\textsubscript{AUCG}).

Research hypothesis 3.2 tested the effect of exercise condition on the HRV awakening response, as calculated by the log transformed area under the curve of the HF power epochs (HF\textsubscript{AUCG}). Mixed-effects models with a random intercept were fit (no other random effects structures were identifiable or converged). Results demonstrated no effect for condition on the HF\textsubscript{AUCG} ($\beta_{\text{Condition}} = 0.06$, [95%CI = -0.02 to 0.13], $p = 0.138$, Conditional $R^2 = 0.592$, Marginal $R^2 = 0.042$).

RH3.3: The relation between CAR/EAR derivates and HF\textsubscript{AUCG}

To test the association between HF\textsubscript{AUCG} and all CAR and EAR indices, variables were scaled to [0,1]. First, models were fit with a simple association and then second, fit with an interaction effect for exercise condition. There were no simple associations, main effects of condition, or interaction effects between HF\textsubscript{AUCG} and any serum CAR derivative: CAR\textsubscript{b$_{\Delta}$} ($\beta_{\text{Simple}} = -0.23$, [95%CI = -0.58 to 0.13], $p = 0.215$, Conditional $R^2 = 0.386$, Marginal $R^2 = 0.059$; $\beta_{\text{Condition}} = 0.15$, [95%CI = -0.13 to 0.44], $p = 0.296$; $\beta_{\text{Interaction}}$
= -0.07, [95%CI = -0.76 to 0.63], p = 0.848, Conditional R² = 0.423, Marginal R² =
0.135), CARb_{AUCG}: (β_{Simple} = -0.12, [95%CI = -0.59 to 0.34], p = 0.601, Conditional R² =
0.534, Marginal R² = 0.013; β_{Condition} = 0.32, [95%CI = -0.07 to 0.70], p = 0.106; β_{Interaction}
= -0.41, [95%CI = -1.11 to 0.29], p = 0.252, Conditional R² = 0.585, Marginal R² =
0.081); CARb_{AUCI}: (β_{Simple} = -0.32, [95%CI = -0.72 to 0.08], p = 0.121, Conditional R² =
0.349, Marginal R² = 0.096; β_{Condition} = 0.17, [95%CI = -0.20 to 0.54], p = 0.371; β_{Interaction}
= -0.09, [95%CI = -0.86 to 0.67], p = 0.808, Conditional R² = 0.395, Marginal R² =
0.165).

Similarly, there were no simple associations, main effects of condition, or
interaction effects between HF_{AUCG} and any EAR derivatives: EARs_{Δ} (β_{Simple} = -0.22,
[95%CI = -0.62 to 0.18], p = 0.283, Conditional R² = 0.437, Marginal R² = 0.045;
β_{Condition} = 0.15, [95%CI = -0.17 to 0.48], p = 0.354; β_{Interaction} = -0.20, [95%CI = -1.20 to
0.79], p = 0.689, Conditional R² = 0.466, Marginal R² = 0.065), EARs_{AUCG}: (β_{Simple} =
0.12, [95%CI = -0.32 to 0.57], p = 0.581, Conditional R² = 0.488, Marginal R² = 0.013;
β_{Condition} = 0.10, [95%CI = -0.28 to 0.49], p = 0.605; β_{Interaction} = 0.09, [95%CI = -0.65 to
0.84], p = 0.804, Conditional R² = 0.542, Marginal R² = 0.114); EARs_{AUCI}: (β_{Simple} = -
0.16, [95%CI = -0.51 to 0.19], p = 0.368, Conditional R² = 0.443, Marginal R² = 0.028;
β_{Condition} = 0.06, [95%CI = -0.32 to 0.44], p = 0.756; β_{Interaction} = 0.16, [95%CI = -0.88 to
1.20], p = 0.762, Conditional R² = 0.504, Marginal R² = 0.053).

RH3.4: The relation between HF_{AUCG}, CARb_{AUCI} and EARs_{AUCI} with AUC_{exercise}

To assess the ability of HF_{AUCG}, CARb_{AUCI}, and, EARs_{AUCI} to predict
AUC_{exercise}, ordinary least squares regressions were fit with HF_{AUCG} only, after
covarying for CARb_{AUCI} only, after covarying for EAR_{AUCI} only, and including all three predictors in the model. No combination of HF_{AUCG}, CARb_{AUCI}, and EAR_{AUCI} was significantly associated with AUCG_{exercise} (all coefficients for all models p > 0.05).
CHAPTER V

DISCUSSION

There were several specific aims addressed in this dissertation, each with multiple research questions. In Specific Aim 1, the effect of a laboratory-controlled evening exercise session on the cortisol awakening response the following morning was investigated. Specifically, the extent to which the exercise session altered the dynamics of the cortisol rhythm in the first hour after waking, the association between the exercise-induced cortisol concentration with the CAR and indices of the CAR, and the degree to which salivary and serum CAR indices were related were all tested. It was demonstrated that a) prior evening exercise resulted in a lower cortisol concentration across the awakening period; b) the cortisol output caused by the evening exercise was not significantly associated with the cortisol output during the awakening period; and c) only the salivary CAR index of total cortisol output (CAR_{SAUCG}) was associated with the corresponding serum index of the CAR (CAR_{SAUCG}).

In Specific Aim 2, the extent to which there was a cortisone awakening response (EAR) and the effect of exercise on the cortisone dynamics during the 1-hour post-awakening period and EAR indices, the relation between the exercise-induced cortisol concentration with the EAR, and the relation between the serum indices of the CAR and the EAR were all investigated. It was demonstrated that a) cortisone does exhibit an awakening response similar to cortisol, and evening exercise decreases both the salivary cortisone concentrations and the increase in salivary across the waking period;
b) no indices of the EAR were associated with serum cortisol output during the evening exercise activity; and c) both area under the curve indices of the EAR are associated with the corresponding serum CAR indices.

In Specific Aim 3, the extent to which there was an HRV awakening response and whether this was affected by evening exercise, the relation between the HRVAR and CAR and EAR derivatives, and the association between the HRVAR, CAR, and EAR, and the cortisol output observed during evening exercise were all investigated. It was demonstrated that a) 5-minute epochs of high-frequency power measures do not appear to demonstrate an awakening response during the first-hour post-awakening, but lnHF is significantly lower following an evening exercise session; b) the area under the curve of these epoched HF power measures (HF_{AUCG}) was not significantly altered by evening exercise; c) HF_{AUCG} was not associated with any CAR or EAR metric; and d) combinations of HF_{AUCG}, CAR_{AUCI}, and EAR_{AUCI} did not significantly predict the cortisol output during exercise on the prior evening.

**Contextualizing Biomarker Concentrations**

In the present study, the mean [SD] serum cortisol concentration at midnight (63.32 nmol.l^{-1} [32.00 nmol.l^{-1}]) and in the morning (413.69 nmol.l^{-1} [124.78 nmol.l^{-1}]) under the control condition are in agreement with previously reported concentrations using a similar serum enzyme immunoassay (mean (SD): serum cortisol at midnight: 72 nmol.l^{-1} [5.52 nmol.l^{-1}]; serum cortisol in the morning = 622.90 nmol.l^{-1} [49.1 nmol.l^{-1}]) in a group of 89 adults (Restituto et al., 2008). Regarding the calculated serum CAR indices, to the best of my knowledge, Wilhelm et al. (2007) is the only other investigation
to have measured the CAR in blood, using a similar procedure employed in the current
study. The mean and (standard error of the mean; SEM) reported by Wilhelm and
colleagues (after conversion from μg.dl⁻¹ to nmol.l⁻¹) was 123.59 nmol.l⁻¹ (18.48 nmol.l⁻¹).
This value is comparable, although slightly higher, to the mean (SEM) for CARbΔ in the
present study: 87.90 nmol.l⁻¹ (13.84 nmol.l⁻¹).

The median concentration of cortisol obtained in the saliva samples in this study
was 30.47 nmol.l⁻¹ (interquartile range: 18.78 nmol.l⁻¹ to 40.59 nmol.l⁻¹), which is
marginally higher than the ranges reported by Miller et al. (R. Miller et al., 2013) when
using a similar assay kit from the same manufacturer. However, this is perhaps to be
expected since the present study includes saliva samples from only the post-waking
period when cortisol concentrations are expected to be highest and are closer in
agreement with the peak salivary cortisol concentrations reported elsewhere
(Westermann et al., 2004).

With regards to the calculated CAR indices, CARsΔ values were higher
(mean[SD] = 20.01 nmol.l⁻¹ [13.40 nmol.l⁻¹]) than the average CAR values reported by
Clow et al. (2004). However, it should be noted that there are considerable differences
between the biochemical analyses which can significantly impact calculated salivary
cortisol concentrations (R. Miller et al., 2013). Moreover, the present study used frequent
sampling across the awakening period at precise times relative to awakening, potentially
permitting a more accurate quantification the actual awakening and peak value and
leading to a larger calculated increase. Further, and perhaps as a result of the precise
timing of sampling, 100% of participants exhibited a positive change in salivary cortisol
from awakening, thereby eliminating the inclusion of any negative CARsΔ values in the calculation of average responses. This slightly higher CAR is further reflected in the relative change in cortisol values from awakening to peak (mean[SD] = 119.91% [138.47%]), although this mean value is within the range frequently cited as being a typical CAR (i.e., 50%-150% increase; Clow et al., 2004).

Lastly, the mean (range) of the ratio of salivary cortisone to salivary cortisol has been reported (Debono et al., 2016) to be approximately 6.4 (2.4 to 14.6). A similar calculation of the concentrations in the present study resulted in a slightly lower median ratio (range) of 2.57 (1.21 to 4.81), although it must be noted that the reference ratio in Debono et al. (2016) was calculated from concentrations determined by LC-MS/MS. This is again a consideration when comparing the predicted peak salivary cortisone values from Debono et al. (2016) (median [range]: 27.1 nmol.l-1 [18.0 nmol.l-1 to 45.1 nmol.l-1]) and Perogamvros et al. (2009) (median [range]: 34.6 nmol.l-1 [18.6 nmol.l-1 to 47.0 nmol.l-1]), compared to the mean values in the present study (median [range] = 75.87 nmol.l-1 [12.97 nmol.l-1 to 136.19 nmol.l-1]). However, in general, it appears that the enzyme immunoassays used in the present study had excellent assay performance, and the resultant cortisol and cortisone concentrations are within physiologically reasonable ranges.

Covariates and Sensitivity Analyses

All models reported in Chapter IV are unadjusted models in that they do not include the effects of any covariates. In the proposed methodology, no a priori hypotheses were made regarding covarying for any specific variables. However, the
present data contains numerous potential covariates that can be included in post-hoc models: age, height, body mass, body fat percentage, aerobic fitness level, mean power output and heart rate during the exercise for the experimental visit, Beck Depression Inventory score, four-factor socioeconomic status, questionnaire derived sleep variables such as the Pittsburgh sleep quality score and chronotype (mid-sleep time on free days), as well as wrist-worn actigraphy-derived sleep metrics from the overnight visit (e.g., wake time and sleep efficiency). Some of the relevant models that include select covariates are described below.

As described in Chapter IV and demonstrated in Figure 9, participant CAR008 was the only participant that did not exhibit a robust cortisol response to exercise. As part of the medical history questionnaire, this participant reported using Flonase (active ingredient: fluticasone propionate) to treat seasonal allergies since childhood. The participant agreed to suspend the use of the medication for a week before the experimental visits. The participant was randomized in the first experimental visit to the control condition, in which the participant had a night of relatively disturbed sleep, indicating it was due to allergy-related symptoms. The participant also had disturbed sleep during the second experimental visit (exercise trial) but did not express experiencing allergy-related symptoms. Therefore, it is unclear whether the use of Flonase was suspended before the first, but not second experimental visit, potentially explaining the blunted HPA-axis response to exercise (Schwindt et al., 2010). Curiously, the participant does appear to exhibit the expected diurnal profile. Regardless, given the reported long-term use of Flonase, the participant may have exogenous glucocorticoid-
induced secondary adrenal insufficiency (Priftis et al., 2009), ultimately resulting in the blunted cortisol response to exercise. Therefore sensitivity analyses were completed, wherein all models in each specific aim were refit with the exclusion of this participant. No differences were observed in the direction or significance of any effects, with the exception that all EAR indices became statistically significantly associated with the serum CAR counterpart (i.e., $\text{EAR}_{\Delta} \sim \text{CARb}_{\Delta}$ was statistically significant following the removal of $\text{CAR}008$). Because all other results remained interpretably similar, the results as presented in Chapter IV are discussed below.

The Effect of Exercise on the Dynamics of Cortisol and Cortisone During the Post-Waking Period

In this study, we have demonstrated for the first time that acute, laboratory-based, moderate to high-intensity and moderate duration exercise in the late evening can significantly decrease the cortisol output during the first hour after waking. A recent study reported an increase in the CAR following soccer-specific training completed at 20:00h (Bonato, La Torre, et al., 2020). However, a close reading of this manuscript suggests that the “CAR” assessment was a single salivary sample taken 30 minutes post-waking, and the comparison was made to pre-exercise values at 20:00h; an increase in cortisol concentration is therefore expected based solely on circadian variation. Interestingly, however, and possibly in contrast to the results of the present study, the single morning spot sample was lower after a small-sided game intervention compared to a high-intensity interval training intervention, despite the HIIT invention inducing a greater increase in cortisol concentrations (Bonato, La Torre, et al., 2020). An additional
study examining a late-evening soccer intervention suggests no effect on the CAR (Ucar et al., 2018).

The present results also contrast with the reported relation between the previous day’s training load and the CAR, which demonstrated a positive association between Banister’s training impulse score and CARΔ (Anderson et al., 2018). However, in the present study, a laboratory-controlled exercise bolus was applied to the participants, designed to elicit a robust cortisol response compared to a control condition using a within-subjects design. In contrast, Anderson et al. (2018) assessed free-living exercise participation, wherein participants self-selected the exercise intensity and duration, and analyses were conducted at the between participant level. Thus, it is conceivable that participants who regularly participate in higher-intensity or longer-duration exercise have an increased CAR, while an acute exercise bout that elicits a robust cortisol response results in an acute reduction in the cortisol output the following morning.

The results of this study agree with recent observations of the modification in the cortisol dynamics after waking in overtrained athletes compared to healthy athlete control participants (Anderson, Wideman, et al., 2021). Overtrained athletes displayed a significantly blunted cortisol output, similar to the effect observed in the current study, confirming early observations of morning cortisol output in overtrained athletes (Gouarné et al., 2005) and more recent work observing the effect of progressively intensified swimming training on urinary cortisol in elite athletes (Izov et al., 2020). Importantly, however, these CAR assessments were completed in the overtrained athletes in a basal state, removed from the effects of acute exercise. These results also agree with the
findings of Serpell et al., who reported a progressive blunting of the CAR during a 4-day pre-season rugby training camp (Serpell et al., 2019). However, in contrast, a 7-day soccer training period has previously demonstrated an increase in the CAR despite an approximated 60% increase in training load (Minetto et al., 2008).

Given the results of the present study within the current state of the literature, it appears that the CAR may be positively associated with exercise loads typical for a specific individual but can be significantly reduced given large acute exercise loads or sustained exercise loads above which the individual can reasonably recover (i.e., the process of overreaching).

The Interaction of Exercise, Sleep, and the CAR

A recent investigation demonstrated that a sleep hygiene program resulted in a significantly lower CAR in the morning following a late-night soccer game (Bonato, Merati, et al., 2020), suggesting that acute exercise interacts with sleep-related factors when predicting the CAR. This association between previous day physical activity and sleep duration has been previously reported (Anderson, Corneau, et al., 2021). In general, sleep efficiency in the present study in both the exercise and control conditions was poor, which is to be expected given the novelty of the sleep environment and contacts with researchers during blood sampling. Even so, post-hoc exploratory analyses in the present study suggest that after covarying for sleep efficiency ($\beta_{\text{Efficiency}} = 112.14$, [95%CI = 2.34 to 221.94], $p = 0.045$), there was no longer a significant effect of exercise condition on the serum cortisol output. Despite Anderson et al. (2021) not observing an interaction effect between physical activity and sleep quality as assessed by the number of
awakenings, the authors did observe a negative effect on the CAR when sleep duration and physical activity were high. In further agreement with this observation, when covarying for total sleep duration in the present study, we also find a significant positive effect on serum cortisol ($\beta_{\text{SleepDuration}} = 245.91$, [95%CI = 123.37 to 368.46], $p < 0.001$), while the effect of exercise condition remains negative, although no longer statistically significant ($\beta_{\text{Condition}} = -16.17$, [95%CI = -36.50 to -3.97], $p = 0.116$).

Previous studies suggest that individuals reporting low sleep quality (Tsai et al., 2019) and individuals with insomnia (Backhaus et al., 2004; Castro-Diehl et al., 2015) have a lower CAR. It is, therefore, reasonable to suspect that it is not the exercise per se that is affecting the cortisol dynamics of the following morning. Instead, the impact of acute late-evening exercise on cortisol output is at least partially mediated by sleep quality. Late-evening exercise can impact sleep duration and time in slow-wave sleep (Vein et al., 1991) and late-evening sporting competitions have been demonstrated to impair sleep quality (Eagles et al., 2014; Fullagar et al., 2016; Juliff et al., 2018) and sleep duration and onset (Sargent & Roach, 2016). However, others have suggested that evening or late-night exercise does not in-fact significantly affect overall sleep quality (C. Thomas et al., 2020; Youngstedt et al., 1999). Formal mediation analyses are therefore required to explore this possibility.

Late evening exercise may disrupt sleep through alterations in thermal homeostasis. A decrease in core body temperature is typically initiated before sleep onset and continues to decrease during sleep (Campbell & Broughton, 1994), and the rate of decrease has been suggested to be an indicator of sleep onset (Murphy & Campbell,
Exposure to high ambient temperatures (and presumably the initiation of thermoregulatory mechanisms) before sleep has been demonstrated to affect sleep quality (Shapiro et al., 1989), and evidence suggests that evening exercise will result in elevated nocturnal core body temperatures (O’Connor et al., 1998; Yamanaka et al., 2015). However, more recent studies dispute the notion that evening exercise will disrupt nocturnal (Juliff et al., 2018; D. Miller et al., 2020) or awakening (Juliff et al., 2018) core body temperature. Furthermore, even though O’Connor et al. (1998) clearly demonstrated an increase in nocturnal core temperature with exercise, this did not appear to impair sleep quality. Others have posited that the increase in core body temperature following exercise is followed by a rapid decrease in core body temperature, facilitating a faster sleep transition (Gilbert et al., 2004), although this too has been recently challenged (Youngstedt et al., 2021).

The substantial fluid loss as indicated by changes in nude body mass following exercise suggest that concentrations of arginine vasopressin may also be elevated for an extended period of time following cessation of exercise. This would occur in conjunction with, but not independent from, changes in core body temperature. Arginine vasopressin can act as a co-secretagogue with CRH to stimulate the HPA-axis (Plotsky, 1991), but can also affect sleep independently from cortisol (Born et al., 1992). It is noted that there was an overall reduction in sleep quality following the evening exercise, but, unfortunately, core body temperature and vasopressin were not measured in the present study, and thus its relation to the reduced sleep quality cannot be determined.
The HPA-axis itself however has an intricate and bidirectional association with sleep (Buckley & Schatzberg, 2005). For example, administration of cortisol and ACTH has been demonstrated to significantly reduce time in rapid eye-movement sleep and increases time in slow-wave sleep, compared to a placebo (Born et al., 1989), while sleep itself has an inhibitory effect on cortisol (Weitzman et al., 1983). Nonetheless, the decreased sleep quality following the exercise intervention may be a result of the large exercise-induced cortisol output, and not necessarily a result of changes to core body temperature or hydration status. Of course, both mechanisms could also be occurring simultaneously. A replication of the current study in combination with AVP (or copeptin), core body temperature, and polysomnography measurements may be able to clarify this issue.

**Nocturnal and Awakening Transition Cortisol Profiles**

To further understand the effect of exercise on the nocturnal and post-waking cortisol concentrations, post-hoc exploratory figures were generated (Figure 20). It can be observed in this figure that cortisol concentrations are elevated in the exercise condition until approximately 02:00 when they return to the baseline levels observed in the control condition.
Figure 20. Composite figure demonstrating the mean cortisol values across the control and exercise profiles until awakening (A) and the profiles windowed every hour until 05:00h (B). Note that the y-axis scale in Figure 20B has been allowed to freely vary between hour-by-hour panels.
From 02:00h onwards, the concentration of cortisol continues to stay below baseline concentrations through the early morning, as depicted by the average cortisol concentrations by exercise condition in Figure 21, where time is centered at the point of waking for each individual in each condition. Because this reduced cortisol concentration was mirrored in the salivary cortisol and salivary cortisone concentrations during the waking period, the potential mechanisms driving these effects in RH1.1 and RH2.1 will be discussed below in combination.

![Figure 21. The average concentration of cortisol for the exercise and control conditions, centered at waking for each individual in each condition.](image)

**Mechanisms of Lowered Cortisol Concentrations**

The reduction in cortisol concentrations following acute exercise is not a novel finding. Resting cortisol concentrations have been previously reported to be lower than baseline concentrations 24-hours following a laboratory-based exhaustive exercise...
session (Anderson et al., 2016). This pattern – i.e., initial increases in cortisol concentrations in response to the exercise followed by decreases in resting cortisol concentrations - has also been observed in real-world exercise investigations, with decreases in cortisol concentrations below baseline observed in the days following competition for collegiate American football (W. J. Kraemer et al., 2009), rugby (Cunniffe et al., 2010; Elloumi et al., 2003), marathon (Bae, Kratzsch, et al., 2019) and Ironman races (Hooper et al., 2019; Neubauer et al., 2008), and a functional fitness competition (Tibana et al., 2019). An analysis of this body of work suggests several plausible explanations for the observation of lower cortisol following late-evening exercise.

First, consider that the levels of circulating cortisol are a function of both the rate of appearance (Ra = production and secretion) and the rate of disappearance or clearance (Rd), the latter of which is further a function of the rate of hormone metabolism (R_M) and cellular uptake (R_U). Therefore, decreases in cortisol concentration could result from a decrease in adrenal cortisol synthesis and secretion, increases in metabolic clearance or cellular uptake, or a combination of these factors (Equation 1).

\[
[Cortisol] = R_a - R_d = R_a - (R_M + R_U)
\]

Equation 1. The concentration of circulating steroid is a function of the rate of appearance (Ra) and rate of clearance (Rd), which is influenced by specific hormone metabolism (R_M) and cellular uptake (R_U).

Cortisol is primarily metabolized via hepatic 5α-reductase and 5β-reductase activity and via the renal conversion of cortisol to cortisone via 11β-HSD2 before urinary
excretion. These mechanisms are complex but have been demonstrated to be physiologically adaptive; for example, the elevated cortisol concentrations in critically ill patients compared to healthy controls have been shown to be in part a result of decreased cortisol metabolism (Boonen et al., 2013). As yet, no study investigated whether intense exercise can acutely increase this enzymatic activity in the hours following acute exercise, and thus it remains a conceivable explanation. Differences in the R_d between conditions may be able to be assessed in the current study via deconvolution analysis, similarly to previous investigations (i.e., Kerrigan et al., 1993).

More plausible than an alteration in cortisol metabolism, however, is that there is an increased cellular uptake of cortisol. Cortisol can increase metabolic substrate availability through hepatic gluconeogenesis and inhibiting insulin-related pathways in skeletal muscle (Kuo et al., 2015) and by increasing lipolysis (Djurhuus et al., 2002). We demonstrated a decrease in blood glucose concentration as a result of the exercise session, which required an average energy expenditure of 658.93 kcals. Because the caloric intake was standardized between study conditions for each individual, the caloric difference in substrate availability following the exercise trial relative to the control condition is substantial. Decreases in circulating cortisol may therefore be a result of increased cellular uptake to counteract the disrupted metabolic state. Cellular uptake may also increase to aid in the myofilament catabolism and repair process (Viru & Viru, 2004) via, for example, activation of the transcription factor FOXO (Schakman et al., 2008, 2013).
Cortisol also demonstrates robust anti-inflammatory effects (Beck et al., 2009; Busillo & Cidlowski, 2013; Rhen & Cidlowski, 2005), and thus there may be an increased cellular uptake and receptor binding to counteract any exercise-induced inflammation (Peake et al., 2017). Relatedly, Duclos et al. (2003) have previously demonstrated that acute exercise can increase the sensitivity of monocytes to glucocorticoids, and thus the altered cortisol dynamics may be partly explained by acute or systemic inflammation caused by exercise.

Second, the decrease in cortisol concentration during the early morning and post-waking period could be a function of decreased adrenal cortisol output. The results of this study are remarkably similar to early studies of day-time exercise on nocturnal cortisol output (Kern et al., 1995). Results from Kern and colleagues demonstrated that although average nocturnal cortisol concentrations were not affected by exercise, concentrations in the first half and second halves of the sleep period were higher and lower, respectively, following long-duration moderate intensity cycling compared to a control condition; mirroring the results presented here. The authors of this work postulated that this may have been a result of the exercise-induced cortisol increases having a delayed negative feedback on the HPA-axis, decreasing concentrations in the second half of the night (Kern et al., 1995).

As previously described, the HPA-axis is influenced by multiple negative feedback loops, influencing both the synthesis and secretion of CRH at the level of the hypothalamus and the synthesis and secretion of ACTH at the anterior pituitary (Gjerstad et al., 2018). Therefore, the substantially elevated cortisol concentrations induced by the
exercise intervention may be influencing multiple physiologic levels of the HPA-axis and inducing a “rebound” effect, ultimately resulting in reduced adrenal cortisol output. The feedback loops of the HPA-axis are complex, operating on a number of overlapping time scales, and include rapid and delayed genomic and non-genomic effects (Keller-Wood, 2011). Yet a clear delayed and proportional feedback mechanism in healthy adults has been demonstrated, whereby the decrease in ACTH 75 minutes after exogenous cortisol administration was correlated to the magnitude of the cortisol bolus (Won et al., 1986). Given this, and since the average peak cortisol concentration in the current study occurred at 20:21h, inhibition of ACTH production would not likely be observed until ~21:36h or later, potentially explaining the delayed yet eventual decrease in cortisol concentrations. Additional analyses of the serum samples obtained in the current study for ACTH may help to clarify this mechanism.

This negative feedback and suppression of the HPA-axis has also been proposed by Cunniffe et al. (2010) as an explanation for the reduced basal cortisol concentrations in rugby athletes 38 hours following a competition. Teleologically, the reduction in cortisol output was speculated to permit a more anabolic endocrine milieu, due to the ability of cortisol to inhibit testosterone production by influencing both the hypothalamic-pituitary-gonadal axis and steroidogenesis at the testicular Leydig cells (Daly et al., 2005; Hackney et al., 2017). This should also be investigated further, especially considering evidence of a testosterone awakening response (Kuzawa et al., 2016) that is inverted relative to the CAR (i.e., a sharp decline in testosterone upon awakening).
Thirdly, reduced cortisol secretion may be explained in relation to the role of cortisol as a circadian regulating hormone (Spencer et al., 2018). The substantial increases in circulating cortisol concentrations may result in a dysregulation of peripheral circadian processes (Yamamoto et al., 2005). The adrenal cortex is itself regulated by cortisol exposure, inducing transcription/translation of Per1 (Ota et al., 2012). The highest concentrations of cortisol are typically observed in the period soon after waking. However, in the current study, we have exposed participants to a substantial endogenous glucocorticoid bolus in the late evening, when cortisol concentrations are typically decreasing. This exposure may have disrupted the typical adrenal circadian processes, and thus the early morning period is characterized by a decreased cortisol output.

Fourth, while closely associated with circadian regulation, the present observations may be a result of alterations to sleep architecture. The typical circadian decreases of cortisol observed during the early sleep period occur concomitantly with slow-wave sleep (Buckley & Schatzberg, 2005). A recent meta-analysis has suggested that evening exercise results in a relative reduction in stage 1 sleep and a relative increase in slow-wave sleep (Stutz et al., 2019), consistent with previous reviews that reported increases in slow-wave sleep following acute exercise (Youngstedt et al., 1997). Despite a statistical difference, however, the clinical relevance of these effects has nonetheless been questioned (Stutz et al., 2019), and more recent well-controlled studies suggest that 30 minutes of moderate-intensity cycling 90 minutes before sleep has no effect on slow-wave sleep (D. Miller et al., 2020). Although comparisons between adults and children are made cautiously, evidence does suggest that evening exercise of higher intensity (30
minutes at 85-90% of maximum heart rate) can increase slow-wave sleep in children, but not moderate (30 minutes at 65-70% of maximum heart rate) exercise (Dworak et al., 2008). This intensity effect on slow-wave sleep has been at least partly replicated in inactive and overweight men (Larsen et al., 2019); although, again, these results should be interpreted with caution. These caveats notwithstanding, if the greater physiological load imposed by the present exercise intervention did result in participants spending more time in slow-wave sleep, this would potentially be inhibitory on the HPA-axis and may serve to at least partially explain the decreased cortisol concentrations observed after the early sleep period.

Fifth, the reduced cortisol output following exercise can be contextualized within the framework of the “boost” hypothesis of the CAR. In this hypothesis, increased cortisol concentrations in the post-waking period are hypothesized to increase substrate availability to meet the demands of the upcoming day (Adam et al., 2006). Therefore, it is plausible that a decreased cortisol output during this period is a protective mechanism, whereby reduced cortisol output limits metabolic substrate availability and thereby physiologically compels an individual to reduce metabolic output over the following day, thus conserving resources during the recovery and repair process. However, in disagreement with this hypothesis, recent evidence suggests that the CAR_{AUCI} is not associated with the amount of moderate to vigorous activity that occurs later the same day in children, after controlling for whether the observation occurred on a weekend or weekday (Naya et al., 2021). Since participant physical activity was not monitored
following the experimental condition, this hypothesis cannot be explored further using the current data, although this should be considered in future work.

Within the “boost” hypothesis framework, a decrease in the CAR may also indicate physiological or psychological maladaptation. As previously discussed, a blunted CAR has been previously observed in overtrained athletes (Anderson, Wideman, et al., 2021), but also in individuals suffering from burnout (Oosterholt et al., 2015) and chronic fatigue syndrome (Hall et al., 2014; Nater et al., 2008; Nijhof et al., 2014; Roberts et al., 2004), although some burnout patients have also expressed greater cortisol levels (Grossi et al., 2005). Far from implying that a single exercise bout can induce a long-term and debilitating condition such as burnout or chronic fatigue syndrome, the present results do suggest that a single episode can induce a similar - but likely transitory - blunting effect on the CAR. Repeated exposures to severe stressors of this nature may then result in the effect becoming more permanent. Future studies should continue to monitor participants for multiple days following the exercise intervention to quantify the time required to return to a baseline CAR. Evidence from single timepoint cortisol analyses suggests this should be within 48-hours (Anderson et al., 2016), although the dynamical nature of the CAR may exhibit a more sustained suppression.

It is possible that one or a combination of these factors, or a yet unconceived explanation, is driving these results. Because we found that these effects are present in both blood and saliva, less time-intensive and less invasive replications of this study could be completed, using only salivary sampling. Such studies could then conceivably
include components to answer these outstanding questions, such as polysomnography or multiple days of sampling post-exercise.

**The Effect of Exercise on Indices of the CAR and EAR.**

The absence of any significant differences in any serum CAR indices between conditions is a curious finding, given the effect of exercise observed in the growth model. A potential explanation for this finding is the mathematical derivation of these indices. The mixed-effects growth model fits a quadratic model across time for the observed cortisol or cortisone concentration, with a separate intercept estimated for each individual, and then assesses average differences in cortisol or cortisone concentrations after accounting for this quadratic change across time. In comparison, the CAR indices first summarize the dynamics of the waking hormonal profile and, in the case of AUCG and AUCI, incorporate the effect of time (or exclude time from the calculation entirely, in the case of evenly spaced sampling intervals as completed in the current study) into the metric. While the growth model summarizes the change across time via a continuous quadratic function, the calculation of the AUCG and AUCI indices uses linear associations between each time point to approximate the integral. The differences of these summarized metrics between conditions are then tested, allowing the baseline (i.e., the value of the index in the control condition) to vary between individuals.

These fundamental differences in analysis technique may contribute to the discrepancy in the results, particularly for the CARb\textsubscript{AUCG} metric – which, based on the main effect of condition in the growth model, would be expected to be lower following exercise. However, differences were observed between conditions for salivary CAR and
salivary EAR indices of the awakening response. Therefore, a more plausible explanation is that while exercise appears to affect total cortisol concentrations (as measured in serum), it differentially impacts the free portion of the hormone that is subsequentially quantified in saliva.

According to the free hormone hypothesis, the free portion of the hormone is biologically active, as it is able to freely diffuse into target cells. Most cortisol is bound to carrier proteins (CBG and albumin), with only a small portion of the hormone unbound. Increasing total cortisol concentrations (especially beyond the binding capacities for these carrier proteins), changing the concentration of CBG, and affecting the affinity of CBG for cortisol, will all substantially affect the free portion of the hormone. Although there is some evidence to suggest that the free portion of the hormone demonstrates circadian variation (Anderson, Berry, et al., 2021), alternative explanations are relevant here.

The CBG–cortisol affinity has been demonstrated to be significantly impacted by temperature, whereby temperature increases will reduce the affinity between the hormone and binding protein, consequently increasing the availability of the free portion of the hormone (Cameron et al., 2010). Although not measured in this current study, participants likely experienced a significant increase in core body temperature, indicated by the larger decreases in nude body mass following the exercise trial. In addition, some evidence suggests that late-night exercise can increase core body temperature throughout the nocturnal period (O’Connor et al., 1998), which may subsequently increase the free portion of the hormone. However, not all studies have demonstrated this alteration in
nocturnal core temperature (D. Miller et al., 2020), so this effect of altered core body temperature on free cortisol should be investigated further.

It may also be possible that high exercise loads acutely impacts the concentrations of CBG and thus the free portion of the hormone. Evidence from animal models suggests that acute exercise can reduce concentrations of corticosteroid binding globin in female rats (Tinnikov, 1999) and although a much more physiological severe condition, patients that experience septic shock have a decrease in CBG and albumin of 35% and 29% respectively (Ho et al., 2006). Conversely, Del Corral et al. (2016) demonstrated that an ~18-minute graded exercise test to volitional fatigue was sufficient to increase CBG concentrations by 30% 20 minutes after exercise, replicating previous findings in humans (Crewther et al., 2010) and animal models (Qian et al., 2011).

The increase in CBG has been proposed to act as a buffer to the increased total cortisol output. The half-life of CBG has been reported to be approximately 10 hours (Lewis et al., 2015), thus allowing the possibility that as cortisol concentrations decrease across the sleeping period, a greater proportion becomes bound (i.e., less free hormone), potentially explaining the decrease in salivary CAR and EAR indices. This may also be a physiological rationale for the observed interaction of condition with linear time for salivary cortisol – a decreased rate of cortisol increase in the saliva due to increased binding capacity in the serum, even during an increase in serum total cortisol concentrations. An analysis of serum CBG across the control and exercise conditions may further elucidate this proposed mechanism.
The affinity between CBG and cortisol is also acutely affected by inflammation. Neutrophil elastase, an enzyme secreted from stimulated neutrophils, can cleave CBG and decrease the affinity for cortisol (Bikle, 2021; Perogamvros et al., 2012). With prolonged exercise, lipopolysaccharide-stimulated neutrophil elastase release is reduced, hypothesized to be, coincidently, a result of increased cortisol concentrations in response to exercise and the associated anti-inflammatory actions (Bishop et al., 2003). Similarly, following an international rugby competition, bacterial-stimulated neutrophil elastase release was demonstrated to be acutely reduced (Cunniffe et al., 2010). If the same phenomena occurred in the present study (i.e., reduce neutrophil elastase activity), it is plausible that systemic CBG affinity for cortisol increases, again increasing the net binding capacity and reducing the free hormone increase in the morning following exercise. It is interesting to consider, however, that the systemic effects of this elastase activity may be distinct from local actions, as this mechanism has been proposed as a method of targeting cortisol delivery to specific sites of inflammation (Hammond et al., 1990).

Lastly, to our understanding, this is the first study to explicitly test for and observe a cortisone awakening response (EAR). One previous study also observed an EAR (Bakusic et al., 2019), although the study’s primary purpose was intended to demonstrate the utility of a liquid chromatography-tandem mass spectroscopy (LC-MS/MS) technique for simultaneously quantifying salivary cortisol and cortisone. Even so, the present study is the first to demonstrate the strong association between the EAR and serum CAR metrics, as discussed below.
In sum, the present results demonstrate that the free cortisol component may be a better indicator of physiological strain compared to total cortisol in serum. This is a welcomed finding, as salivary collection is substantially more logistically feasible compared to blood sampling via intravenous catheter. However, additionally analyses, including the quantification of any differences in CBG between trials, should be completed prior to abandoning the possibility that blood-derived factors are less germane for monitoring physiological responses to exercise.

The Association Between Exercise-Induced Cortisol Output and the CAR and EAR

The lack of association between the cortisol output during exercise (AUCG_{exercise}) and any CAR or EAR index was in contrast to the hypothesized relationship. A hypothesized function of the CAR is to “test” the adrenal output capacity upon waking to respond more appropriately to stressful events later in the day. This hypothesis is in part founded on the positive correlation observed between the CAR and adrenal cortisol output induced during pharmacologically-induced maximal adrenal activation via 0.25 mg ACTH (Synacthen) administration (Schmidt-Reinwald et al., 1999). The failure to replicate this finding using endogenous HPA-axis activation provides evidence against this hypothesized model, although some have suggested that there are differences between exogenous and exercise-induced activation of the HPA-axis (Kanaley et al., 2001).

However, it should be noted that the present analysis observed the association between AUCG_{exercise} and the CAR or EAR on the morning following the HPA-axis activation and not a CAR or EAR index that was more temporally independent of the
exercise-induced HPA-axis activation. Because we observed a disruption to the CAR following exercise, it is possible that the association between the CAR and $AUC_{\text{exercise}}$ may be observed if the CAR samples were collected before the exercise session. As part of this study protocol, saliva samples on the evening and morning preceding the experimental visit were also collected. These samples have yet to be analyzed but will further elucidate the association between the CAR and EAR with close to maximal adrenal capacity. Further, the present analyses were conducted using only observations from the exercise condition. The small sample size (i.e., $n = 11$) substantially limits the statistical power of these analyses and thus these interpretations and conclusions are made with caution.

The Association Between Serum Cortisol and Salivary Cortisol and Salivary Cortisone Indices of the CAR

This study demonstrated that the salivary CAR index of total cortisol output (i.e., $CAR_{SAUCG}$) was positively associated with the serum cortisol equivalent (i.e., $CAR_{b_{AUCG}}$). However, indices representing the actual awakening response of cortisol – that is, the change in concentration induced by the process of awakening (i.e., $CAR_A$ and $CAR_{AUCI}$) were not significantly associated between the biological fluids. In contrast, $CAR_{AUCG}$ and $CAR_{AUCI}$ indices in serum were associated with the equivalent index in saliva when calculated using salivary cortisone (i.e., the EAR indices).

Although cortisol concentrations in saliva are independent of flow rate (Riad-Fahmy et al., 1982; Vining et al., 1986), a lag may be introduced due to serum CBG concentration binding characteristics (Schlotz et al., 2008) or through the time course of
converting cortisol to cortisone. To elucidate further the association between serum cortisol and salivary cortisol and salivary cortisone, and to assess whether these associations were a result of a significant lag between the two compartments, cross-correlations with a lag of up to 1 hour were computed (Figure 22).

![Cross-correlations between serum cortisol and salivary cortisol (A) and salivary cortisone (B). Gold dashed lines indicate the approximate 95% confidence interval for the correlation coefficients.](image)

Figure 22. Cross-correlations between serum cortisol and salivary cortisol (A) and salivary cortisone (B). Gold dashed lines indicate the approximate 95% confidence interval for the correlation coefficients.

The cross-correlations demonstrated that the strongest relationship between the biological compartments is at a zero-lag. This effect has also been previously demonstrated for salivary cortisone (Debono et al., 2016), although only at a resolution of
1 hour. We extend these findings to demonstrate that the delay between serum cortisol represented by salivary cortisone is likely <15 minutes. Interestingly, the cross-correlation between serum cortisol and salivary cortisone remains significant out to a 1-hour lag, whereas salivary cortisol remains significantly correlated only at a 45-minute lag. This may be a result of the longer half-life of cortisone in saliva (Perogamvros et al., 2011).

The association between serum cortisol and salivary cortisone has been observed in the past. The rationale for salivary cortisone being a more accurate representation of serum free cortisol (Perogamvros et al., 2011) results from the high activity of the enzyme 11β-HSD2 in the salivary glands, which readily converts cortisol to cortisone as it passes into the salivary fluid. We have demonstrated a positive linear association between serum cortisol and salivary cortisone in the present study, and while serum cortisol and salivary cortisol are also positively related, a quadratic function best describes this association. Although the present biochemical analyses were completed using enzyme-linked immunoassay techniques, the same non-linear association between salivary and serum cortisol has been observed previously using radioimmunoassay (Aardal & Holm, 1995), chemiluminescent enzyme immunoassay (Del Corral et al., 2016), and LC-MS/MS (Perogamvros et al., 2009).

The use of saliva to measure the CAR is preferable due to its non-invasiveness and relative simplicity in the collection (i.e., no phlebotomy requirements). Therefore, such salivary collections can be completed in a wide variety of settings, including one’s home under normal sleep circumstances. Due to the stability of steroids within the saliva,
these samples can be transported to the laboratory without needing rapid storage in ultra-low freezers. The most pertinent question then becomes not whether the CAR measurement should be completed using blood or saliva samples, but rather which steroid – cortisol or cortisone – should be analyzed in the saliva.

We have demonstrated here that two of three EAR metrics are related to their serum counterpart, while only CAR_{SAUCG} was related to the corresponding serum index. To further test whether utilizing the EAR instead of the CAR when assessing salivary awakening responses improves the representation of the associated CAR index measured in the serum, additional post-hoc models were computed. In these models, the serum CAR metric was predicted by the salivary cortisone metric after controlling for the association with the salivary cortisol metric. In this manner, the association of the EAR with the serum CAR was tested above and beyond any association with the salivary CAR metric. In both the CAR_{AUCG} and CAR_{AUCI} models, the inclusion of the salivary CAR metric did not improve the prediction of the serum CAR metric, but the salivary EAR metric remained a significant predictor of the serum CAR even after controlling for the salivary CAR metric.

Thus, the combination of the results described in Chapter IV and these additional post-hoc analyses convincingly demonstrates that if saliva samples are used to quantify the CAR, and the best estimate of the serum CAR_{AUCG} or CAR_{AUCI} is desired, salivary samples should be assayed for cortisone instead of cortisol. This may also be true for CAR_{Δ}, although these models did not reach statistical significance, and as such, we
cannot make this claim. However, as a point of interest, this should also be considered in future work.

**The Heart Rate Variability Awakening Response and the Effect of Exercise.**

In this study, we failed to detect a temporal trend in HRV parameters across the waking period, suggesting that HRV does not express a direct awakening response in the same manner as cortisol or cortisone. It was, however, demonstrated that the lnHF was significantly lower across the waking period following evening exercise.

The lack of an HRV awakening response has been previously reported by (Stalder et al., 2011), who observed a decrease in HF from the pre-awakening to post-awakening period, but no additional changes across the waking period. It was hypothesized in this dissertation proposal that the lack of a continued change across this period of time might have been a result of participants being permitted to freely ambulate after the first 15 minutes, which could have substantially impaired the ability to accurately measure HRV and thus lead to an inability to detect a trend in the HRV response to awakening. In the present study, participants remained supine for the entire 1-hour post waking period in an effort to address this concern. Even so, there was no apparent effect of time during the post-waking period, suggesting that even under controlled postural conditions, HRV does not exhibit an awakening response on the same time scale as the HPA-axis.

As noted, Stalder et al. (2011) reported decreases in HF after waking compared to pre-awakening but no further changes in the post-awakening period. In this study, RR-intervals were continuously recorded across the entire experimental period, permitting the ability to replicate these findings. However, even if these results are replicated, because
of the practically instantaneous response of the ANS to perturbations compared to the relatively leisurely rate of HPA-axis activation and eventual cortisol secretion, it remains possible that HRV does exhibit a response to awakening, but that it occurs on a time-scale that is orders of magnitude faster than the CAR or EAR. This possibility should be investigated further, using short or extremely short RR time series in the peri-awakening period.

Despite the lack of an awakening response, this study did demonstrate a reduction in post-waking HRV following evening exercise, an effect that has been previously demonstrated in soccer players (Ucar et al., 2018). The utility of this assessment window has also been demonstrated through the association of HRV measured immediately after waking with subjective ratings of fatigue in Japanese National Team badminton players (Iizuka et al., 2020). Further, Flatt et al. demonstrated that weekly training load was significantly correlated with log-transformed RMSSD measured soon after waking. This suggests that morning HRV may be a valuable metric for monitoring acute fatigue and help to guide exercise prescription, even in the absence of any discernable awakening response.

However, monitoring HRV during the sleep period has been suggested to be a better alternative to early morning assessments and may be able to provide information on specific sleep stages (D. Herzig et al., 2017). Evening exercise of 90 minutes at moderate intensity has been demonstrated to reduce nocturnal HRV (RMSSD) and increase nocturnal HR, while running for 30 minutes at a vigorous intensity did not affect HRV but did increase nocturnal HR (Myllymäki et al., 2012). Likewise, HRV during the
estimated first slow-wave sleep period has been reported to decrease following only a
short supra-maximal exercise challenge (Haddad et al., 2009). The timing of the exercise
session appears to be relevant as well, as evening exercise can increase nocturnal HR but
have no effect on HRV, whereas morning exercise may increase nocturnal HRV
(Yamanaka et al., 2015). This lack of difference in nocturnal HRV was further
demonstrated when comparing HRV in the sleep period after the highest and lowest
training load in soccer players (Costa et al., 2019). In sum, HRV monitoring during sleep
may yield more relevant information regarding physiological strain and recovery
compared to the sampling methods employed in the current study, but additional research
is needed to clarify this point.

Limitations

Despite the numerous strengths of this study, including the exact quantification of
the exercise load, the randomized cross-over design where each participant serves as their
own control, and the exact timing of awakening and the collection of waking samples,
several limitations should be acknowledged. First, although there are substantial benefits
to a laboratory-controlled environment, the sleep experienced by participants is not
necessarily comparable to the sleep duration or sleep quality one may expect if they were
to sleep in their usual sleep environment. The extensive nature of the current protocol did
not permit additional sleep trials to allow habituation to the laboratory sleep environment.
In addition, even if the sleep environment per se was not disruptive, participants did sleep
with the intravenous line in the forearm, some of whom may have had difficulty sleeping
due to the worry or concern of the catheter itself or participants’ preferred sleeping
positions becoming disrupted during the overnight blood draws. This was reflected in the present study by the relatively low sleep efficiency scores in both trials.

Second, although all attempts were made to limit light exposure to participants during the sleep and awakening periods, some participants did use the bathroom throughout the night and were consequentially exposed to fluorescent lighting. Rodent models have demonstrated an increase in mPer1 RNA (a critical circadian clock gene) after 10 minutes of light exposure during what would typically be dark periods (Shigeyoshi et al., 1997). Even when using the bathroom during typical sleeping hours, no participant was exposed to these artificial light sources for this long and on visual inspection of the data, this did not appear to impact the cortisol profiles obtained significantly. These sleep-laboratory-associated limitations certainly limit the generalizability of these findings to at-home sleep environments; however, due to the randomized cross-over design, these limitations are unlikely to affect the present results.

Third, although wrist-worn accelerometry provides some insight into the nature of sleep quality, we are limited in describing potential sleep-associated mechanisms that may influence neuroendocrine changes. Ideally, polysomnography may elucidate the influence of specific sleep stages, the disruption to these sleep stages caused by exercise, and the eventual effects on endocrine awakening responses. Further, although the present study used volitional sleep offset and began awakening sampling immediately, it is possible that actual sleep offset as defined by polysomnography would occur before this time.
Fourth, the exercise intervention employed in this study was designed to elicit the maximal cortisol response to exercise that was reasonably possible. As such, the exercise intensity was set using an objective standard (i.e., power output) based on maximal capacity measured in a cool environment, but the exercise intervention itself was completed in a hot and humid environment. The addition of this environmental stressor was included to aid in total physiological load and thus maximize cortisol output, but it also resulted in participants not maintaining the prescribed load for the duration of the 1-hour exercise bout. Indeed, the load was reduced for all participants within the first 15 minutes of the protocol. Even though the mean heart rate and cortisol responses suggest that the exercise protocol was effective in achieving the desired aims and all parameters associated with the exercise session are recorded and can be included as covariates in future secondary analyses, the need to modify the load throughout the trial resulted in inter-participant variance in the objective load experienced. Our results demonstrate that perhaps only a 30-minute exercise bout, under the same conditions, is required to maximize circulating cortisol output and might be more reasonably manageable by participants from the same population. Moreover, although we attempted to use a continuous exercise protocol, interval-style protocols may be a more practical solution for participants to maximize total work.

Fifth, food intake was controlled between conditions, requiring participants to consume the same meal between trials, within the same time window. Fluid intake, however, was permitted _ad libitum_, following the nude body mass assessment after exiting the environmental chamber. Given the greater reduction in body mass following
the exercise trial compared to the control condition, one would presume that participants consumed more fluid following the exercise trial than the control trial, although this was not recorded. Due to the influence of hydration on the HPA-axis, there may have been intra- and inter-individual differences here that may have influenced the results. However, *ad libitum* fluid intake does increase the generalizability and improves the practical utility of these findings. Relatedly, the present study used a saline infusion KVO throughout the duration of the experimental visit. Since whole blood was drawn and saline was infused, it was unavoidable that some degree of hemodilution likely occurred. However, the KVO was consistent between trials; it is, therefore, doubtful that this could explain the difference observed between conditions. In future studies, alternative biomarker sampling protocols (i.e., saliva sampling only) could be used to avoid this potential limitation and confirm the present results.

Sixth, an *a priori* power analysis was completed to determine the sample size for this study, based on detecting the effect of condition in the multi-level growth model. However, we used additional model structures to test other research hypotheses, including using only the exercise condition observations in the linear regression model to test the association between the CAR/EAR indices and the cortisol response to exercise. For these research hypotheses, fewer samples were used to fit the models, and thus this study is likely underpowered to detect those associations if they were present. Therefore, those research hypotheses should be studied further, using the variance observed in the current data to calculate and guide the required sample size.
Lastly, all biochemical assessments used in the present study were completed using immunoassays. Recommendations and guidelines in recent years have encouraged the use of liquid chromatography-tandem mass spectroscopy (LC-MS/MS) to quantify steroids (Casals & Hanzu, 2020; Haq et al., 2020; Monaghan et al., 2014). The use of LC-MS/MS avoids common issues with immunoassays, such as cross-reactivity with other steroids. The salivary cortisol ELISAs used in the present study report relatively low cross-reactivity for cortisone (0.8%), and the immunoassay used to quantify salivary cortisone has a reported cortisol cross-reactivity of <0.1%. However, the serum cortisol assay used reports a serum cortisone cross-reactivity of 78%, potentially affecting the strength of the association between serum cortisol and salivary cortisone. However, cortisol concentrations are approximately 4.3-fold greater in serum than cortisone (Debono et al., 2016), and so it is unclear the extent to which this would affect the present analyses. Regardless, future investigations should replicate and contrast the present findings with those obtained using LC-MS/MS.

Conclusions and Future Directions

In this study, we demonstrated that 1) cortisol concentrations are significantly reduced during the awakening period following evening exercise, 2) the free portion of circulating cortisol may be differentially affected by exercise as indicated by effects on the CAR and EAR measured in saliva, and 3) HRV does not exhibit a discernable awakening response over the 1-hour post-awakening period but is significantly decreased the morning after exercise.
Despite the limitations already described, the results of this study have the potential to significantly improve our understanding of the CAR and its utility in monitoring the physiological strain of exercise. Since we demonstrated a similar and perhaps more informative effect in saliva than serum, this further supports the notion that non-invasive biomarkers can be utilized in this capacity. Moreover, we demonstrated that salivary cortisone also exhibits an awakening response and may be preferable to salivary cortisol in future studies and applications of endocrine awakening responses in the exercise sciences.

Currently, wearable technologies are relatively effective at capturing and quantifying the work and load that has already been completed. Moreover, advancements in technology that permit a non-intrusive assessment of recovery processes - such as sleep - are commercially available. However, additional physiological assessments are required to accurately quantify how the interaction of both exercise load and recovery become manifest within an individual and establish their readiness for additional exercise training. In moving towards this vision, it should be a goal of applied exercise science to not only inform an individual of the exercise load they have completed previously or their recovery status but use methods – similar to those described and utilized in this study – to gauge the readiness of the individual to exercise and then proactively recommend and prescribe the appropriate exercise dose. This dynamical periodization is currently being explored but is considered the next evolution in exercise monitoring systems.

Specific to the future applications of the present findings, there have been continual and promising advancements in non-invasive, wearable cortisol monitors (H.-
B. Lee et al., 2020; Sankhala et al., 2020), although there are still many barriers to their current use (Upasham et al., 2021; Zea et al., 2020). Suppose, however, that these wearable cortisol sensors become a viable option for continuous and timestamped cortisol quantification during nocturnal and post-waking periods. In that case, the results of this study may play an essential role in the routine assessment of the CAR (or the EAR, if cortisone sensors prove to be a reasonable alternative) to inform an individual of their current physiological state. This information could then potentially be utilized in a data-driven approach to exercise monitoring and prescription. Of course, the translation of the present results to a technology that uses an alternative biological fluid (i.e., sweat) will require additional considerations of the specific composition of the fluid and thus further research.

An exercise monitoring system that employs non-invasive assessments of HPA-axis and ANS function, therefore, has the potential to be not only descriptive but also prescriptive. This proposed system could be employed by athletes in order to maximize athletic performance or could conceivably also be readily accessible to the general population – thereby further reducing barriers to lifelong exercise adherence and, by doing so, helping to maximize health and wellbeing across the lifespan.

Further, because we demonstrated here a modulation in awakening cortisol and cortisone concentrations, possibly as a result of HPA-axis feedback mechanisms or alterations in the free cortisol concentrations, acute exercise tests may be a feasible option for the detection of some chronic health conditions. The reactivity of the CAR in response to an evening exercise intervention may provide additional information or
context with regards to the functioning of the HPA-axis and may be a more physiologically relevant alternative to reactivity assessed via a test such as a dexamethasone suppression test. Further research is required to extend the findings in healthy participants here to other populations.

To this end, beyond the recommendations made to address the study limitations and open questions, it is clear that if the CAR, EAR, or HRV is to be utilized as a method of monitoring the physiological readiness of an individual each morning and thus lead to improved exercise prescriptions, then the results of this study should also be replicated under more real-world conditions. This study design may take the form of salivary samples collected within an individual’s home under more ordinary sleeping circumstances following a laboratory-controlled exercise session. Alternatively, more real-world exercise training across various exercise modalities could be used as the study intervention rather than laboratory-controlled, cycle ergometer exercise. Future studies should also investigate whether the present results can be replicated when the exercise occurs earlier in the day and further removed from the CAR, EAR, or HRV assessment. Since the present study was delimited to only males of reasonable fitness and normative body composition, these same questions should be explored across various aerobic fitness levels and body compositions (including inactive persons) and certainly within females.
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https://doi.org/10.18637/jss.v067.i01


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### APPENDIX A. ABBREVIATIONS, INITIALIZATIONS, AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUCG</strong>&lt;sub&gt;exercise&lt;/sub&gt;</td>
<td>The area under the curve with respect to a &quot;0&quot; for blood cortisol concentrations from the start of exercise (19:00) to the end of recovery period (20:30). 6 samples.</td>
</tr>
<tr>
<td><strong>CARb&lt;sub&gt;Δ&lt;/sub&gt;</strong></td>
<td>The maximum increase in blood cortisol concentration, from the first sample, to the highest cortisol concentration in the proceeding hour. Calculated as simple difference between these two samples.</td>
</tr>
<tr>
<td><strong>CARb&lt;sub&gt;AUCG&lt;/sub&gt;</strong></td>
<td>The area under the curve with respect to a &quot;0&quot; concentration for blood cortisol concentrations from first awakening sample to the final awakening sample. 5 samples.</td>
</tr>
<tr>
<td><strong>CARb&lt;sub&gt;AUCI&lt;/sub&gt;</strong></td>
<td>The area under the curve with respect to the first sample concentration for blood cortisol concentrations from first awakening sample to the final awakening sample. 5 samples.</td>
</tr>
<tr>
<td><strong>CARs&lt;sub&gt;Δ&lt;/sub&gt;</strong></td>
<td>The maximum increase in salivary cortisol concentration, from the first sample, to the highest cortisol concentration in the proceeding hour. Calculated as simple difference between these two samples.</td>
</tr>
<tr>
<td><strong>CARs&lt;sub&gt;AUCG&lt;/sub&gt;</strong></td>
<td>The area under the curve with respect to a &quot;0&quot; concentration for saliva cortisol concentrations from first awakening sample to the final awakening sample. 5 samples.</td>
</tr>
<tr>
<td><strong>CARs&lt;sub&gt;AUCI&lt;/sub&gt;</strong></td>
<td>The area under the curve with respect to the first sample concentration for saliva cortisol concentrations from first awakening sample to the final awakening sample. 5 samples.</td>
</tr>
<tr>
<td><strong>EARs&lt;sub&gt;Δ&lt;/sub&gt;</strong></td>
<td>The maximum increase in salivary cortisone concentration, from the first sample, to the highest cortisol concentration in the proceeding hour. Calculated as simple difference between these two samples.</td>
</tr>
<tr>
<td><strong>EARs&lt;sub&gt;AUCG&lt;/sub&gt;</strong></td>
<td>The area under the curve with respect to a &quot;0&quot; concentration for saliva cortisone concentrations from first awakening sample to the final awakening sample. 5 samples.</td>
</tr>
<tr>
<td><strong>EARs&lt;sub&gt;AUCI&lt;/sub&gt;</strong></td>
<td>The area under the curve with respect to the first sample concentration for saliva cortisone concentrations from first awakening sample to the final awakening sample. 5 samples.</td>
</tr>
<tr>
<td><strong>HF&lt;sub&gt;AUCG&lt;/sub&gt;</strong></td>
<td>The area under the inverted HF curve with respect to a value of &quot;0&quot; for the five peri- and post-awakening epochs.</td>
</tr>
<tr>
<td><strong>HRVAR</strong></td>
<td>Generalized change in HRV following awakening. Each HRV metric is calculated in the five-minute epoch surrounding each blood sample collection (5, 5-minute epochs).</td>
</tr>
<tr>
<td><strong>AIC</strong></td>
<td>Akieke Information Criteria. Metric to assess model fit. Lower values (closer to 0) indicate improved model fit.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Neural Structure</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>ACC</td>
<td>Anterior cingulate cortex</td>
</tr>
<tr>
<td>AM</td>
<td>Amygdala</td>
</tr>
<tr>
<td>AN</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>APi</td>
<td>Anterior pituitary</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>BF</td>
<td>Basal Forebrain</td>
</tr>
<tr>
<td>dIPFC</td>
<td>Dorsolateral prefrontal cortex</td>
</tr>
<tr>
<td>DMN</td>
<td>Dorsomedial hypothaalmic nucleus</td>
</tr>
<tr>
<td>DMNV</td>
<td>Dorsal motor nucleus of the vagus</td>
</tr>
<tr>
<td>HC</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>Hyp.</td>
<td>Hypothalamus</td>
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<tr>
<td>Insula</td>
<td>Insula</td>
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<tr>
<td>IPFC</td>
<td>lateral prefrontal cortex</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
</tr>
<tr>
<td>LDT</td>
<td>Lateraldorsal tegmental nucleus</td>
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<tr>
<td>LN</td>
<td>Lateral nucleus</td>
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<tr>
<td>LT</td>
<td>Lateral hypothalamus</td>
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<tr>
<td>mPFC</td>
<td>Medial prefrontal cortex</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleus ambiguus</td>
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<tr>
<td>NTS</td>
<td>Nucleus tractus solitarius</td>
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<tr>
<td>OC</td>
<td>Optic chiasm</td>
</tr>
<tr>
<td>OVLTT</td>
<td>Organum vasculosum laminae terminalis</td>
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<tr>
<td>PAG</td>
<td>Periaquedntal gray</td>
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<tr>
<td>PC</td>
<td>Posterior cortex</td>
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<tr>
<td>PCC</td>
<td>Posterior cingulate cortex</td>
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<tr>
<td>PN</td>
<td>Posterior nucleus</td>
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<tr>
<td>PON</td>
<td>Preoptic nucleus</td>
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<tr>
<td>PP</td>
<td>Posterior pituitary</td>
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<td>Pedunculopontine nucleus</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
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<td>Abbreviation</td>
<td>Neural Structure</td>
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<td>--------------</td>
<td>---------------------------------------</td>
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<tr>
<td>Raphe</td>
<td>Raphe nuclei</td>
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<tr>
<td>RVLM</td>
<td>Rostal ventrolateral medulla</td>
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<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
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<td>SFO</td>
<td>Subfornical organ</td>
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<tr>
<td>SON</td>
<td>Supraoptic nucleus</td>
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<tr>
<td>Thal.</td>
<td>Thalamus</td>
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<tr>
<td>TMN</td>
<td>Tuberomammillary nucleus</td>
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<tr>
<td>VLPO</td>
<td>Ventrolateral preoptic nucleus</td>
</tr>
<tr>
<td>VMN</td>
<td>Ventromedial hypothalamic nucleus</td>
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</table>
APPENDIX B. SHARED NEURAL STRUCTURES FOR THE REGULATION OF AWAKENING, CAR, HRV, AND EXERCISE CORTISOL SECRETION.

Neural structures associated with the ascending arousal system, activated during waking.
Neural structures associated with the cortisol awakening response.
Neural structures associated with heart rate variability (Level 2).
Neural structures associated with heart rate variability (Level 3).
Neural structures associated with heart rate variability (Level 4).
Neural structures associated with heart rate variability (Level 5).
Neural structures associated with the cortisol response to exercise.
A composite image and shared neural structures associated with CAR, HRV, ascending arousal system, and cortisol response to exercise.
APPENDIX C. POWER CALCULATIONS AND SIMULATIONS

<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Power (No Effect)</th>
<th>Power (Small/Medium Effect)</th>
<th>Power (Large Effect)</th>
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<td>39.5</td>
<td>97.5</td>
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<td>15.5</td>
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<td>10.5</td>
<td>35.5</td>
<td>96.5</td>
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<td>10</td>
<td>10</td>
<td>38</td>
<td>100</td>
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<td>11</td>
<td>50</td>
<td>94.5</td>
<td>100</td>
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<tr>
<td>12</td>
<td>38.5</td>
<td>23</td>
<td>95.5</td>
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<td>16</td>
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<td>88.5</td>
<td>94.5</td>
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<td>0.5</td>
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<td>95</td>
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<td>82</td>
</tr>
<tr>
<td>20</td>
<td>10.5</td>
<td>99</td>
<td>98.5</td>
</tr>
</tbody>
</table>
APPENDIX D. MEDICAL HISTORY QUESTIONNAIRE

Medical History Form

Participant ID: ___________________________ Date: ___________________________

Occupation: ___________________________ Sex: ___________________________ Age: ___________________________ DOB: ___________________________

PLEASE ANSWER ALL OF THE FOLLOWING QUESTIONS AND PROVIDE DETAILS FOR ALL "YES" ANSWERS IN THE SPACES AT THE BOTTOM OF THE FORM.

YES NO

1. Have you ever had surgery?

2. Are you currently on any medications?

3. Are you currently exercising >10 hours per week?

4. Are you actively attempting to gain or lose body weight?

5. Do you have any known sleep pathologies (e.g., insomnia, restless leg syndrome, etc.)?

6. Have you been diagnosed or treated with any of the following conditions?
   - High blood pressure
   - Kidney Problems
   - Bladder Problems
   - Heart Problems
   - Diabetes
   - Elevated Cholesterol
   - Intestinal Tract Disorders
   - Urinary Tract Disorders

7. What is your ethnicity?
   - Hispanic or Latino or Spanish Origin
   - Not Hispanic or Latino or Spanish Origin

8. What is your race?
   - American Indian or Alaskan Native
   - Native Hawaiian or Other Pacific Islander
   - Asian
   - White
   - Black or African American
   - Other

9. Do you consider yourself as?
   - Sedentary (no exercise)
   - Inactive-occasional light activity (walking)
   - Active-regular light activity and/or occasional vigorous activity (heavy lifting, running, etc.)
   - Heavy Work-regular vigorous activity

10. List your regular physical activities

    Activity: ___________________________ How often do you do it? ___________________________
    How long do you do it? ___________________________ How long ago did you start? ___________________________
    Activity: ___________________________ How often do you do it? ___________________________
    How long do you do it? ___________________________ How long ago did you start? ___________________________
    Activity: ___________________________ How often do you do it? ___________________________
    How long do you do it? ___________________________ How long ago did you start? ___________________________

ADDITIONAL DETAILS: ____________________________________________________________

__________________________________________________________

__________________________________________________________
APPENDIX E. BECK DEPRESSION INVENTORY II

Please pick the statement in each group that best describes the way you have been feeling during the last two weeks, including today. If more than one statement seems to apply equally well, pick the highest number for that group.

1. Sadness
   0  I do not feel sad
   1  I feel sad much of the time
   2  I am sad all of the time
   3  I am so sad or unhappy that I can’t stand it

2. Pessimism
   0  I am not discouraged about my future
   1  I feel more discouraged about my future than I used to be
   2  I do not expect things to work out for me
   3  I feel my future is hopeless and will only get worse

3. Past Failure
   0  I do not feel like a failure
   1  I have failed more than I should have
   2  As I look back, I see a lot of failures
   3  I feel I am a total failure as a person

4. Loss of Pleasure
   0  I get as much pleasure as I ever did from the things I enjoy
   1  I don’t enjoy things the way I used to
   2  I get very little pleasure from the things I used to enjoy
   3  I can’t get any pleasure from the things I used to enjoy

5. Guilty Feelings
   0  I don’t feel particularly guilty
   1  I feel guilty over many things I have done or should have done
   2  I feel quite guilty most of the time
   3  I feel guilty all of the time

6. Punishment Feelings
   0  I don’t feel I am being punished
   1  I feel I may be punished
   2  I expect to be punished
   3  I feel I am being punished
7. **Self-Dislike**  
0 I feel the same about myself as ever  
1 I have lost confidence in myself  
2 I am disappointed in myself  
3 I dislike myself  

8. **Self-Criticalness**  
0 I don’t criticize or blame myself more than usual  
1 I am more critical of myself than I used to be  
2 I criticize myself for all of my faults  
3 I blame myself for everything bad that happens  

9. **Crying**  
0 I don’t cry any more than I used to  
1 I cry more than I used to  
2 I cry over every little thing  
3 I feel like crying, but I can’t  

10. **Agitation**  
0 I am no more restless or wound up than usual  
1 I feel more restless or wound up than usual  
2 I am so restless or agitated that it’s hard to stay still  
3 I am so restless or agitated that I have to keep moving or doing something  

11. **Loss of Interest**  
0 I have not lost interest in other people or activities  
1 I am less interested in other people or things than before  
2 I have lost most of my interest in other people or things  
3 It’s hard to get interested in anything  

12. **Indecisiveness**  
0 I make decisions about as well as ever  
1 I find it more difficult to make decisions than usual  
2 I have much greater difficulty in making decisions than I used to  
3 I have trouble making any decisions  

13. **Worthlessness**  
0 I do not feel I am worthless  
1 I don’t consider myself as worthwhile and useful as I used to  
2 I feel more worthless as compared to other people  
3 I feel utterly worthless
14. Loss of Energy
0  I have as much energy as ever
1  I have less energy than I used to have
2  I don’t have enough energy to do very much
3  I don’t have enough energy to do anything

15. Changes in Sleeping Pattern
0  I have not experienced any change in my sleeping pattern
1  I sleep somewhat more *or* somewhat less than usual
2  I sleep a lot more *or* a lot less than usual
3  I sleep most of the day *or* I wake up 1-2 hours early and can’t get back to sleep

16. Irritability
0  I am no more irritable than usual
1  I am more irritable than usual
2  I am much more irritable than usual
3  I am irritable all the time

17. Changes in Appetite
0  I have not experienced any change in appetite
1  My appetite is somewhat less than usual *or* somewhat greater than usual
2  My appetite is much less than before *or* much greater than usual
3  I have no appetite at all *or* I crave food all the time

18. Concentration Difficulty
0  I can concentrate as well as ever
1  I can’t concentrate as well as usual
2  It’s hard to keep my mind on anything for very long
3  I find I can’t concentrate on anything

19. Tiredness or Fatigue
0  I am no more tired or fatigued than usual
1  I get more tired or fatigued more easily than usual
2  I am too tired or fatigued to do a lot of the things I used to do
3  I am too tired or fatigued to do most of the things I used to do

20. Loss of Interest in Sex
0  I have not noticed any recent change in my interest in sex
1  I am less interested in sex than I used to be
2  I am much less interested in sex now
3  I have lost interest in sex completely
APPENDIX F. CHRONOTYPE QUESTIONNAIRE

Munich ChronoType Questionnaire (MCTQ)

In this questionnaire, you report on your typical sleep behaviour over the past 4 weeks. We ask about work days and work-free days separately. Please respond to the questions according to your perception of a standard week that includes your usual work days and work-free days.

I have a regular work schedule (this includes being, for example, a housewife or househusband):
Yes □ No □
I work on 1□ 2□ 3□ 4□ 5□ 6□ 7□ days per week.

Is your answer “Yes, on 7 days” or “No”, please consider if your sleep times may nonetheless differ between regular ‘workdays’ and ‘weekend days’ and fill out the MCTQ in this respect.

Please use 24-hour time scale (e.g. 23:00 instead of 11:00 pm)!

Workdays
Image 1: I go to bed at _______ o’clock.
Image 2: Note that some people stay awake for some time when in bed!
Image 3: I actually get ready to fall asleep at _______ o’clock.
Image 4: I need _______ minutes to fall asleep.
Image 5: I wake up at _______ o’clock.
Image 6: After _______ minutes I get up.
I use an alarm clock on workdays: Yes □ No □
If “Yes”: I regularly wake up BEFORE the alarm rings: Yes □ No □

Free Days
Image 1: I go to bed at _______ o’clock.
Image 2: Note that some people stay awake for some time when in bed!
Image 3: I actually get ready to fall asleep at _______ o’clock.
Image 4: I need _______ minutes to fall asleep.
Image 5: I wake up at _______ o’clock.
Image 6: After _______ minutes I get up.
My wake-up time (image 5) is due to the use of an alarm clock: Yes □ No □
There are particular reasons why I cannot freely choose my sleep times on free days:
Yes □ if “Yes”: Child(ren)/pet(s) □ Hobbies □ Others □, for example: __________________________
No □

Participant ID: __________________________
MCTQ Core, English, Version 2015-01
©Till Roenneberg & co-workers
Work Details

In the last 3 months, I worked as a shift worker.

No ☐ Yes ☐ (please continue with “My work schedules are…”).

My usual work schedule ...

… starts at _______ o’clock.
… ends at _______ o’clock.

My work schedules are …

… very flexible ☐ … a little flexible ☐ … rather inflexible ☐ … very inflexible ☐

I travel to work …

… within an enclosed vehicle (e.g. car, bus, underground). ☐
… not within an enclosed vehicle (e.g. on foot, by bike). ☐
I work at home. ☐

For the commute to work, I need ___ hours and ___ minutes.
For the commute from work, I need ___ hours and ___ minutes.

Time Spent Outdoors

On average, I spend the following amount of time outdoors in daylight (without a roof above my head):

on workdays: _____ hours _____ minutes
on free days: _____ hours _____ minutes
Stimulants

Please give approximate/average amounts!

<table>
<thead>
<tr>
<th>Activity</th>
<th>per</th>
<th>day / week / month</th>
</tr>
</thead>
<tbody>
<tr>
<td>I smoke ____ cigarettes ...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I drink ____ glasses of beer ...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I drink ____ glasses of wine ...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I drink ____ glasses of liquor/whiskey/gin etc. ...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I drink ____ cups of coffee ...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I drink ____ cups of black tea ...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I drink ____ cans of caffeinated drinks (soft-drinks) ...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I take sleep medication ____ times ...</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous and moderate activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?
   □ Yes
   □ No  ➔  Skip to PART 2: TRANSPORTATION

The next questions are about all the physical activity you did in the last 7 days as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work?
   Think about only those physical activities that you did for at least 10 minutes at a time.
   ___ days per week
   □ No vigorous job-related physical activity  ➔  Skip to question 4

3. How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?
   ___ hours per day
   ___ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads as part of your work? Please do not include walking.
   ___ days per week
   □ No moderate job-related physical activity  ➔  Skip to question 6

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.
5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?

___ hours per day
___ minutes per day

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.

___ days per week
☐ No job-related walking ➔ Skip to PART 2: TRANSPORTATION

7. How much time did you usually spend on one of those days walking as part of your work?

___ hours per day
___ minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?

___ days per week
☐ No traveling in a motor vehicle ➔ Skip to question 10

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?

___ hours per day
___ minutes per day

Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the last 7 days, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?

___ days per week
☐ No bicycling from place to place ➔ Skip to question 12
11. How much time did you usually spend on one of those days to bicycle from place to place?
   
   _____ hours per day
   _____ minutes per day

12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?
   
   _____ days per week

   ☐ No walking from place to place

   → Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

13. How much time did you usually spend on one of those days walking from place to place?
   
   _____ hours per day
   _____ minutes per day

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shoveling snow, or digging in the garden or yard?
   
   _____ days per week

   ☐ No vigorous activity in garden or yard

   → Skip to question 16

15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?
   
   _____ hours per day
   _____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?
   
   _____ days per week

   ☐ No moderate activity in garden or yard

   → Skip to question 18
17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?

___ hours per day
___ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?

___ days per week

☐ No moderate activity inside home ➔ Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY

19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?

___ hours per day
___ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?

___ days per week

☐ No walking in leisure time ➔ Skip to question 22

21. How much time did you usually spend on one of those days walking in your leisure time?

___ hours per day
___ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

___ days per week

☐ No vigorous activity in leisure time ➔ Skip to question 24

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.
23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?
   
   _____ hours per day
   _____ minutes per day

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?

   _____ days per week
   ☐ No moderate activity in leisure time  ➡️ Skip to PART 5: TIME SPENT SITTING

25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?

   _____ hours per day
   _____ minutes per day

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?

   _____ hours per day
   _____ minutes per day

27. During the last 7 days, how much time did you usually spend sitting on a weekend day?

   _____ hours per day
   _____ minutes per day

This is the end of the questionnaire, thank you for participating.

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.
APPENDIX H. PITTSBURG SLEEP QUALITY INDEX QUESTIONNAIRE

Name: ____________________________ Date: ______________

Pittsburgh Sleep Quality Index (PSQI)

Instructions: The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. **Please answer all questions.**

1. During the past month, what time have you usually gone to bed at night? ______________

2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night? __________

3. During the past month, what time have you usually gotten up in the morning? ______________

4. During the past month, how many hours of **actual sleep** did you get at night? (This may be different than the number of hours you spent in bed.) ______________

5. During the past month, how often have you had trouble sleeping because you...

<table>
<thead>
<tr>
<th></th>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Cannot get to sleep within 30 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Wake up in the middle of the night or early morning</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. Have to get up to use the bathroom</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d. Cannot breathe comfortably</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e. Cough or snore loudly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f. Feel too cold</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>g. Feel too hot</td>
<td></td>
<td></td>
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<tr>
<td>h. Have bad dreams</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Have pain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>j. Other reason(s), please describe:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6. During the past month, how often have you taken medicine to help you sleep (prescribed or “over the counter”)?

7. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

<table>
<thead>
<tr>
<th></th>
<th>No problem at all</th>
<th>Only a very slight problem</th>
<th>Somewhat of a problem</th>
<th>A very big problem</th>
</tr>
</thead>
</table>

8. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?

<table>
<thead>
<tr>
<th></th>
<th>Very good</th>
<th>Fairly good</th>
<th>Fairly bad</th>
<th>Very bad</th>
</tr>
</thead>
</table>

9. During the past month, how would you rate your sleep quality overall?

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10. Do you have a bed partner or room mate?</td>
<td>No bed partner or room mate</td>
<td>Partner/room mate in other room</td>
<td>Partner in same room but not same bed</td>
<td>Partner in same bed</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-----------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>Not during the past month</td>
<td>Less than once a week</td>
<td>Once or twice a week</td>
<td>Three or more times a week</td>
</tr>
</tbody>
</table>

If you have a room mate or bed partner, ask him/her how often in the past month you have had:

- Loud snoring
- Long pauses between breaths while asleep
- Legs twitching or jerking while you sleep
- Episodes of disorientation or confusion during sleep
- Other restlessness while you sleep, please describe:
APPENDIX I. SOCIOECONOMIC BACKGROUND SURVEY (HOLLINGSHEAD INDEX)

Please describe the primary occupation of your father (if currently retired, then indicate their most recent occupation before retirement). Please include occupation and setting e.g. “teacher, secondary school” or “senior manager, pharmaceutical company”.

Please indicate the highest level of education your father completed:
- Advanced degree (Ph.D., law, medicine, veterinary school)
- Master’s degree
- Some graduate or professional school
- College graduate (4 year degree)
- College graduate (2 year degree)
- Part college or post high school training
- High school graduate
- Part high school
- Elementary (grammar) school graduate
- Part elementary (grammar) school

Please describe the primary occupation of your mother (if currently retired, then indicate their most recent occupation before retirement). Please include occupation and setting e.g. “teacher, secondary school” or “senior manager, pharmaceutical company”.

Please indicate the highest level of education your mother completed:
- Advanced degree (Ph.D., law, medicine, veterinary school)
- Master’s degree
- Some graduate or professional school
- College graduate (4 year degree)
- College graduate (2 year degree)
- Part college or post high school training
- High school graduate
- Part high school
- Elementary (grammar) school graduate
- Part elementary (grammar) school
APPENDIX J. ACCELEROMETER LOG

ID: ___________________

ACCELEROMETER LOG
While wearing the accelerometer, please note any points in which you removed the accelerometer (example: while showering). Please record, as precisely as possible, what time you went to sleep, and what time you woke up. If you woke up during the night for extended periods (>10mins) please also record these wake and sleep times. If you were in bed prior to sleeping or after waking, please only record the times that you fell asleep or awoke, respectively.

<table>
<thead>
<tr>
<th>Date</th>
<th>What time did you wake up?</th>
<th>What time did you go to sleep?</th>
<th>Please note any times you removed the accelerometer, and a brief description of why.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example: 01/16/19</td>
<td>6:15am</td>
<td>11:05pm</td>
<td>8:30-8:43pm - shower</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX K. DIETARY LOG

DIETARY FOOD RECORD INSTRUCTIONS

Please record dietary intake for every day until your next visit.

- **All** foods and beverages consumed should be recorded.

- **Be very specific.** Make sure you include:
  - the **type** of food/beverage
  - the **amount** of each food/beverage
  - the **preparation method** (i.e., fried, baked)
  - the **brand name** of the food (if applicable)
  - the **time** it was eaten
  - the restaurant you ate it at (i.e., Subway, Applebees, Red Robin)

- Record food/beverage consumption **after each meal/snack** instead of waiting until the end of the day.

- **Save labels** from packages of food you eat and return them with your food record forms (this will greatly assist and enhance our analysis of your true nutrient intake).

- Use nutrient descriptors (e.g., low-fat, low-carb, fat-free, light, reduced calorie, etc.).

- **Include miscellaneous items** such as condiments (ketchup, salad dressing, mayonnaise, jams, creams, sugar), and chewing gum.

- Refer to the serving size pictures if you are unsure of the portions when eating away from home.

- If you are unsure how to record something, please note this and discuss with the dietitian at your nutrition meeting.
<table>
<thead>
<tr>
<th>Time</th>
<th>Detailed food/beverage description: brand name, restaurant, method of preparation, flavor, condiments, etc.</th>
<th>Amount</th>
<th>Calories (if known; from food label? Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:30 am</td>
<td>Oatmeal</td>
<td>1 bowl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low fat milk</td>
<td>8 oz</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Omelette with ham and cheddar cheese</td>
<td>3 eggs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Orange juice</td>
<td>12 oz</td>
<td></td>
</tr>
<tr>
<td>11:30 am</td>
<td>Turkey sandwich</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regular chips</td>
<td>1 bag</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tomato soup</td>
<td>1 ½ cups</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dunkin Donuts Coffee</td>
<td>medium</td>
<td></td>
</tr>
<tr>
<td>2:00 pm</td>
<td>Yogurt</td>
<td>1 container</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Granola</td>
<td>Handful</td>
<td></td>
</tr>
<tr>
<td>6:20 pm</td>
<td>Salad</td>
<td>1 large</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chicken breast chunks</td>
<td>Large handful</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frozen mixed vegetables</td>
<td>1 cup</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red wine</td>
<td>1 glass</td>
<td></td>
</tr>
<tr>
<td>9:05 pm</td>
<td>Popcorn</td>
<td>2 cups</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ice cream</td>
<td>¾ cup</td>
<td></td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>1 bottle</td>
<td></td>
</tr>
</tbody>
</table>

Comments:
<table>
<thead>
<tr>
<th>Time</th>
<th>Detailed food/beverage description: brand name, restaurant, method of preparation, flavor, condiments, etc.</th>
<th>Amount</th>
<th>Calories (if known; from food label? Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:30 am</td>
<td>Regular oatmeal- Quick 1 minute Quaker oats made with 2 cups water</td>
<td>1 cup</td>
<td>dry</td>
</tr>
<tr>
<td></td>
<td>Hood LightBlock 1% lowfat milk, vitamins A, C, &amp; D (fortified)</td>
<td>8 oz</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Omelette with 2 Tbsp Hillshire Farm diced ham and 2 oz Cabot 50% reduced fat sharp cheddar cheese</td>
<td>3 whole</td>
<td>eggs (large)</td>
</tr>
<tr>
<td></td>
<td>Tropicana Pure Premium orange juice with calcium &amp; vitamin D</td>
<td>12 oz</td>
<td></td>
</tr>
<tr>
<td>11:30 am</td>
<td>Turkey sandwich: Arnold 100% whole wheat bread Butterball extra thin sliced honey roasted smoked turkey</td>
<td>2 slices</td>
<td>7 slices</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Tbsp</td>
<td>1 leaf</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 leaf</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lays classic potato chips</td>
<td>1 oz</td>
<td>bag</td>
</tr>
<tr>
<td></td>
<td>Campbell’s Select soup-Tomato Garden flavor</td>
<td>1 ½ cups</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dunkin Donuts Medium Coffee made with cream, 1 splenda packet</td>
<td>14 oz</td>
<td></td>
</tr>
<tr>
<td>2:00 pm</td>
<td>Dannon fruit on the bottom yogurt- strawberry</td>
<td>1- 6 oz</td>
<td>container</td>
</tr>
<tr>
<td></td>
<td>Bear Naked Banana Nut flavored Granola</td>
<td>¼ cup</td>
<td></td>
</tr>
<tr>
<td>6:20 pm</td>
<td>Salad: 2 cups romaine lettuce medium red tomato cucumber Pepperidge Farm Zesty Italian croutons, Newman’s Own Ranch dressing</td>
<td>1 large</td>
<td>3 slices</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 slices</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Tbsp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tyson premium chunk chicken breast (canned chunks)</td>
<td>4 oz.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mixed frozen vegetables (Bird’s Eye broccoli, cauliflower, and carrots) boiled in salt (1t) water then sautéed in 1 Tbsp Stonehouse California olive oil</td>
<td>1 cup</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RED wine, Yellow Tail Australian Merlot</td>
<td>9-10 oz.</td>
<td></td>
</tr>
<tr>
<td>9:05 pm</td>
<td>Act II light butter popcorn</td>
<td>2 cups</td>
<td>popped</td>
</tr>
<tr>
<td></td>
<td>Hood regular ice cream- cookies n’ cream</td>
<td>3/4 cup</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dasani water</td>
<td>500 mL</td>
<td>bottle</td>
</tr>
</tbody>
</table>

Comments:
<table>
<thead>
<tr>
<th>Time</th>
<th>Detailed food/beverage description: brand name, restaurant, method of preparation, flavor, condiments, etc.</th>
<th>Amount</th>
<th>Calories (if known; from food label? Y/N)</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

**Comments:** Note: Each participant will receive seven copies of this form.
WHAT DO YOU DRINK?
Liq.In⁷ record

NAME OF INTERVIEWER / INVESTIGATOR: ____________________________

DATE OF PLACEMENT: ____________________________

SAMPLE POINT: ____________________________

RESPONDENT ID / STUDY ID: ____________________________

RESPONDENT DAY OF BIRTH: __/__/____

RESPONDENT GENDER: MALE FEMALE

REMEMBER:
At any moment during this study, if you have any doubt/question on any aspect, please feel free to contact ________ by phone ________ or via email ________.
INSTRUCTIONS FOR COMPLETING THE DIARY

Thank you very much for participating in our study. We would like to ask you to record in detail everything you drink in the coming 7 days. For this purpose we have prepared "a fluid diary" for you. The completion of this diary won’t take you more than a few minutes each day.

To assist you with the completion of this diary, more information is provided below. Please read the instructions carefully before starting to complete the diary.

When completing in this diary, please note that:

• The diary is composed of 7 recording pages, ONE page for each day.

• At any moment of the day and night, please record about ALL the beverages you drank, regardless the time of day or whether it was at home or not.

• For each moment of the day you can record several beverages. Each beverage must be recorded on a separate line.

• Please fill in your diary regularly throughout the day - if possible - without leaving it until the end of the day. Otherwise, there is a risk that you may forget a few important details. It is very important that you record EVERY beverage that you have consumed.

HOW TO FILL THE BEVERAGES DIARY?

• For each day, please write the date of recording in the blanks provided at the top of the age (e.g. date: 04/03/2014)

• Each consumed beverage should be entered on a separate line. If 3 lines for one moment of the day is not sufficient, you may use the lines of the following moment of the day.

• For each beverage consumed, please record the following information:

A. TIME: report the actual time of consumption using 24 hour clock (e.g. 14h35 or 2PM35)

B. MOMENT OF DAY: this indicates the moment of the day you had this beverage. Please consider every moment during the day or night when you drank something

C. WHAT: enter the code of the fluid type you had. A detailed list of the different types of fluid can be found below on the page above the recording page. Please include all types of beverages eg. juice, water, tea, beer, wine. Note there are several options for some types of beverages eg bottled water, tap water, water from a cooler etc.

D. BEVERAGE BRAND: if known, enter the brand name and/or flavour of the beverage in column D

E. WHICH CONTAINER: to assist you in recording this information, you have at your disposal a concise photo board above each recording page, as well as a more extensive one at the end of this booklet (page 18). Enter the code of the container from which you drank the fluid entered in columns C and D. If you can’t find the specific container, record a code resembling the volume of container you drank from

F. HOW MUCH: tick of the amount you drunk of this fluid example: if you drank one full can of cola, please tick of all of it. If you drank only half of the can of cola, please tick of half of it. If you drank more than 1 can, write on a separate line the 2nd can.

G. ADDITIONS: tick of all the additives you added to this fluid. If you did not add anything, please select the answer “no additives”

H. WHERE: tick of where you consumed this drink

THANK YOU FOR YOUR COOPERATION!!
<table>
<thead>
<tr>
<th>Column C: What?</th>
<th>WATER Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>1</td>
</tr>
<tr>
<td>Water from water cooler/Fountain</td>
<td>2</td>
</tr>
<tr>
<td>Bottled non-carbonated water</td>
<td>3</td>
</tr>
<tr>
<td>Bottled carbonated water</td>
<td>4</td>
</tr>
<tr>
<td>Flavoured non-carbonated bottled water</td>
<td>5</td>
</tr>
<tr>
<td>Flavoured carbonated bottled water</td>
<td>6</td>
</tr>
<tr>
<td>HOT DRINKS Code</td>
<td></td>
</tr>
<tr>
<td>Coffee</td>
<td>7</td>
</tr>
<tr>
<td>Hot chocolate, Cacao, Cappuccino</td>
<td>8</td>
</tr>
<tr>
<td>Tea</td>
<td>9</td>
</tr>
<tr>
<td>Herbal/Fruit Tea (e.g. Green tea)</td>
<td>10</td>
</tr>
<tr>
<td>Other hot drink</td>
<td>11</td>
</tr>
<tr>
<td>MILK DRINKS Code</td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>12</td>
</tr>
<tr>
<td>Flavoured Milk</td>
<td>13</td>
</tr>
<tr>
<td>Drinking yoghurt</td>
<td>14</td>
</tr>
<tr>
<td>Smoothies (with Milk/Yoghurt)</td>
<td>15</td>
</tr>
<tr>
<td>Other milk drinks</td>
<td>16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Column D: Which container?</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Glass/ tumbler/ beaker (excl. wine glass)</th>
<th>Wine glass</th>
<th>Plastic/ Foam/ paper cup (cold beverages)</th>
<th>Plastic/ Foam/ paper cup (hot beverages)</th>
<th>Teacup/ mug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Portion Size:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Code:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small glass 175 ml 6 oz</td>
<td>0103</td>
<td>0105</td>
<td>0110</td>
<td>0008</td>
</tr>
<tr>
<td>Glass 300 ml 10 oz</td>
<td></td>
<td>0009</td>
<td>0011</td>
<td>0211</td>
</tr>
<tr>
<td>Large glass 475 ml 16 oz</td>
<td></td>
<td>0212</td>
<td>0204</td>
<td>0209</td>
</tr>
<tr>
<td>220 ml 8.7 oz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Small' at McDonald 473 ml 16 oz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Medium' at McDonald 710 ml 24 oz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Large' at McDonald 946 ml 32 oz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium (=&quot;Tall&quot; at Starbucks) 350 ml 12 oz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large (=&quot;Grande&quot; at Starbucks) 470 ml 16 oz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extra large 67.9 oz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal mug 250 ml 10 oz</td>
<td>0304</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For an exhaustive list of containers, see page 18 of this booklet.

<table>
<thead>
<tr>
<th>Column E: Which container?</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1101</td>
<td>1111</td>
<td>1405</td>
<td>1416</td>
<td>1209</td>
<td>1307</td>
<td>1312</td>
<td>1318</td>
<td>0904</td>
<td>1003</td>
</tr>
</tbody>
</table>
**RECORDING PAGE DAY 1**

Enter the date: __/__/20__

Respondent ID: ______________________

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME</td>
<td>MOMENT OF DAY</td>
<td>WHAT</td>
<td>BEVERAGE BRAND, SPECIFICATIONS OR FLAVOR</td>
<td>WHICH CONTAINER</td>
<td>HOW MUCH</td>
<td>ADDITIONS</td>
<td>WHERE</td>
</tr>
<tr>
<td>Enter time of consumption</td>
<td>The moment of day you had this fluid</td>
<td>Enter the code type of fluid</td>
<td>Enter the exact brand of the product mentioned in C</td>
<td>See photo board and enter the code for the container from which you drank</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2:30 PM</th>
<th>Example</th>
<th>3</th>
<th>Desani</th>
<th>1212</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>After wake up, Before breakfast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breakfast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In the morning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lunch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In the afternoon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinner</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In the evening</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: All participants will receive seven copies of this page.
Details for Column E.  

*What was the size of the container / bottle from which you drank?*

In this section you can find an extensive photo board of containers/receptacles of different sizes. Based on the container you select (column E) and the amount you consumed (column F), the total amount consumed of a beverages will be estimated.

**HOW TO USE THE PHOTO BOARD?**

1. Look for the page of a specific type of container/receptacle

<table>
<thead>
<tr>
<th>Type of container</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass cups</td>
<td>19</td>
</tr>
<tr>
<td>Wine/Stem glasses</td>
<td>20</td>
</tr>
<tr>
<td>Mugs</td>
<td>21</td>
</tr>
<tr>
<td>Disposable glass (plastic/foam/paper)</td>
<td>22</td>
</tr>
<tr>
<td>Cups</td>
<td>23</td>
</tr>
<tr>
<td>Bowls</td>
<td>24</td>
</tr>
<tr>
<td>Tetra pack containers</td>
<td>25</td>
</tr>
</tbody>
</table>

2. Look for the applicable size or the approximate of the one you used.
   The pencil can serve as a size reference.
   *If the receptacle from which you drank the product is not listed, please look for a size, independently of the container type, comparable to the one you used.*

3. Enter the container/receptacle size code where you drank the beverage in the recording sheet.

For example, if at breakfast you drank *still water* in a *glass*, you will go to *page 19* and will look for the applicable glass size. Suppose it was a *250ml* one, you will Record code “0104” in the column E.
GLASS CUPS

30 ml. 1 oz. (0101)  
44 ml. 1.4 oz. (0102)  
175 ml. 6 oz. (0103)  
240 ml. 8.1 oz. (0104)  
300 ml. 10.1 oz. (0105)  
325 ml. 10.9 oz. (0106)

350 ml. 11.8 oz. (0107)  
380 ml. 12.8 oz. (0108)  
450 ml. 15.2 oz. (0109)  
475 ml. 16 oz. (0110)
Wine glasses

74 ml. 2.5 oz (0501)
130 ml. 4.3 oz (0502)
175 ml. 5.9 oz (0503)
150 ml. 5 oz (0504)
180 ml. 6 oz (0505)
220 ml. 8.7 oz (0506)
473 ml. 16 oz (0507)
500 ml. 16.9 oz (0508)
Mugs

354 ml.  
11.8 oz.  
(0601)

475 ml.  
16 oz.  
(0602)

1 Lt  
35.2 oz.  
(0603)
Disposable cup
(Plastic / foam / paper)

90 ml 3.2 oz. (0201)
148 ml 5 oz. (0202)
207 ml 6.9 oz. (0203)
240 ml 8.1 oz. (0204)
266 ml 8.9 oz. (0205)
Vaso bajo - 266 ml 8.9 oz. (0206)
296 ml 10 oz. (0207)

355 ml 11.9 oz. (0208)
473 ml 15.9 oz. (0209)
591 ml 19.9 oz. (0210)
710 ml 23.9 oz. (0211)
946 ml 31.9 oz. (0212)
Cups

50 ml
1.6 oz.
(0301)

100 ml
3.3 oz.
(0302)

180 ml
6 oz.
(0303)

250 ml
10.1 oz.
(0304)

300 ml
10.1 oz.
(0305)

450 ml
15.2 oz.
(0306)

500 ml
16.9 oz.
(0307)
Bowls

300 ml
10.1 oz.
(0401)

450 ml
15.2 oz.
(0402)

600 ml
20.2 oz.
(0403)
Tetra Pack containers

200ml
6.7 oz.
(1101)

220ml
7.4 oz.
(1102)

236ml
7.9 oz.
(1103)

240ml
7.9 oz.
(1104)

250 ml
8.4 oz.
(1105)

310 ml
10.4 oz.
(1106)

500 ml
16.9 oz.
(1107)

750 ml
25.3 oz.
(1108)

946 ml
31.9 oz.
(1109)

950 ml
32.1 oz.
(1110)

1 Lt
33.8 oz.
(1111)

1.892 Lt
63.9 oz.
(1112)
Glass containers

- 237 ml (8 oz.) (1201)
- 245 ml (ej: cuartito) (8.2 oz.) (1202)
- 250 ml (1203)
- 255 ml (8.6 oz.) (1204)
- 275 ml (9.2 oz.) (1205)
- 281 ml (9.4 oz.) (1206)
- 300 ml (ej: media) (10.1 oz.) (1207)
- 325 ml (10.9 oz.) (1208)
- 330 ml (11.1 oz.) (1209)
- 333 ml (11.2 oz.) (1210)
- 354 ml (11.8 oz.) (1211)

- 355 ml (11.9 oz.) (1212)
- 413 ml (13.9 oz.) (1213)
- 450 ml (15.2 oz.) (1214)
- 470 ml (15.8 oz.) (1215)
- 473 ml (15.9 oz.) (1216)
- 500 ml (16.9 oz.) (1217)
- 750 ml (25.3 oz.) (ej: caguama) (1218)
- 1 litro (33.8 oz.) (1219)
- 1.25 lt (42.2 oz.) (1220)
Plastic containers
(1 of 3, continue in the next page)

300 ml
10.1 oz.
(1301)

330 ml
11.1 oz.
(1302)

355 ml
11.9 oz.
(1303)

400 ml
13.5 oz.
(1304)

450 ml
15.2 oz.
(1305)

473 ml
15.9 oz.
(1306)

500 ml
16.9 oz.
(1307)

600 ml
20.2 oz.
(1308)

710 ml
23.9 oz.
(1309)

750 ml
25.3 oz.
(1310)

946 ml
31.9 oz.
(1311)

1 Lt
33.8 oz.
(1312)

1.280 Lt
43.2 oz.
(1313)
Plastic containers
(2 of 3, continue in the next page)

80 grs
3.0 oz
(1333)

100 grs
3.7 oz
(1334)

110 grs
3.7 oz
(1335)

116 ml
3.9 oz
(1336)

150 ml / gr
5 oz
(1337)

170 ml / grs
5.7 oz
(1338)

215 ml / grs
7.2 oz
(1339)

220 ml
7.4 oz
(1340)

245 ml / grs
8.2 oz
(1341)

250 ml / grs
8.4 oz
(1342)

300 ml
10.1 oz
(1343)

330 ml
11.1 oz
(1344)

450 ml / grs
15.2 oz
(1345)

495 ml / grs
16.7 oz
(1346)

500 ml / grs
16.9 oz
(1347)
Familiar plastic containers

(3 of 3)
Thermos

350 ml
11.8 oz.
(0901)

450 ml
15.2 oz.
(0902)

500 ml
16.9 oz.
(0903)

550 ml
18.5 oz.
(0904)

1 Lt
33.8 oz.
(0905)
Canteen

- 400 ml.
  - 13.52 oz.
  - (1001)
- 500 ml.
  - 16.9 oz.
  - (1002)
- 600 ml.
  - 20.28 oz.
  - (1003)

- 750 ml.
  - 25.4 oz.
  - (1004)
- 1 l.
  - 33.8 oz.
  - (1005)
APPENDIX M. ADHERENCE QUESTIONS

Screening Form

Participant ID: ____________________________ Date: ____________________________
Condition: ____________________________ Age: ____________ DOB: ____________________________
Height: ____________________________
Weight: ____________________________
Food order: ____________________________

PLEASE ANSWER ALL OF THE FOLLOWING QUESTIONS AND PROVIDE DETAILS FOR ALL "YES"
ANSWERS IN THE SPACES AT THE BOTTOM OF THE FORM.

YES NO
1. Did you collect saliva at all time points (7pm last night, and immediately, 30 mins, and 45 mins after waking this morning)?
2. Did you engage in moderate or vigorous exercise yesterday?
3. Have you engaged in any moderate or vigorous activity today?
4. Have you had anything to eat after 5pm today?
5. Have you worn the accelerometer every day since receiving it?
6. Did you wake at a regular time this morning?
7. What time did you go to sleep last night?
8. What time did you wake up this morning?

ADDITIONAL DETAILS:
____________________________________________________________________________
____________________________________________________________________________
____________________________________________________________________________

280
APPENDIX N. RANDOM NUMBER COUNTDOWN
Minute
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
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29
30
31
32
33
34
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36
37
38
39
40
41
42
43
44
45
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47
48
49
50
51
52
53
54
55

Exercise Random Values
912 (909, 906, 903, 900, 897)
225 (222, 219, 216, 213, 210)
612 (609, 606, 603, 600, 597)
486 (483, 480, 477, 474, 471)
321 (318, 315, 312, 309, 306)
471 (468, 465, 462, 459, 456)
646 (643, 640, 637, 634, 631)
317 (314, 311, 308, 305, 302)
251 (248, 245, 242, 239, 236)
224 (221, 218, 215, 212, 209)
659 (656, 653, 650, 647, 644)
496 (493, 490, 487, 484, 481)
251 (248, 245, 242, 239, 236)
173 (170, 167, 164, 161, 158)
924 (921, 918, 915, 912, 909)
925 (922, 919, 916, 913, 910)
592 (589, 586, 583, 580, 577)
336 (333, 330, 327, 324, 321)
797 (794, 791, 788, 785, 782)
750 (747, 744, 741, 738, 735)
583 (580, 577, 574, 571, 568)
285 (282, 279, 276, 273, 270)
828 (825, 822, 819, 816, 813)
226 (223, 220, 217, 214, 211)
704 (701, 698, 695, 692, 689)
307 (304, 301, 298, 295, 292)
202 (199, 196, 193, 190, 187)
324 (321, 318, 315, 312, 309)
323 (320, 317, 314, 311, 308)
713 (710, 707, 704, 701, 698)
476 (473, 470, 467, 464, 461)
166 (163, 160, 157, 154, 151)
542 (539, 536, 533, 530, 527)
456 (453, 450, 447, 444, 441)
510 (507, 504, 501, 498, 495)
431 (428, 425, 422, 419, 416)
579 (576, 573, 570, 567, 564)
220 (217, 214, 211, 208, 205)
733 (730, 727, 724, 721, 718)
196 (193, 190, 187, 184, 181)
105 (102, 99, 96, 93, 90)
947 (944, 941, 938, 935, 932)
659 (656, 653, 650, 647, 644)
498 (495, 492, 489, 486, 483)
985 (982, 979, 976, 973, 970)
525 (522, 519, 516, 513, 510)
349 (346, 343, 340, 337, 334)
546 (543, 540, 537, 534, 531)
620 (617, 614, 611, 608, 605)
795 (792, 789, 786, 783, 780)
920 (917, 914, 911, 908, 905)
823 (820, 817, 814, 811, 808)
791 (788, 785, 782, 779, 776)
482 (479, 476, 473, 470, 467)
809 (806, 803, 800, 797, 794)

281

Control Random Values
905 (902, 899, 896, 893, 890)
186 (183, 180, 177, 174, 171)
847 (844, 841, 838, 835, 832)
145 (142, 139, 136, 133, 130)
844 (841, 838, 835, 832, 829)
555 (552, 549, 546, 543, 540)
787 (784, 781, 778, 775, 772)
425 (422, 419, 416, 413, 410)
990 (987, 984, 981, 978, 975)
304 (301, 298, 295, 292, 289)
257 (254, 251, 248, 245, 242)
720 (717, 714, 711, 708, 705)
458 (455, 452, 449, 446, 443)
434 (431, 428, 425, 422, 419)
721 (718, 715, 712, 709, 706)
107 (104, 101, 98, 95, 92)
469 (466, 463, 460, 457, 454)
652 (649, 646, 643, 640, 637)
312 (309, 306, 303, 300, 297)
806 (803, 800, 797, 794, 791)
537 (534, 531, 528, 525, 522)
260 (257, 254, 251, 248, 245)
772 (769, 766, 763, 760, 757)
419 (416, 413, 410, 407, 404)
618 (615, 612, 609, 606, 603)
490 (487, 484, 481, 478, 475)
506 (503, 500, 497, 494, 491)
495 (492, 489, 486, 483, 480)
117 (114, 111, 108, 105, 102)
872 (869, 866, 863, 860, 857)
478 (475, 472, 469, 466, 463)
790 (787, 784, 781, 778, 775)
736 (733, 730, 727, 724, 721)
588 (585, 582, 579, 576, 573)
155 (152, 149, 146, 143, 140)
310 (307, 304, 301, 298, 295)
312 (309, 306, 303, 300, 297)
432 (429, 426, 423, 420, 417)
731 (728, 725, 722, 719, 716)
479 (476, 473, 470, 467, 464)
130 (127, 124, 121, 118, 115)
587 (584, 581, 578, 575, 572)
452 (449, 446, 443, 440, 437)
808 (805, 802, 799, 796, 793)
740 (737, 734, 731, 728, 725)
935 (932, 929, 926, 923, 920)
872 (869, 866, 863, 860, 857)
314 (311, 308, 305, 302, 299)
993 (990, 987, 984, 981, 978)
665 (662, 659, 656, 653, 650)
765 (762, 759, 756, 753, 750)
302 (299, 296, 293, 290, 287)
838 (835, 832, 829, 826, 823)
688 (685, 682, 679, 676, 673)
538 (535, 532, 529, 526, 523)


APPENDIX O. SCREENING DATA COLLECTION FORM

Screening Visit

Participant ID: ___________________________ Height (cm): ___________________________
Date: ___________________________ Mass (kg): ___________________________
Time: ___________________________ HR: ___________________________
Screening Number: ___________________________ BP: ___________________________
DOB: ___________________________

<table>
<thead>
<tr>
<th>Clock Time</th>
<th>Event</th>
<th>Completed</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Participant reports to laboratory</td>
<td>✅</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Complete Written Informed Consent</td>
<td>✅</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medical History Questionnaire</td>
<td>✅</td>
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<tr>
<td></td>
<td>Beck Depression Inventory II</td>
<td>✅</td>
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<tr>
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<td>Munich Chronotype Questionnaire</td>
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<td>IPAQ</td>
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<tr>
<td></td>
<td>Pittsburg Sleep Quality Index</td>
<td>✅</td>
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<td></td>
<td>Hollingshead Index</td>
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<td>BodPod</td>
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<td>Skinfold Assessment</td>
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<td>HRV Orthostatic Challenge</td>
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<td>Polar HR Started</td>
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<td>Fluid log</td>
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<td>Cryovials for saliva collection</td>
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<td>Schedule experimental visit</td>
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### Screening Visit

Participant ID: 
Date: 
Time: 
Screening Number: 

#### VO2max Assessment

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<th>RPE</th>
<th>HR</th>
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#### Skinfold Assessment

- **Chest**
- **Subscapular**
- **Tricep**
- **Abdominal**
- **Suprailiac**
- **Midaxillary**
- **Thigh**

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<thead>
<tr>
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<th>2</th>
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#### Pre-exercise HRV Orthostatic Challenge

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<th>Standing</th>
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<tr>
<td>End</td>
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#### Post-exercise HRV Orthostatic Challenge

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<th>Standing</th>
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<tr>
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## APPENDIX P. EXPERIMENTAL DATA COLLECTION FORM

### Experimental Visit

<table>
<thead>
<tr>
<th>Clock Time</th>
<th>Running Clock</th>
<th>Sample Number</th>
<th>Event</th>
<th>Completed</th>
<th>Blood</th>
<th>Saliva</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:00</td>
<td></td>
<td></td>
<td>Participant reports to laboratory</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19:00</td>
<td></td>
<td></td>
<td>Returns cryovials, ActiGraph watch, and food, hydration, and watch logs</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>20:00</td>
<td></td>
<td></td>
<td>Provides urine sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:15</td>
<td></td>
<td></td>
<td>Participant completes questionnaires</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>Hb recording starts / first plasma draw</td>
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<td></td>
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<tr>
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<td>Exercise or Control Begins</td>
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<tr>
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<td>Exercise or Control</td>
<td>Hb</td>
<td>Hct</td>
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<tr>
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<td>4</td>
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<td>Hb</td>
<td>Hct</td>
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<tr>
<td>20:00</td>
<td>75</td>
<td>6</td>
<td>End of Exercise, begin rest in Environmental Chamber</td>
<td>Hb</td>
<td>Hct</td>
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<tr>
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<td>8</td>
<td>Rest in Environmental Chamber</td>
<td>Hb</td>
<td>Hct</td>
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<tr>
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<tr>
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<tr>
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<td>Blood</td>
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Complete clock time, running clock, and sleep state. Clock time and running clock should reflect time participant wakes.

Nude body mass
### Experimental Visit

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<th>Participant ID:</th>
<th>Height (cm):</th>
<th>Date:</th>
<th>Mass (kg):</th>
<th>Condition (EX or CON):</th>
<th>Time exercise started:</th>
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<table>
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<th>Time (mins)</th>
<th>Power (W)</th>
<th>RPE</th>
<th>HR</th>
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<td>30-60</td>
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<td>45</td>
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<td></td>
<td>300-</td>
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APPENDIX Q. DATA REDUCTION APPROACHES TO WAKING SAMPLE COLLECTION.

Delta ($\Delta$) scores are calculated as the simple difference between the first sample and greatest sample across the sampling period. In the example above, this occurs at 30 minutes post-waking.

The area under the curve relative to the ground (AUCG) is calculated as the integrated area under the curve with reference to a “0” score (e.g. concentration or power) via the trapezoidal method (J. C. Pruessner et al., 2003), assuming constant time intervals.

$$AUCG = \sum_{i=1}^{n-1} \frac{(s_{i+1} + s_i)}{2}$$

Where $s$ denotes an individual sample.

The area under the curve relative to the increase (AUCI) is calculated as the integrated area under the curve with reference to the first sample score (e.g. concentration or power) via the trapezoidal method (J. C. Pruessner et al., 2003), assuming constant time intervals.

$$AUCI = \left( \sum_{i=1}^{n-1} \frac{(s_{i+1} + s_i)}{2} \right) - (n - 1) \cdot m_1$$

Where $s$ denotes an individual sample and $n$ denotes the number of samples.
Heart Rate Variability (HRV) processing workflow. The entire RR time series is recorded, from 6:45pm until 1:05 hours after waking (A). The data is reduced to the 5, 5 minute epochs of interest (B), centered around each cortisol awakening sample (wake +0 mins, +15 mins, +20 mins, +45 mins, and +60 mins). Each epoch is cleaned (low threshold filter) and detrended (smoothing priors) prior to analysis (C). Each epoch is converted to the frequency domain by fast Fourier transformation and HF power (HFpow) is calculated (D). A quadratic function is fit to HFpow for the waking period (E). The area under the HRV curve is calculated on the raw values and the modelled values by the trapezoidal approach (F). A, C, and D, are completed via Kubios Premium (v3.1.0), B, E, and F are completed using R Statistical Software.
APPENDIX S. MODELS, SAMPLE MODEL RESULTS, AND DEPICTIONS

To test RH1.1, RH2.1, or RH3.1, a mixed-effects random intercept model may demonstrate the best model fit. The model formulation would then be:

\[ \text{Analyte}_{ij} = \beta_{0j} + \beta_{1j} \times \text{time}_{ij} + \beta_{2j} \times \text{time}^2_{ij} + R_{ij} \]

\[ \beta_{0j} = \gamma_{00} + \gamma_{01} \times \text{Condition}_j + U_{0j} \]

\[ \beta_{1j} = \gamma_{10} + \gamma_{11} \times \text{Condition}_j \]

\[ \beta_{2j} = \gamma_{20} + \gamma_{21} \times \text{Condition}_j \]

Below are potential model results, aligned with the respective depiction of the awakening response for the given analyte.

Example 1:
No effect of exercise.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>14.71</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Condition</td>
<td>0.03</td>
<td>0.884</td>
</tr>
<tr>
<td>Time</td>
<td>19.14</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Time^2</td>
<td>-17.86</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Time * Condition</td>
<td>0.32</td>
<td>0.902</td>
</tr>
<tr>
<td>Time^2 * Condition</td>
<td>-0.13</td>
<td>0.96</td>
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</table>
**Example 2:**
No effect of exercise on first value, but an effect of exercise on linear change over time.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>p</th>
</tr>
</thead>
<tbody>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Condition</td>
<td>-1.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time</td>
<td>35.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time$^2$</td>
<td>-25.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time * Condition</td>
<td>-7.35</td>
<td>0.025</td>
</tr>
<tr>
<td>Time$^2$ * Condition</td>
<td>5.99</td>
<td>0.067</td>
</tr>
</tbody>
</table>

**Example 3:**
No effect of exercise on first value, but an effect of exercise on quadratic change over time.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Condition</td>
<td>-0.42</td>
<td>0.063</td>
</tr>
<tr>
<td>Time</td>
<td>24.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time$^2$</td>
<td>-18.92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time * Condition</td>
<td>-10.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time$^2$ * Condition</td>
<td>-10.19</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Example 4:
Exercise lowers starting value, but the same pattern of change occurs over time.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>15.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Condition</td>
<td>-1.94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time</td>
<td>22.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time²</td>
<td>-18.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time * Condition</td>
<td>0.36</td>
<td>0.9</td>
</tr>
<tr>
<td>Time² * Condition</td>
<td>-0.22</td>
<td>0.938</td>
</tr>
</tbody>
</table>

Example 5:
Exercise increases starting value, and has an effect of linear and quadratic change over time.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>15.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Condition</td>
<td>-0.38</td>
<td>0.047</td>
</tr>
<tr>
<td>Time</td>
<td>19.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time²</td>
<td>-19.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time * Condition</td>
<td>-11.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time² * Condition</td>
<td>14.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>